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The zebrafish as a model system for analyzing mammalian and native α-crystallin promoter function

Mason Posner Corresp., [1] , **Kelly Murray** [1] , **Hayden Eighinger** [1] , **Amy Drossman** [1] , **Zachary Haley** [1] , **Justin Nussbaum** [2] , **Larry L David** [3] , **Kirsten J Lampi** [4]

[1] Department of Biology/Toxicology, Ashland University, Ashland, Ohio, United States of America

[2] Department of Biology, Lakeland Community College, Kirtland, Ohio, United States

[3] Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon, United States

[4] Department of Integrative Biosciences, Oregon Health and Science University, Portland, Oregon, United States

Corresponding Author: Mason Posner
Email address: mposner@ashland.edu

Previous studies have used the zebrafish to investigate the biology of lens crystallin proteins and their roles in development and disease. However, little is known about zebrafish α-crystallin promoter function, how it compares to that of mammals, or whether mammalian α-crystallin promoter activity can be assessed using zebrafish embryos. We injected a variety of α-crystallin promoter fragments from each species combined with the coding sequence for green fluorescent protein (GFP) into zebrafish zygotes to determine the resulting spatiotemporal expression patterns in the developing embryo. We also measured mRNA levels and protein abundance for all three zebrafish α-crystallins. Our data showed that mouse and zebrafish αA-crystallin promoters generated similar GFP expression in the lens, but with earlier onset when using mouse promoters. Expression was also found in notochord and skeletal muscle in a small percentage of embryos. Mouse αB-crystallin promoter fragments drove GFP expression primarily in zebrafish skeletal muscle, with less common expression in notochord, lens, heart and in extraocular regions of the eye. A short fragment containing only a lens-specific enhancer region produced no GFP expression, suggesting that these lens responsive elements in the mouse are not used in the zebrafish. The two paralogous zebrafish αB-crystallin promoters produced subtly different expression profiles, with the αBa promoter driving expression equally in notochord and skeletal muscle while the αBb promoter resulted primarily in skeletal muscle expression. Messenger RNA for zebrafish αa, aBa and αBb were all detected by 1 day post fertilization (dpf). Parallel reaction monitoring (PRM) mass spectrometry was used to detect αA, αBa, and αBb peptides in digests of zebrafish embryos. In whole embryos, αA-crystallin was first detected by 2 dpf, peaked in abundance by 4-5 dpf, and was localized to the eye. αBa was also detected in whole embryo at nearly constant levels from 1-6 dpf, was also localized primarily to the eye, and its abundance in extraocular tissues

decreased from 4-7 dpf. In contrast, due to its low abundance, no αBb protein could be detected in whole embryo, or dissected eye and extraocular tissues. Our results show that mammalian α-crystallin promoters can be efficiently screened in zebrafish embryos and that their controlling regions are well conserved, although their use in each species may reflect evolutionary changes in developmental roles for α-crystallins. An ontogenetic shift in zebrafish αBa-crystallin promoter activity provides an interesting system for examining the evolution and control of tissue specificity. Future studies that combine these promoter based approaches with the expanding ability to engineer the zebrafish genome via techniques such as CRISPR/Cas9 will allow the manipulation of protein expression to test hypotheses about lens crystallin function and its relation to lens biology and disease.

The zebrafish as a model system for analyzing mammalian and native α-crystallin promoter function

Mason Posner[1], Kelly Murray[1], Hayden Eighinger[1], Amy Drossman[1], Zachary Haley[1], Justin Nussbaum[2], Larry David[3] and Kirsten Lampi[4]
[1]Department of Biology, Ashland University, Ashland, OH, USA
[2]Department of Biology, Lakeland Community College
[3]Department of Biochemistry and Molecular Biology, Oregon Health and Science University
[4]Department of Integrative Biosciences, Oregon Health and Science University

Corresponding author:
Mason Posner[1]
Email address: mposner@ashland.edu

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Introduction:

The zebrafish has become a valuable model system for examining lens development, disease and the function of lens crystallin proteins. Multiple studies have identified genes and proteins involved in lens formation (Yang et al., 2004; Yang & Cvekl, 2005; Vihtelic, 2008) and taken advantage of zebrafish embryo transparency to produce detailed imagery of lens development (Greiling, Aose & Clark, 2010). Changes in the lens proteome during its development have also been described (Greiling, Houck & Clark, 2009; Greiling, Aose & Clark, 2010; Wages et al., 2013). The crystallin protein content of the zebrafish lens has been detailed (Posner, Kantorow & Horwitz, 1999; Runkle et al., 2002; Wistow et al., 2005; Smith et al., 2006; Posner et al., 2008), and functional studies have examined zebrafish crystallin chaperone-like activity and stability in comparison to mammals (Dahlman et al., 2005; Koteiche et al., 2015). Multiple ocular diseases, such as glaucoma, diabetic retinopathy, macular degeneration and cataract have been modeled in the zebrafish (Morris, 2011; Gestri, Link & Neuhauss, 2012; Chhetri, Jacobson & Gueven, 2014). In total these studies illustrate the benefits of using the zebrafish to study lens biology and provide insights into the normal function and dysfunction of the vertebrate lens.

One area of zebrafish lens biology that has not been well explored is the activity and function of lens crystallin promoters. Kurita et al. (2003) cloned the zebrafish αA-crystallin promoter region and used it to drive the expression of diphtheria toxin in the lens to study developmental connections between lens and retina. Davidson et al. (2003) used a *Xenopus* γ-crystallin promoter to express green fluorescent protein (GFP) in the zebrafish lens. Goishi et al. (2006) constructed a zebrafish αA-crystallin promoter/GFP plasmid to show how the zebrafish *cloche* mutant, which lacks a functional DNA-binding transcription factor implicated in vascular development (Reischauer et al., 2016), might downregulate αA-crystallin expression. To our knowledge, no work since these studies has utilized zebrafish crystallin promoters, and no study has characterized the temporal or spatial expression of reporter genes linked to these promoters.

The function of mammalian α-crystallin promoters has been the subject of multiple studies. Examination of the shared promoter region between *αB-crystallin* and *HspB2* in the mouse identified specific regions that enhance αB-crystallin expression. For example, an enhancer spanning -426/-259 was required for extralenticular expression while a more proximal region from -164/+44 produced reporter gene expression in lens (Dubin et al., 1991; Gopal-Srivastava, Kays & Piatigorsky, 2000; Swamynathan & Piatigorsky, 2002). To recapitulate endogenous expression of mouse αB-crystallin, four kilobases of the 5'-flanking promoter sequence was needed (Haynes, Duncan & Piatigorsky, 1996). A region spanning -111 to +46 of the mouse αA-crystallin promoter was shown to drive expression of GFP in both cultured lens cells and in the mouse, with this expression enhanced by inclusion of a distal enhancer approximately 8 kilobases upstream of the gene (Yang & Cvekl, 2005; Yang et al., 2006). While no published studies report the use of mouse lens crystallin promoters in the zebrafish, Hou et al. (2006) showed that a fragment of the human βB1-crystallin promoter produced

transgenic expression of GFP in the zebrafish lens. A subsequent study used this human promoter to drive the expression of novel proteins in the zebrafish lens to study the function of aquaporin water channels (Clemens et al., 2013). The evolutionary conservation of lens crystallin gene regulation is not surprising considering the similar expression of lens crystallin proteins between zebrafish and mammals (Posner et al., 2008; Greiling, Houck & Clark, 2009). This conservation suggests that mammalian α-crystallin promoters could be functionally assessed in the zebrafish, providing a faster and less expensive system than traditional mouse transgenic approaches. The growing development of zebrafish gene editing techniques would greatly expand the capabilities of this system. Data on crystallin promoter activity would also facilitate the expression of introduced proteins in zebrafish lens and other tissues.

A comparison of mouse and zebrafish α-crystallin promoter activity can also help detail the evolution of tissue specific expression. Past studies have examined the evolution of crystallin gene expression at different timescales. For example, sequence comparisons have detailed the recruitment of crystallins during the initial evolution of the vertebrate lens, finding that the α-crystallins are related to extra-lenticular small heat shock proteins (Wistow & Piatigorsky, 1988). A subsequent gene duplication event was followed by divergence in expression between the two resulting paralogs (αA and αB-crystallin). A more recent evolutionary change in the regulation of α-crystallins was investigated in the blind mole rat, in which the αB-crystallin promoter has specifically lost lens activity, presumably reflecting the degenerated eyes of this subterranean species (Hough et al., 2002). In this present study we further examine evolutionary changes in α-crystallin expression by comparing promoter activity of the two divergently expressed zebrafish αB-crystallin paralogs. While the expression of these proteins is already known in adults, an examination of their gene's promoter activities and protein abundance during early development can identify possible ontogenetic shifts in expression. The structure, stability, chaperone-like activity and expression pattern of zebrafish αBb-crystallin is similar to the mouse ortholog (Dahlman et al., 2005; Smith et al., 2006). We predicted that this conservation would extend into early development. However, it is an open question whether the altered expression of the lens-specific zebrafish αBa-crystallin begins in early development, or appears later in ontogeny.

