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Lengsin expression and function during zebrafish lens formation

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Abstract

A zebrafish ortholog of human lengsin was identified by EST analysis of an adult lens cDNA library. During zebrafish development, *lengsin* transcription is first detected at 24 hours post-fertilization (hpf). Immunolocalization, using polyclonal antiserum generated against a Lengsin bacterial fusion protein, detects lens-specific protein in whole-mount embryos at 30 hpf. Lengsin expression in zebrafish follows the temporal expression of the α A- α B1- and β B1-crystallin proteins in the lens. At 72 hpf, Lengsin is localized to a subpopulation of differentiating secondary fiber cells, while no expression is detected in the lens epithelial cells or central lens fibers. In the adult lens, Lengsin is restricted to a narrow band of cortical fibers and co-localizes with actin at the lateral faces of these interdigitating cells. Stable transgenic lines, using a 3 kb *lengsin* genomic fragment to regulate EGFP expression, recapitulate the Lengsin temporal and spatial expression patterns. Lengsin function in zebrafish lens formation was examined by antisense morpholino-mediated translation and mRNA splice inhibition. At 72 hpf, the *lengsin* morphant lenses are reduced in size and exhibit separations within the cortex due to defects in secondary fiber morphogenesis. The location of the morphant lens defects correlates with the Lengsin protein localization at this age. These results demonstrate Lengsin is required for proper fiber cell differentiation by playing roles in either cell elongation or the establishment of cell interactions.

Keywords

ocular lens; secondary fiber cells; lens development; zebrafish; morpholino

1. Introduction

During zebrafish eye development, lens placode cells delaminate forming a solid cell mass, which is equivalent to the mammalian lens vesicle and visible at 21–24 hours post-fertilization [hpf; (Li et al., 2000; Dahm et al., 2007)]. The nascent lens detaches from the surface ectoderm and differentiates into either epithelial or primary fiber cells (Soules and Link, 2005; Dahm et al., 2007). At 30 hpf, lens epithelial cells are visible and primary fiber cell elongation has begun (Soules and Link, 2005). By 36 hpf, secondary fiber differentiation is morphologically evident in the proximally located lens transition zone (Soules and Link, 2005; Dahm et al., 2007). Similar to the lens of other species (reviewed in Bassnett, 2002), zebrafish fiber cell differentiation is characterized by marked cell elongation and organelle degradation necessary to maintain the transparent lens organelle free zone (Dahm et al., 2007). During this maturation

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process, the secondary fibers establish contacts with the apical membranes of the overlying epithelial cells and lateral membranes of the previously formed, and more centrally located, fiber cells (reviewed in Kuszak, 1995).

The morphological changes characterizing maturation of the differentiating fiber cells depend on the coordinated regulation of genes encoding a cohort of lens structural proteins. High levels of MIP (major intrinsic protein) and Mp19 in lens fiber cell membranes have been implicated in adhesion and reducing the interfiber space (Gruijters, 1989; Reizer et al., 1993; Michea et al., 1994; Taylor et al., 1995; Gonen et al., 2001; Grey et al., 2003; Fan et al., 2005). In addition, intermediate filament proteins such as Vimentin and the beaded filament proteins CP49 and Filensin are expressed in different temporal patterns during mammalian lens development (Orri et al., 1993; Remington, 1993; Sandilands et al., 1995; Hess et al., 1998; Blankenship et al., 2001).

Comparison of zebrafish lens EST analysis to human lens gene expression identified an abundant transcript in adult zebrafish corresponding to *lengsin* (Wistow et al., 2002; Vihtelic et al., 2005a). Lengsin belongs to the Glutamine Synthetase enzyme superfamily, although no enzyme activity was detected in recombinant human or mouse proteins (Grassi et al., 2006; Wyatt et al., 2006). In mouse, *Lengsin* (*Glul1*) is restricted to a subpopulation of differentiating secondary fibers and co-localizes with intermediate filament proteins at the plasma membrane (Wyatt et al., 2006; Wyatt et al., 2008).

To examine Lengsin function in the vertebrate lens and characterize its role during zebrafish lens formation, antisense morpholinos were used to knockdown Lengsin protein in zebrafish embryos. A polyclonal antiserum was generated to immunolocalize Lengsin during development and in the adult. Lengsin is detected in differentiating fiber cells and is concentrated at the lateral margins of a subpopulation of cortical fibers. Knockdown of Lengsin resulted in lens tissue separations within the cortex near the equator and secondary fiber cell dysmorphogenesis. These results demonstrate Lengsin functions in fiber cell differentiation and may be required for elongation or the establishment of cell-cell contacts.