Our results suggest that the zebrafish can be used as a time efficient and cost effective model for screening the activity of native and modified mammalian lens crystallin promoters. Our comparative promoter analysis of the two zebrafish α□-crystallins shows a subtle difference in expression between these two paralogs and confirmed an early onset for αBa-crystallin expression, although at low expression levels. We also show that zebrafish αBa-crystallin undergoes an ontogenetic shift in its expression to become lens-specific later in development.

Materials and Methods:

Zebrafish Maintenance and Breeding

AB or ZDR strain zebrafish were housed in 10 L aquaria on a recirculating filtering system maintained at 28-30 °C with a 14:10 h light and dark cycle. Fish were fed twice each day with either commercial flake food or live *Artemia* brine shrimp. Two males and two females were placed in one liter breeding tanks the afternoon prior to morning egg collections. Plastic dividers were used to separate the two sexes until eggs were needed to assure that all embryos were of similar ages. All animal procedures were approved by Ashland University's Animal Care Committee (approval number MP 2015-1).

Comparative analysis of α-crystallin promoter regions

The UCSC Genome Browser (http://genome.ucsc.edu/; Kent et al., 2002) was used to identify conserved regions in the mouse and zebrafish αA- and αB-crystallin promoters (Supplemental Fig. 1). A previous analysis of syntenic relations was used to assess the rearrangement of gene relationships after duplication of zebrafish αB-crystallin (Elicker & Hutson, 2007).

Promoter expression plasmid construction, embryo injection and assessment of GFP expression

Primers used to amplify regions of each α-crystallin promoter were designed using DNA Main Workbench based on sequences in GenBank and ordered from Sigma Genosys. Each promoter region was then amplified from a bacterial artificial chromosome (BAC) clone obtained from the BACPAC Resources Center (bacpac.chori.org) using Platinum Pfx DNA polymerase (Thermo Fisher; Waltham MA). Amplification conditions were optimized to produce single bands of the expected size, which were then subcloned into the pJET1.2 plasmid (Thermo Fisher) and sequenced to confirm their identity (Functional Biosciences; Madison, WI). Restriction enzyme sites designed into each amplification primer were used to digest and ligate each cloned promoter into the pAcGFP1-1 plasmid (Clontech; Mountain View CA) using enzymes from New England Biolabs (Ipswich, MA). NEB 5 alpha cells (NEB) were transformed with each promotor/GFP construct. Some promoter constructs were produced using an alternate Gibson Assembly approach (NEB). All cloned promoters have been deposited with Addgene.

To prepare promoter expression plasmids for injection into zebrafish embryos, plasmids were linearized with NotI, purified using a QIAquick PCR Purification kit (Qiagen, Valencia, CA) and then dialyzed with TE buffer using a 0.025 μm VSWP membrane (Millipore, Billerica, MA). Injection solutions contained 35 ng/ul of the dialyzed plasmids, 0.2% phenol red and a sufficient volume of 0.1 M KCl to produce 5 microliters of injection mix. Two nanoliters of this solution was injected into one-cell stage zebrafish embyros with a Harvard Apparatus PL-90 picoinjector (Holliston, MA) using needles prepared with a Sutter P97 Micropipette Puller (Novato, CA). Injection pressures were adjusted to inject 1 nl of plasmid solution with each 20 ms pulse. Injected embryos and uninjected controls were incubated at 28 °C in fish system water and transferred to 0.2

mM PTU at 24-30 hours post fertilization to block melanin production and facilitate observation of GFP expression.

The presence of any GFP expression was examined using an Olympus IX71 inverted microscope and imaged with a SPOT RT3 camera (Diagnostic Instruments, Sterling Heights, MI). Live embryos were anesthetized in tricaine and imaged at 100X or 200X total magnification using UV illumination and GFP filter. Confocal images were captured on a Leica SP5 microscope after embryos were anesthetized and fixed in 4% paraformaldehyde. Embryos for confocal imaging were mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA). Image series were then rendered as three-dimensional surface projections using Volocity imaging software (Perkin Elmer, Waltham, MA)

Semi quantitative RT-PCR and qPCR analysis of α-crystallin expression in embryos

Whole embryos or dissected embryos separated into isolated eyes and extraocular tissues were collected and frozen in 0.5 mL RNAlater solution (Thermo Fisher). Typically 10 to 15 embryos or eyes were collected for each timepoint. Total RNA from each sample was purified using an RNEasy Minikit (QIAGEN) and quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Resulting total RNA (300-350 ng) was used to synthesize cDNA using the Protoscript II First Strand cDNA Synthesis Kit (NEB) according to the manufacturer's protocol. Complimentary DNA equivalent to 60-70 ng of initial purified RNA was then amplified in a T100 thermal cycler (Biorad) with OneTaq Hot Start 2x Master Mix (New England Biolabs) for 30-40 cycles using primers specific to each zebrafish α-crystallin. A tubulin gene was used as an internal control to insure equal starting amounts of cDNA in each reaction. Primer sequences used are shown in Table 1a. Products were separated and visualized in 1% agarose gels with a 100 bp ladder at 100 V for 20-35 minutes. Three biological replicates were collected and analyzed for each reported timepoint and tissue.

Quantitative PCR was used to confirm specific variations in α-crystallin gene expression identified by semi quantitative RT-PCR. Primers designed by Elicker & Hutson (2007; Table 1b) were used to amplify the same cDNA samples as those used in RT-PCR, described above. Reactions were performed in triplicate using cDNA equivalent to 25 ng of initial purified RNA in 20 μl reactions with Power SYBR Green PCR Master Mix (Thermo Fisher) at an annealing temperature of 56°C for 40 cycles in an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher). Elongation factor (EF-1α) primers were used as an internal control and lack of template was used as a negative control.

Proteomic analysis of α-crystallin content in zebrafish

A pair of lenses from adult zebrafish were dissected, placed in 100 µl of 50 mM ammonium bicarbonate buffer, and probe sonicated (3 x 5 sec with cooling on ice between treatments) to produce a uniform suspension. The protein concentration was then determined using a BCA assay (Thermo Fisher) using BSA as a standard. A 50 µg portion of protein was then reduced, alkylated, and trypsinized in the presence of ProteaseMax™ detergent using the manufacturer's recommended protocol (ProMega).

Following digestion, trifluoroacetic acid was added at a final 0.5% concentration, the sample centrifuged at 16,000 x g for 5 min, and the supernatant transferred to an autosampler vial. One µg of digest was then loaded onto an Acclaim PepMap 0.1 x 20 mm NanoViper C18 peptide trap (Thermo Fisher) for 5 min at a 5 µl/min flow rate in a 0.1% formic acid mobile phase. Peptides were then separated using a PepMap RSLC C18, 2 µm particle, 75 µm x 25 cm EasySpray column (Thermo Fisher) and 7.5–30% acetonitrile gradient over 60 min in mobile phase containing 0.1% formic acid at a 300 nl/min flow rate using a Dionex NCS-3500RS UltiMate RSLCnano UPLC system. Data-dependent tandem mass spectrometry data was collected using an Orbitrap Fusion Tribrid mass spectrometer configured with an EasySpray NanoSource (Thermo Fisher). Survey scans from 400-1600 m/z were performed in the Orbitrap mass analyzer at 120,000 resolution, automatic gain control (AGC) setting of 4.0×10^5, 50 msec maximum injection time, and lock mass using a m/z = 445.12 polysiloxane ion. Data-dependent MS2 scans on peptide ions with signal intensities higher than 5,000, ranging from +2 to +6 charge state, and passing the monoisotopic precursor selection filter were selected for higher energy collision dissociation (HCD) with a 30% collision energy using quadrupole isolation with a 1.6 m/z window. Fragment ions were then analyzed in the linear ion trap with an AGC setting of 1.0×10^4, maximum injection time (MIT) of 35 msec, dynamic exclusion enabled, repeat count of 1, exclusion duration of 30 sec, exclusion mass tolerance of +/- 10 ppm, top speed mode, and 3 sec dwell time between Orbitrap survey scans. MS/MS results were then matched to peptide sequences using Sequest HT software within the Protein Discoverer 1.4 suite (Thermo Fisher) using a UniProt database containing the taxon identifier 7955 (*Danio rerio*) generated in July 2016 and containing 58,290 entries. Searches were performed with trypsin specificity, a maximum of 2 missed cleavages, precursor and fragment ion tolerances of 10 ppm and 1 Da, respectively for parent and daughter ions using monoistopic masses. A static modification of +57.02 Da was added to all cysteine residues due to alkylation with iodoacetamide, and a variable modification of +15.99 Da for methionine oxidation. Peptide identifications were filtered with the Percolator node in Protein Discoverer using a reverse sequence database strategy to estimate peptide false discovery. The resulting Protein Discoverer .msf file was then imported into Skyline software (version 3.6.0.10162) (MacLean et al., 2010) to create a spectral library using identified peptides with Percolator q scores ≤0.05, having between 8-25 residues, and no missed cleavages. Three peptides each for entries Q8UUZ6 (α A-crystallin), Q9PUR2 (α Ba-crystallin), and Q6DG35 (α Bb-crystallin) were selected based on manual observation of parent ion intensities and quality of fragment ion spectra. These were then used to create a parallel reaction monitoring method to detect the presence of the three α-crystallins during embryo development.

Uniform suspensions of either whole embryos or dissected embryo eyes and trunks were created using either probe sonication in 50 mM ammonium bicarbonate as above, or by vortexing vigorously for 30 min in 20 µl of 50 mM ammonium bicarbonate buffer containing 0.2% ProteaseMax detergent. Following a protein assay, from 10-50 µg of each suspension was digested using the ProteaseMax protocol as recommended by the manufacturer, and 2 µg of each digest analyzed by LC/MS using the same chromatographic separation and instrument as above, except using a parallel reaction

monitoring method (Bourmaud, Gallien & Domon, 2016) to detect the 9 targeted α-crystallin peptide ions (Supplemental Table 1). Peptides were isolated and fragmented as above, except without data-dependency and by cycling through the list of ions throughout the chromatographic separation so the intensity of fragment ions could be continuously monitored. MS/MS spectra were acquired in the instrument's Orbitrap mass analyzer at a resolution of 30,000, AGC setting of 5×10^4, 100 msec MIT, with a scan range of m/z 200-2000. Skyline was then used to extract intensities for the three most intense fragment ions for each peptide determined from the lens spectral library, and perform peak detection and integration to monitor the relative abundance of α-crystallins during embryo development.

Results:

The location of green fluorescent protein (GFP) expression resulting from injection of mouse and zebrafish promoters into zebrafish zygotes was examined by both standard fluorescent microscopy and confocal microscopy. Representative confocal images of anatomical structures that expressed GFP during this study are shown in Fig. 1. Video fly-throughs of representative structures can be found in Supplemental Videos 1-3. Patterns of expression produced by each promoter can be found in Tables 2 and 3, and are described below. Overall 1489 observations were made of 755 individual injected embryos ranging in age from 24 hours post fertilization (hpf) to 7 days post fertilization (dpf). GFP expression was seen in 73.8% of examined embryos.

Mouse and zebrafish αA-crystallin promoters produced similar GFP expression in zebrafish embryos with a subtle difference in timing

Previous work has shown strong conservation in αA-crystallin DNA sequences, protein stability and chaperone-like activity between zebrafish and mammals (Runkle et al., 2002; Dahlman et al., 2005; Posner et al., 2012). The zebrafish and mouse αA-crystallin orthologs are similarly arranged relative to other genes, with both located in a head-to-head orientation with heat shock factor binding protein gene *hsf2bp* (Fig. 2a). However, mouse and human contain a second gene, *salt inducible kinase 1* between αA and *hsf2bp*, and the intergenic distances are much greater (Wolf et al., 2008). Several sequence regions of the mouse αA-crystallin promoter are conserved in the zebrafish genome (Supplemental Fig. 1a). Here we show that a mouse αA-crystallin promoter fragment (-111 to +46) combined with enhancer region DCR1 drove green fluorescent protein expression in the zebrafish lens, with much less common expression in skeletal muscle (Fig. 2b-c; Table 2). This pattern was similar to that produced by a 1 kb fragment of the zebrafish αA promoter (Fig. 2d-e). The zebrafish promoter also produced spots of GFP expression dorsal to the yolk that were much less common with the mouse promoter (Fig. 1g-h). A small fraction of embryos injected with the zebrafish and mouse αA-crystallin promoters showed GFP expression in segments of the notochord. There was a subtle difference in onset of expression between the two promoters, with GFP driven by the mouse αA promoter noticeable by 30 hours post fertilization (hpf) while the zebrafish αA promoter became active between 30 and 48 hpf (Table 3). Reverse-transcription PCR analysis found zebrafish αA-crystallin mRNA present by 24 hpf with an increase at 2 days post fertilization (dpf) and then constant

levels through 6 dpf (Fig. 2f). Quantitative PCR indicated a two-fold increase in mRNA from 1 to 2 dpf (data not shown). Separate RT-PCR analysis of eyes and the rest of the body indicated the presence of αA-crystallin mRNA in both tissues, but with higher levels of expression in the eye at both 4 and 6 dpf (Fig. 2g).

Mouse αB-crystallin promoter drove GFP expression in zebrafish embryos, but a previously identified lens enhancer was not active

Previous studies have shown that an upstream enhancer region (-426/-257) of the mouse αB-crystallin promoter is required for extralenticular expression, while a more proximal region (-164/+44) is sufficient for driving lens expression (Dubin et al., 1991; Gopal-Srivastava & Piatigorsky, 1994) (Fig. 3a). Genomic sequence alignment shows two areas of conservation between mouse and zebrafish in this proximal promoter region (Supplemental Fig. 1b). We cloned three mouse αB-crystallin promoter fragments into a GFP plasmid to examine whether these functional regions had similar effect in zebrafish. Our results indicate that the mouse αB-crystallin promoter drives GFP expression in zebrafish embryos, and that the resulting spatial patterns reflect the functional regions first identified in mouse. For example, the 0.8 and 1.4 kb promoters produced GFP expression in skeletal muscle and lens, as seen in mouse (Fig. 3b-g). However, while skeletal muscle expression was seen as early as 30 hpf, lens expression was not found until 5 dpf. This result is consistent with the reduced early expression of zebrafish αB-crystallins in the zebrafish lens compared to mammals (Greiling, Houck & Clark, 2009; Wages et al., 2013). The 0.8 and 1.4 kb mouse αB-crystallin promoters also produced GFP expression in the notochord (Fig. 3b-c) and in eye tissues peripheral to the lens. Heart expression was also seen with a late onset at 5 dpf, similar to lens expression (Fig. 3g). We expected the mouse 0.25 kb αB-crystallin promoter to drive GFP exclusively in the lens, but instead found no GFP expression in any tissue through 7 dpf (Fig. 3e).

The two zebrafish αB-crystallins produced similar, yet subtly different patterns of GFP expression

The presence of two αB-crystallins in the zebrafish is likely the result of an ancient genome duplication event at the base of teleost evolution (Van de Peer, Taylor & Meyer, 2003). This duplication resulted in a divergence in chromosomal arrangement. Zebrafish αBa is located on chromosome 15 along with several distant genes with which its ortholog shows syntenic relationship in mammals (Elicker & Hutson, 2007). Zebrafish αBb has maintained the same tail-to-tail organization with fellow heat shock protein Hspb2 as is found in mammals, however the intergenic region in the zebrafish is much larger, at 6 kilobases compared to 1 kb in the mouse (Fig. 4a).

Previous studies indicated that the expression pattern of zebrafish αBa- and αBb-crystallin differs in adults, and proteomic analysis showed a difference in the timing of expression onset (Posner, Kantorow & Horwitz, 1999; Smith et al., 2006; Wages et al., 2013). However, no study has characterized developmental patterns produced by the two respective promoter regions. We produced a GFP-linked 3 kb fragment of the zebrafish αBa-crystallin promoter and a series of GFP-linked fragments spanning the

expanded αBb-crystallin promoter. We found no difference between the timing of onset for any of these zebrafish promoters, with GFP first appearing between 30 and 48 hpf (Table 3). We also found no difference in timing or spatial expression between the αBb-crystallin promoter fragments, suggesting that sequences upstream of 1 kb do not regulate expression of this gene. The spatial expression of GFP produced by all of the zebrafish αB-crystallin promoters was similar, with expression common in skeletal muscle and notochord (Fig. 4 b-c). However, the prevalence of GFP in these two tissues differed, with the zebrafish αBa promoter driving GFP equally (54.9% in skeletal muscle and 56.3% in notochord) while zebrafish αBb promoters were much more active in skeletal muscle than notochord (95.5% versus 10.3%; Fig. 4d). Both zebrafish αB promoters produced very rare GFP expression in lens (7 embryos out of 357 observed; Fig. 4e), some expression in the eye peripheral to the lens (22 embryos) and three αBb promoter-injected embryos, out of 267, produced GFP expression in the heart. Overall these data suggest that the divergent expression of the two zebrafish αB-crystallin promoters previously identified in adults appears later in development than the 1-7 dpf window examined in this present study.