2. Methods

2.1 Animals

Zebrafish (*Danio rerio*) were raised and maintained in the University of Notre Dame zebrafish facility using standard husbandry techniques (Westerfield, 1993). Lighting was maintained on a 14/10 hour (hr) light/dark cycle. Embryos were collected from either pair- or mass-matings and staged according to hrs post-fertilization (hpf) at 28.5°C and by morphological criteria (Kimmel et al., 1995). All experiments were conducted in accordance with the protocols approved by the university animal care and use committee and the guidelines in the ARVO statement on the use of animals for research.

2.2 RT-PCR

The zebrafish *lengsin* (zgc: 136604; *lgs*) cDNA sequence (GenBank accession no. **NM_001031674**) was used to design oligonucleotide primer pairs annealing to sequences within the different *lengsin* exons (Table 1). To examine potential *lengsin* alternative transcripts, cDNA was synthesized from 7 days post-fertilization (dpf) larval eye and adult lens total RNA (First Strand cDNA Synthesis, Invitrogen, Carlsbad, CA). For the developmental time course and adult tissue analysis of *lengsin* gene expression, amplifications using primers F19 and B21 (591 bp product; Table 1) were performed by one-step RT-PCR (Invitrogen) using RNA extracted from whole embryos at 8, 12, 18, 24, 30, 36 and 48 hpf and adult brain, caudal fin and a mixture of internal organs. In addition, adult eyes were dissected

into the following tissue groups for *lengsin* gene expression analysis: lens, anterior segments lacking lens, retinas, and posterior segments lacking retinas (Vihtelic et al., 2005a). The PCR consisted of 38 (embryonic tissues) or 35 (adult tissues) cycles at 94°C for 30 sec, 58°C for 30 sec, 68°C for 1 min and a final termination step at 68°C for 10 min. β -actin was amplified as a positive control (forward, 5'-TCAAACGAACGACCAACC-3'; reverse, 5'-AGACACCCTGGCTTACAT-3'). Negative control reactions lacking reverse transcriptase or template were also performed. The PCR products were visualized by agarose gel electrophoresis, cloned using the pCR 4-TOPO vector (Invitrogen) and DNA sequenced (Sequetech, Mountain View, CA) to confirm their identities.

2.3 Polyclonal antisera

Several different rabbit polyclonal antisera were generated for this study including antiserum to detect Lengsin and α B1-, α B2- and β B1- crystallin. For the anti-Lengsin serum, an *E. coli* fusion protein encoding the N-terminal 89 amino acids of zebrafish Lengsin was used as the immunogen (Vihtelic et al., 1999). The sequence corresponding to nucleotides 154-421 (GenBank accession no. **DQ122929**) was PCR amplified from adult lens cDNA using primers containing *Bam*HI and *Xho*I restriction enzyme sites (forward primer, 5'-GGATTCATGCAAGAATCAGAGGAATCCAAAG-3'; reverse primer, 5'-CTCGAGAGAAACCCTGGTACTGGGACTCAC-3'). The *lengsin* cDNA product was cloned using the TOPO vector (Invitrogen) and subcloned into the pET32a expression vector (Novagen, San Diego, CA) using the *Bam*HI and *Xho*I restriction sites. The expression construct was sequenced to confirm the sequence identity and maintenance of the open reading frame. The fusion protein was induced in BL21(DE3) cells (Novagen), purified by metal affinity chromatography (His-Bind Resin; Novagen) and used to immunize rabbits (PickCell Laboratories, Amsterdam, Netherlands). The rabbit antiserum was purified by affinity chromatography using a column prepared with the purified Lengsin fusion protein (AminoLink Plus Immobilization, Pierce, Rockford, IL). The Lengsin antiserum specificity was confirmed by immunoblots of whole embryo, adult lens, and *lengsin* hypomorphic embryo whole-mount immunohistochemistry (Supplemental Figures 1 and 3, respectively; also see *lengsin* morphant analysis described below). Immunoblots were prepared as previously described (Shi et al., 2006).

The antisera to immunolocalize the zebrafish α B1-, α B2- and β B1-crystallins were generated by immunizing rabbits with KLH-conjugated peptides (Proteintech Group, Chicago, IL). The peptide sequences (and their corresponding proteins) included PSWWDSGMSEMRQDRDRFV (α B1-crystallin, **AAD49096**), LTVTGPLKLSDGPER (α B2-crystallin, **NP_001002670**) and MSQTAKSATNQGTDAKEKG (β B1-crystallin, **NP_775338**). The α B1- and β B1-crystallin antisera were purified by affinity chromatography using full-length recombinant proteins expressed and purified from BL21(DE3) bacteria, while the α B2-crystallin antiserum was purified by Protein A chromatography (Pierce). Specificities of the antisera were verified by immunoblot analysis of adult lens extracts (Supplemental Figure 2). Although high background signal precluded the use of the anti- α B2-crystallin serum for whole-mount immunolocalization studies, the signal-to-noise ratio of this polyclonal antiserum was suitable for immunodetection of the protein in frozen tissue sections (Supplemental Figure 2).