When are zebrafish αB-crystallins first expressed?

To help resolve inconsistencies in the literature regarding the timing of expression of zebrafish αB-crystallins we measured mRNA levels from each gene using reverse transcription PCR. We identified transcription of zebrafish αBa-crystallin in whole embryos as early as 1 dpf, but with some variation in abundance (Fig. 5a-b). Two replications showed a spike in mRNA at 1 dpf while a third showed a spike at 3 dpf. Quantitative PCR analysis of two of these time series indicated a 4-fold increase in expression at 1 dpf compared to 2 dpf and a 12-fold increase at 3 dpf compared to 4 dpf (using the same cDNA samples shown in Fig. 5a and b). Reverse transcription PCR of αBb-crystallin showed relatively constant expression from 1-7 dpf (Fig. 5c). Amplification of tubulin was used as an internal control for these experiments (Fig. 5d shows a representative experiment).

Proteomic analysis identified two of three α-crystallins in zebrafish embryos

We used a mass spectrometric parallel reaction monitoring approach to identify the presence of α-crystallins in pooled zebrafish embryos at 1 to 6 dpf as a complement to the promoter expression and RT-PCR data presented above. Two of the three targeted αA-crystallin peptides were detected by 2 dpf and peaked in abundance at 4-5 dpf. The results for αA peptide 52-65 from whole embryo digests are shown in Fig. 6a, while Fig. 6b shows the relative abundance of αA-crystallin in dissected eye and remaining trunks at 4 and 7 dpf. These results indicated that αA-crystallin was largely present only in eye. Its apparent decrease in abundance in whole embryos by 6 dpf was likely due to its dilution by non-ocular proteins during embryonic development. An RT-PCR analysis found αA mRNA in both homogenized eye and extraocular tissues, but at greater abundance in the eye (Fig. 2g). While only one αBa-crystallin was detected in embryo digests, its measurement indicated that αBa-crystallin was present in almost equal abundance from 1-6 dpf (Fig. 6c). While small amounts of αBa-crystallin were detected in 4 dpf trunks, the protein was not detected in trunks by 7 dpf (Fig. 6d). However, αBa-

crystallin was present in eye and appeared to increase from 4 to 7 dpf. The decrease in αBa in trunk and concurrent increase in eye is consistent with its unaltered abundance in whole embryos from 1-6 dpf. No αBb-crystallin peptides were detected in either whole embryos or dissected eyes or trunks (data not shown), although RT-PCR analysis identified mRNA coding for both αBa- and αBb-crystallins in whole embryos (Fig. 5) and in isolated eyes and extraocular tissues (data not shown). The extracted ion chromatograms from these parallel reaction monitoring experiments are shown in Supplementary Figures 2-8.

Discussion:

This present study is the first to show that mammalian α-crystallin promoters will drive expression of green fluorescent protein (GFP) in zebrafish embryos, suggesting that this species could be used as an efficient model for mammalian crystallin expression analysis. We also provide the first data characterizing the activity of the three zebrafish α-crystallin gene promoters. These data detail their spatiotemporal expression and indicate differences between embryonic and adult expression patterns for the duplicated and divergent zebrafish αB-crystallin paralogs. Future studies can use the techniques described here to measure the expression potential of modified lens crystallin promoters. Our data also show how different crystallin promoters could be used to drive the expression of target genes in specific tissues. Lastly, by examining promoter activity, mRNA expression and protein abundance for zebrafish αBa-crystallin we resolve remaining questions about the timing of expression of this gene.

Our data show that mouse α-crystallin promoters successfully drive expression in zebrafish embryos. An interesting question is whether the resulting expression patterns match that expected in the mouse, or alternatively, if the zebrafish embryos read the mouse promoter in their own way. Is mouse promoter activity modified by the signaling molecule environment of the zebrafish? This question is difficult to answer for the mouse αA-crystallin promoter as the GFP expression produced in zebrafish embryos was very similar to that of the native zebrafish ortholog. Similar expression profiles resulting from each αA-crystallin promoter could be due to evolutionarily conserved roles in development, or alternatively that the zebrafish embryo reads the mouse promoter as one of its own. Interestingly, the mouse αA promoter expressed GFP at a slightly younger age than the native zebrafish promoter. This difference was also seen when comparing the mouse and zebrafish αB-crystallin promoters. Some element in the mouse promoter sequences appears to have accelerated the timing of expression. Earlier studies showed that the transcription factors Pax6, c-Maf, and CREB regulate expression of mouse αA-crystallin (Yang & Cvekl, 2005) and that FGF signaling regulates expression of c-Maf (Xie et al., 2016). Considering its similar activity in the zebrafish we hypothesize that teleost fishes use a similar regulatory system, although the presence of two Pax6 genes in zebrafish may alter the details of this regulation (Kleinjan et al., 2008). Analysis of mouse and chicken βB1-crystallin promoter regions showed similar cross-species conservation in regulation, with some differences that indicated additional regulatory elements for lens-specific expression in the mouse

promoter (Chen et al., 2001). The presence of zebrafish αA mRNA outside the eye, but lack of detected αA peptides and weaker GFP expression resulting from αA-promoters, is consistent with the interpretation that αA-crystallin is expressed at low levels in extraocular tissues.

Comparison of mouse and zebrafish αB-crystallin promoter function is complicated, and potentially more interesting, because of the presence of two αB-crystallin paralogs in the zebrafish. Zebrafish αBa-crystallin protein is restricted to the lens in adults while αBb-crystallin is found ubiquitously, similar to the single mammalian ortholog (Posner, Kantorow & Horwitz, 1999; Smith et al., 2006). Since duplicated αB-crystallins are only known from teleost fishes such as the zebrafish, the restriction of expression to the lens likely evolved after the genome duplication event in this taxon (Van de Peer, Taylor & Meyer, 2003). Previous developmental proteomic analyses indicated that neither zebrafish αB-crystallin is prominent in early development (Greiling, Houck & Clark, 2009; Wages et al., 2013). A published quantitative PCR analysis showed no presence of zebrafish αBa-crystallin mRNA through 5 dpf, while αBb mRNA was found starting at 12 hpf (Elicker & Hutson, 2007). Another study found no αBa mRNA in zebrafish embryos through 78 hpf (Mao and Shelden 2006). However, a recent report conflicts with these former findings, identifying zebrafish αBa mRNA at 24 hpf (Zou et al. 2015). Our promoter expression, RT-PCR, qPCR, and proteomic data are in general agreement and provide a comprehensive data set to address this published discrepancy. Zebrafish αBa-crystallin mRNA was detectable in embryos by 26 hpf, earlier than seen in some published studies, but similar to findings by Zou et al. (2015). GFP expression resulting from this gene's promoter was noticeable by 48 hpf and a single native αBa-crystallin peptide was detectable in 1-day old embryos. These data all indicate that zebrafish αBa-crystallin protein is found in zebrafish embryos at least by their first day post fertilization. However, GFP-expression data suggest that promoter activity is rare in the lens, but common in skeletal muscle and notochord, and proteomic and RT-PCR results suggest that αBa expression levels are measurable, but low. Data also indicate a significant shift in the localization of zebrafish αBa-crystallin during development, with expression becoming largely restricted to the lens.

The GFP spatiotemporal expression patterns produced by the two zebrafish αB-crystallin promoters were very similar, first leading us to think that we had erroneously cloned the same promoter twice. Sequencing confirmed the correct identity of each promoter and quantification of our observations showed a divergence in the promoters' activity in skeletal muscle and notochord. Not knowing the precise developmental role of each protein in these tissues it is difficult to hypothesize the functional significance of this finding. It is interesting that the prevalence of skeletal muscle GFP expression was similar between the mouse αB and zebrafish αBb promoters, reflecting their conservation of adult expression pattern, gene sequence and protein function compared to zebrafish αBa-crystallin. However, the late-onset of mouse αB promoter-driven heart expression was not seen with the zebrafish αBb promoter.

The spatiotemporal expression of GFP produced by various lengths of the mouse αB-crystallin promoter in zebrafish embryos suggests that at least some of the control elements of this promoter are conserved between mammals and teleost fish. For example, promoter constructs of 0.8 and 1.4 kb, which contain the identified skeletal muscle enhancing regions of the promoter in mouse, drove GFP expression in zebrafish skeletal muscle. The shorter 250 bp fragment lacking these control regions did not, suggesting that this muscle enhancer region functions within the signaling system of both species. However, zebrafish embryos appeared to use lens expression signaling in a different way. The three mouse promoter fragments all result in lens expression in the mouse. We saw this result in zebrafish embryos for the 0.8 and 1.4 kb fragments, but that expression was delayed compared to notochord and skeletal muscle, perhaps reflecting the later expression of αBb-crystallin in the zebrafish lens, as indicated by our RT-PCR and proteomics data. It is possible that the zebrafish's signaling molecules for lens expression are used at a later development stage, but interact with the same elements in the mouse promoters. This interpretation would lead us to predict that the 250 bp mouse fragment would produce zebrafish lens expression, but this was not the case. It appears that elements in this region are not sufficient, on their own, to produce lens expression in zebrafish, at least through 7 dpf. We did not find similar differences in gene expression when comparing 1, 2, 4 and 5 kb lengths of the zebrafish αBb-crystallin promoter, suggesting that any control elements remain within the first 1,000 basepairs of the promoter and have not been stretched out with the expansion of the intergenic region between αBb-crystallin and HSPB2.

In total the results of this study show that mammalian α-crystallin promoters can be screened efficiently in zebrafish embryos. Controlling regions in these promoters appear to be well conserved, although the way in which they are used has diverged between zebrafish and mammals. Mammalian α-crystallin promoter elements should not be assumed to work identically in zebrafish, as differences in expression may reflect evolutionary changes in how α-crystallins are used during development between mammals and teleost fishes. Our analysis of the three zebrafish promoters provides a new toolset for directing the expression of introduced proteins in various embryonic zebrafish tissues at different stages of development. Comparison of the duplicated zebrafish αB-crystallin promoters provides further insight into the mechanism of tissue specificity as we show that αBa-crystallin promoter activity in embryonic tissues is quite different from the lens restricted expression of αBa-crystallin protein in adults. Our combined analysis of αBa-crystallin promoter activity, mRNA expression and protein abundance also clarifies discrepancies in the literature about when and where this gene is expressed. The ease with which engineered promoters can be injected into zebrafish embryos and their expression patterns visualized makes this model species ideal for analyses of protein expression regulation. Future studies that combine these promoter based approaches with the expanding ability to engineer the zebrafish genome via techniques such as CRISPR/Cas9 will allow the manipulation of protein expression to test hypotheses about lens crystallin function and its relation to lens biology and disease.

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669

Figure 1

Confocal imagery showing representative sites of GFP expression produced by mouse and zebrafish α -crystallin promoters

Examples of lens expression produced with a zebrafish αA promoter (A and B). Various sites of extraocular expression shown as single z-planes (on left) and as 3-dimensional renders (on right) for skeletal muscle produced with a mouse αB promoter (C and D); for notochord produced with a zebrafish αBb promoter (E and F); dorsal to the yolk produced with a zebrafish aA promoter (G and H).

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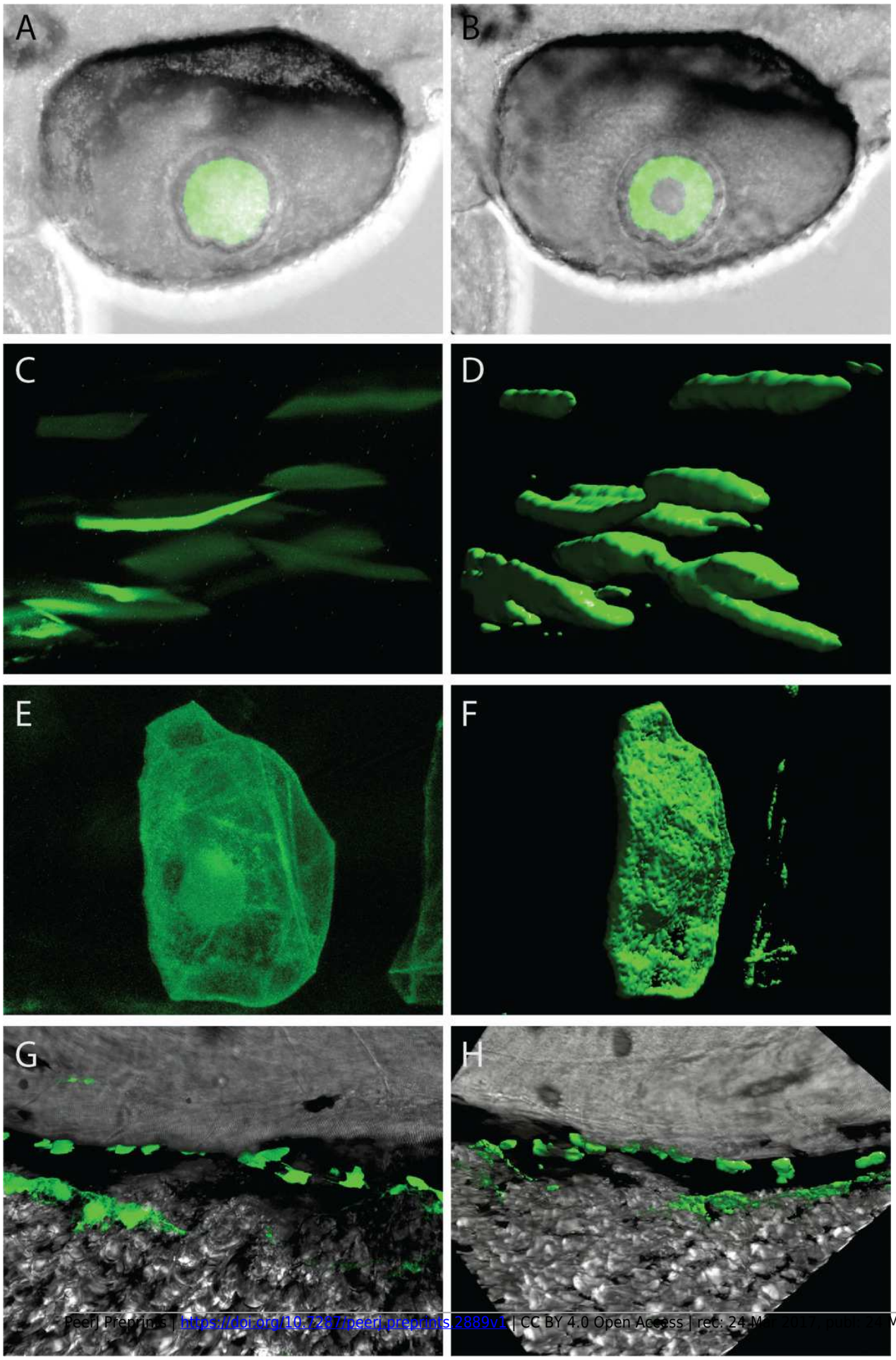
A
B
C
D
E
F
G
H

Figure 2

Comparison of mouse and zebrafish αA-crystallin chromosomal arrangement and their ability to drive GFP expression in zebrafish embryos

The structural and functional conservation of mammalian and zebrafish αA-crystallin is mirrored in their shared syntenic relationship with *hsf2bp* (A). The promoter regions for each gene produced similar temporal and spatial expression patterns (B-E), with expression almost exclusively restricted to the lens. The extent of lens expression varied for both orthologous promoters (compare A to B for mouse and C to D for zebrafish). Reverse-transcriptase PCR showed that zebrafish embryos expressed αA-crystallin mRNA as early as 1 dpf (F), although its promoter did not drive GFP expression until between 30 and 48 hpf (Table 3). A separate analysis showed zebrafish mRNA expression in both eyes (eye) and extraocular tissues (tr) at days 4 and 6 (d4 and d6), but with higher expression in eye (G).

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

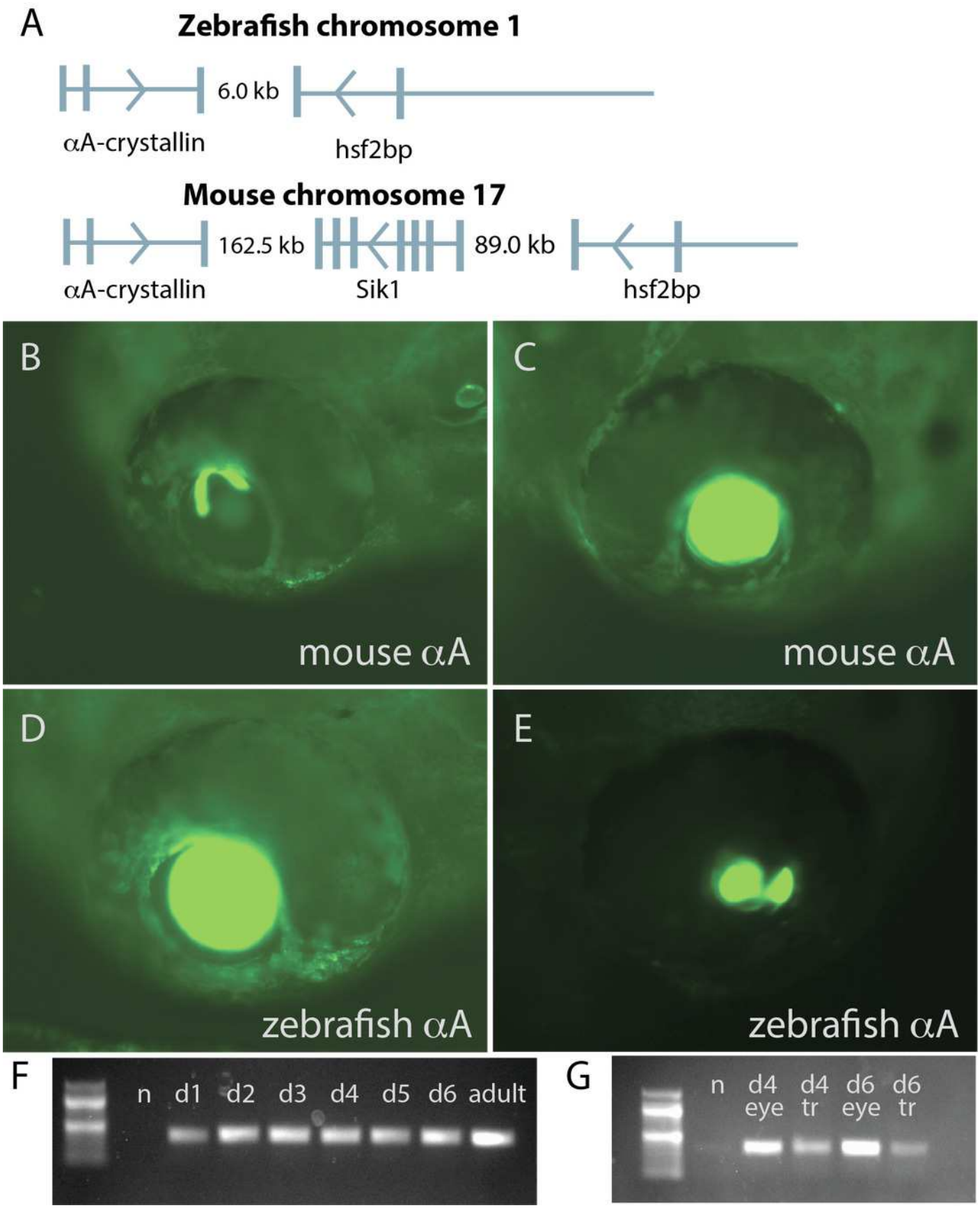

Figure 3

Mouse αB-crystallin promoter fragments produced native expression in zebrafish embryos

Enhancer elements of a promoter upstream of mouse αB-crystallin were previously shown to regulate expression in skeletal muscle (sm), heart and lens (lsr1 and 2) (A; adapted from (Swamynathan & Piatigorsky, 2002)). Fragments containing 0.8 and 1.4 kb lengths of this promoter produced GFP expression in zebrafish embryo notochord (B-C), skeletal muscle (D-E), lens (F) and heart (G; arrows). The 250 basepair length, containing only the mouse lens enhancer, produced no GFP expression in zebrafish embryos (E, embryo at top).

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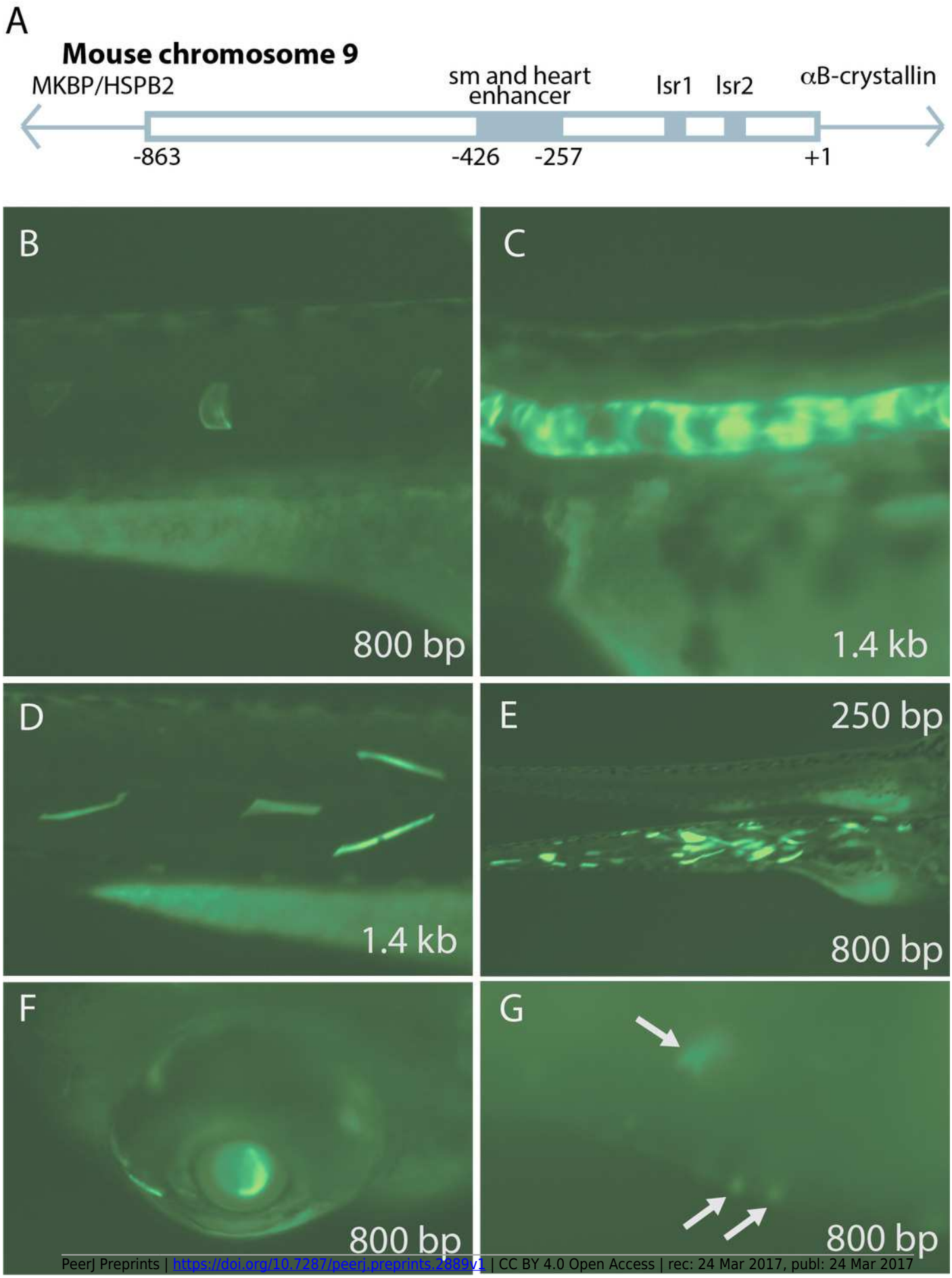

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Figure 4

The paralogous zebrafish αBa- and αBb-crystallin promoters produced similar, but distinct, GFP expression profiles

Zebrafish αBb-crystallin has the same syntenic relationship with *Hspb2* as mouse αB-crystallin, although the intergenic region between the two genes is much larger in the zebrafish (A). The zebrafish αBa-crystallin paralog has moved to a separate chromosome. Both zebrafish paralogs produced GFP expression most often in notochord (B) and skeletal muscle (C). The αBa paralog drove expression in these tissues equally while αBb was more active in skeletal muscle (D). Expression in lens (E) and extralenticular regions of the eye was more rare. Images shown are representative with the details of GFP expression not differing noticeably between paralogs or the promoter length used.

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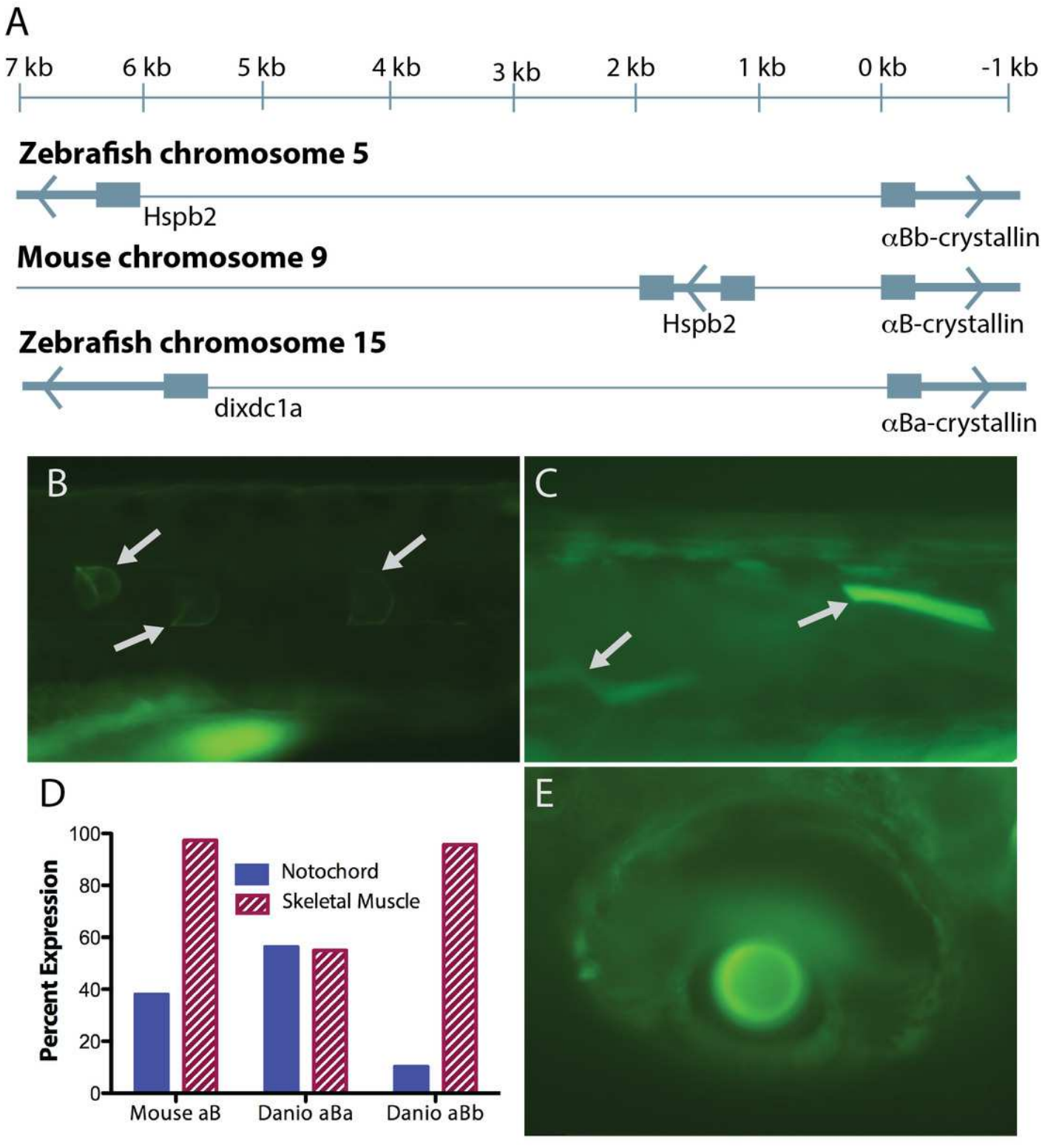

Figure 5

RT-PCR analysis of zebrafish αB-crystallin paralog expression in whole embryos compared to adult

Analysis of three separate biological replicates showed similarly weak expression of αBa-crystallin at days 1 through 6 post fertilization (d1 – d6) with a peak at day 1 in two samples (A) and day 3 in a third (B). Three biological replicates showed similar temporal expression for αBb-crystallin, with relatively constant levels from days 1 through 6 and an increase in the adult (C). Amplification of tubulin was used to confirm equal amounts of mRNA in each reaction (D).

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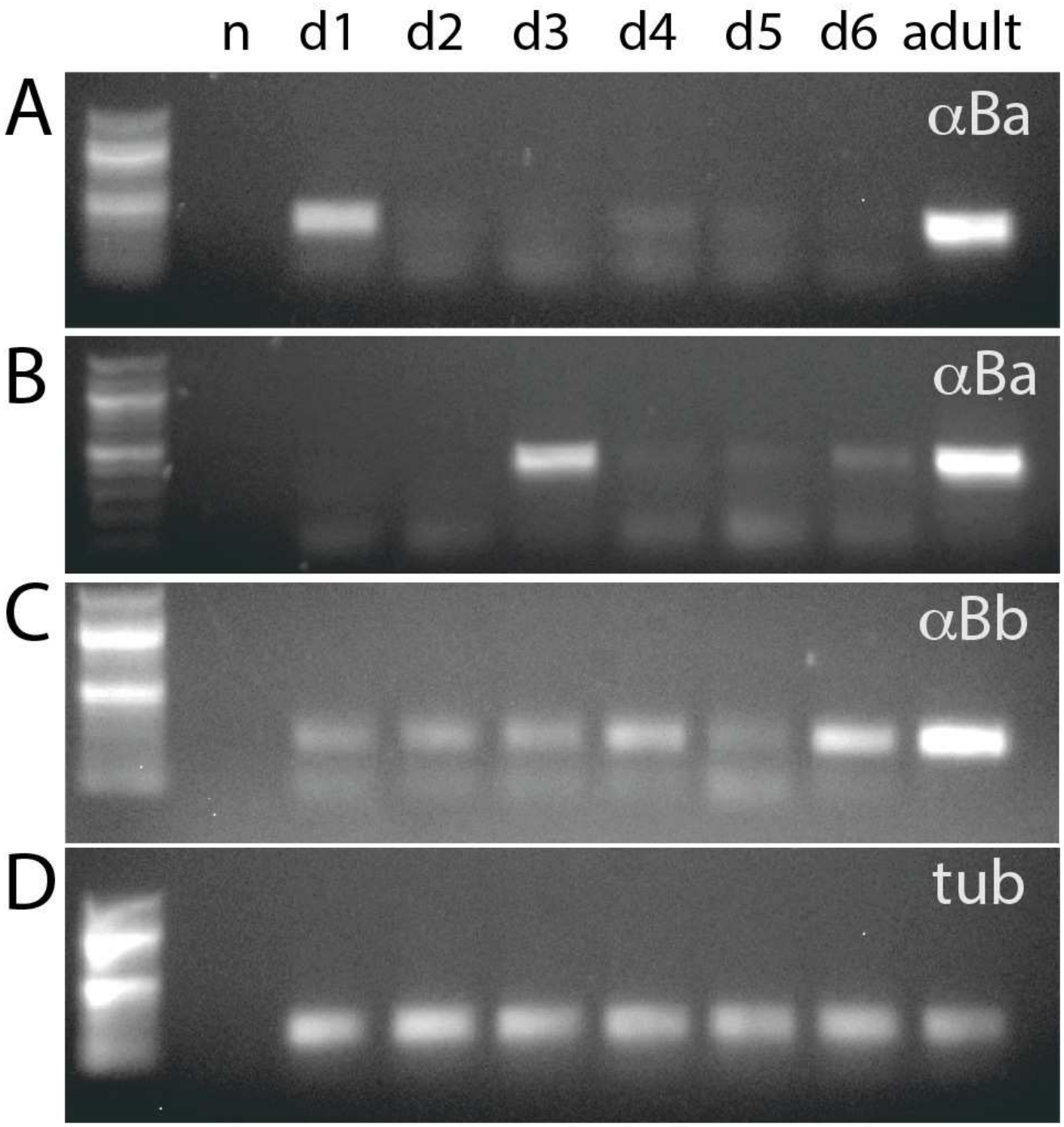

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Figure 6(on next page)

Relative abundance of αA- and αBa-crystallin proteins in zebrafish embryos during development measured by mass spectrometric parallel reaction monitoring of tryptic peptides

(A) Changes in αA-crystallin relative abundance in whole embryos from 1-6 days post fertilization (dpf) by measurement of peak areas for the top 3 fragment ions of peptide 52-65 (NILDSSNSGVSEVR). Orange = y12, blue = y11, and green = y10 fragment ions. The bar labeled library shows the relative proportion of these fragment ions for this peptide identified in a digest from an adult zebrafish lens, while the dotp value above each bar is a measurement of how well the observed fragment ions for this peptide in each embryo digest matched those for this peptide in a spectral library created from an adult lens digest. Note that the relative peak area for the library peptide was arbitrary set to the same value as the largest peak area for ease of comparison. (B) Relative abundance of αA-crystallin in dissected eyes and remaining trunks of either 4 or 7 dpf embryos. The same αA peptide and fragment ions as measured above in A were used. (C) Measurement of αBa-crystallin in whole embryos from 1-6 dpf by measurement of peak areas for the top 3 fragment ions of peptide 79-88 (HFSPDELTVK). Orange = b2, blue = y9, and green = y8 fragment ions. (D) Relative abundance of αBa-crystallin in dissected eyes and remaining trunks of either 4 or 7 dpf embryos. The same αBa peptide and fragment ions as measured in C were used. Extracted ions chromatograms for the fragment ions of these peptides are shown in Supplementary Figure 2.

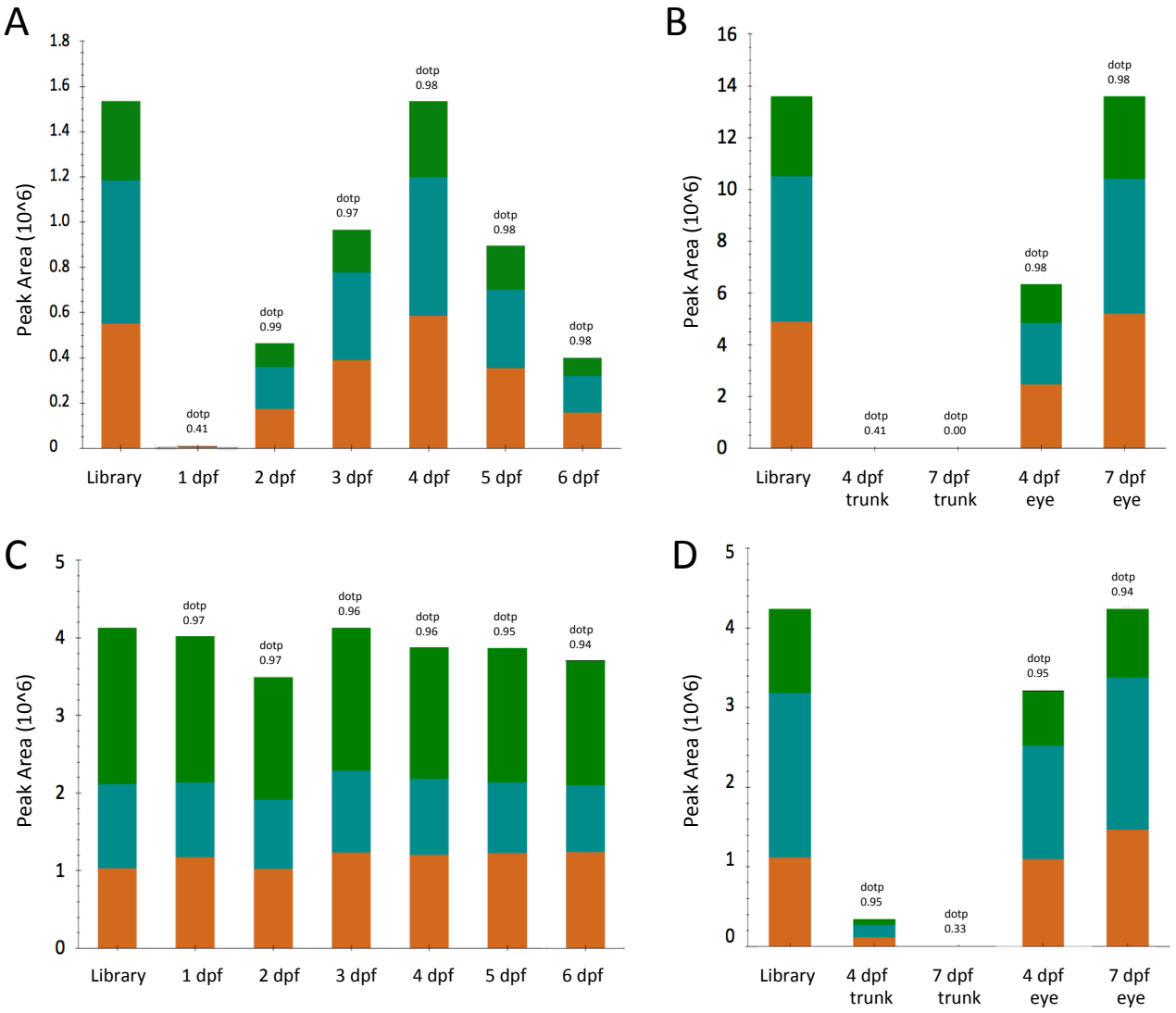

Table 1(on next page)

Primers used for semi-quantitative RT-PCR (A) and qPCR analysis (B) of zebrafish gene expression.

All primers for RT-PCR were used at an annealing temperature of 55°C and qPCR primers were used at an annealing and extension temperature of 56°C.

Table 1. Primers used for semi-quantitative RT-PCR (A) and qPCR analysis (B) of zebrafish gene expression. All primers for RT-PCR were used at an annealing temperature of 55°C and qPCR primers were used at an annealing and extension temperature of 56°C.

A

Gene	Primer Sequence	Product Size (bp)	Accession #
αA-crystallin	F: 5'-AGGAGTTACCAGGTCTGACA-3' R: 5'-ACGGGAGATGTAGCCATGAT-3'	361	NM_152950
αBa-crystallin	F: 5'-GCCGTCAGAGGACAGAGAAGA-3' R: 5'-GTATCGCCGACCCTTGTT-3'	388	NM_131157
αBb-crystallin	F: 5'-GCCGACGTGATCTCCTCATT-3' R: 5'-CCAACAGGGACACGGTATTT-3'	260	NM_001002670
Tubulin	F: 5'-CTGTTGACTACGGAAAGAAGT-3' R: 5'-TATGTGGACGCTCTATGTCTA-3'	198	NM_200185

B

Gene	Primer Sequence	Product Size (bp)	Accession #
αA-crystallin	F: 5'-ATGGCCTGCTCACTCTTTGT-3' R: 5'-CCCACTCACACCTCCATACC-3'	159	NM_152950
αBa-crystallin	F: 5'-CCCAGGCTTCTTCCCTTATC-3' R: 5'-GTGCTTCACATCCAGGTTGA-3'	196	NM_131157
EF-1α	F: 5'-CAGCTGATCGTTGGAGTCAA-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	94	NM_131263

Table 2(on next page)

Location of Promoter Activity

Total embryos shows the number of separate embryos examined after injection with each indicated promoter fragment**.** *Percentage of embryos* shows the proportion of GFP-expressing embryos with observable GFP in each tissue. A “O” indicates no embryos expressed GFP in that tissue.

Table 2. Location of Promoter Activity. *Total embryos* shows the number of separate embryos examined after injection with each indicated promoter fragment**.** *Percentage of embryos* shows the proportion of GFP-expressing embryos with observable GFP in each tissue. A "O" indicates no embryos expressed GFP in that tissue.

Zebrafish Promoters	*Total Embryos*	*Percentage of embryos*				
		Lens	*Eye*	*NC*	*SM*	*Heart*
1kb αA	62	97	O	5	11	O
3kb αBa	90	3	7	56	55	O
1kb αBb	67	O	5	5	90	O
2kb αBb	59	O	6	3	92	3
4kb αBb	51	O	O	20	100	O
5kb αBb	90	4	4	7	97	O

Mouse Promoters	*Total Embryos*	*Percentage of embryos*				
		Lens	*Eye*	*NC*	*SM*	*Heart*
αA	55	70	O	4	30	O
0.25kb αB	35	O	O	O	O	O
0.8 kb αB	50	13	15	33	94	6
1.4 kb αB	67	15	5	42	100	12

Table 3(on next page)

Timeline of Promoter Activity

Numbers indicate percentage of injected embryos expressing GFP in any tissue at indicated timepoints. Lack of expression is noted with an “O” and “--” indicates that no embryos were observed at that timepoint.

Table 3. Timeline of Promoter Activity. Numbers indicate percentage of injected embryos expressing GFP in any tissue at indicated timepoints. Lack of expression is noted with an "0" and "--" indicates that no embryos were observed at that timepoint.

	Hours post fertilization (hpf)					
	24	*30*	*48*	*54*	*72*	*78*
Zebrafish promoters						
1kb αA	0	0	61	83	84	--
3kb αBa	0	0	90	40	52	61
1kb αBb	0	0	63	56	62	26
2kb αBb	0	0	67	--	47	47
4kb αBb	0	0	58	87	90	--
5kb αBb	0	0	100	--	83	50
Mouse promoters						
αA	0	17	27	39	32	--
0.25kb αB	0	0	0	0	0	0
0.8 kb αB	0	19	100	100	91	--
1.4 kb αB	0	33	85	80	72	--