2.4 Immunohistochemistry

For whole-mount immunolabeling of zebrafish embryos, tissues were fixed in ethanolic formaldehyde for 3 hrs at room temperature (Vihtelic et al., 2001; Vihtelic et al., 2005b). The embryos were rehydrated through an ethanol series, washed in PBS (pH 7.4) and water, placed into methanol for 5 min at -20°C, rinsed in water and PBS, and blocked in PBS/5% normal goat serum/1% DMSO/1% Triton X-100/1% Tween-20 for 1 hr at room temperature. The

embryos were incubated in primary antibodies diluted in blocking buffer overnight at room temperature (or 72 hrs at 4°C), washed in blocking buffer (6 × 1 hr) and incubated with fluorophore-conjugated secondary antibodies overnight at room temperature. The immunolabeled embryos were infiltrated with 50% glycerol/PBS prior to image collections.

For frozen section immunolocalizations, embryos and larvae were fixed in ethanolic formaldehyde for 3 hrs at room temperature (Vihtelic et al., 2001; Vihtelic et al., 2005b). The fish were rehydrated, washed in 5% sucrose/PBS and individuals were embedded in 1.5% agarose/5% sucrose/PBS before cryoprotection in 30% sucrose/PBS overnight at 4 °C. The agarose-embedded larvae were infiltrated in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and 30% sucrose/PBS (1:1 mix) for 4 hr at room temperature, followed by incubation in 100% tissue freezing medium overnight at 4°C. The embryonic and larval frontal plane cryosections (16 µm), were dried on SuperFrost Plus slides (Fisher) at 42°C and stored at -20°C. The central lens sections from fish sectioned in this plane provide longitudinal profiles of the lens fiber cells. In addition to the embryonic and larval lens, dissected whole adult lenses were fixed in ethanolic formaldehyde overnight at 4°C, rehydrated and cryoprotected in 30% sucrose/PBS overnight at 4°C. The adult lenses were frozen embedded in tissue freezing medium and sections (20 µm) collected on SuperFrost Plus slides. The sectioned adult lenses provide fiber cell cross sectional profiles.

For all tissues, sections were rehydrated in PBS and blocked in PBS/2% normal goat serum/1% DMSO/0.3% Triton X-100 for 60 min at room temperature. The sections were incubated with primary antibodies in blocking buffer overnight at room temperature, washed in PBS/0.05% Tween-20 and incubated with fluorophore-conjugated secondary antibodies for 1 hr at room temperature.

The primary antibodies included rabbit polyclonal anti-Lengsin (1:500), anti-αA-crystallin (1:500; Shi et al. 2006), anti-αB1-crystallin (1:500), anti-αB2-crystallin (1:500), anti-βB1-crystallin (1:500) and the mouse monoclonal zl-1 (1:1,500; University of Oregon Monoclonal Antibody Facility). The secondary antibodies included goat anti-rabbit or anti-mouse whole IgG AF488 or AF594 conjugates (2 mg/mL, 1:500; Molecular Probes, Eugene, OR). AF594- or AF488-conjugated phalloidin (6.6 µM, 1:50; Molecular Probes) was used to stain filamentous Actin in larval and adult lens. Nuclei were stained using TO-PRO-3 (1 mM, 1:750; Molecular Probes). Coverslips were mounted using ProLong Gold (Invitrogen) and images were recorded using BioRad MRC1024 or Leica TCS SP2 laser scanning confocal microscopes.

2.5 Tg(lengsin:EGFP)nt transgenic lines

The 3,030 bp immediately upstream of the *lengsin* translational start codon were PCR-amplified from AB genomic DNA (Platinum Taq DNA Polymerase High Fidelity; Invitrogen). The forward (5'-CTCGAGAAAACAAAAACCATCACAACAAGC-3') and reverse (5'-GGATCCCCATCTGGCCTAATCAAAGTG-3') primers included *Bam*HI and *Xho*I restriction sites, respectively. The PCR products were A-tailed, cloned into the pCR2.1-TOPO vector (Invitrogen) and DNA sequenced. The *lengsin* promoter sequences were subcloned into pT2KXIG (Kawakami et al., 1998), upstream of the EGFP gene (*lgs:EGFP*) within the defective Tol2 element, using the *Xho*I and *Bam*HI restriction sites. In addition to the *lgs:EGFP* construct, a construct was created that utilized a nuclear-localized EGFP, (*lgs:EGFP-NLS*; **AF242365**; generously provided by Dr. R. Schulz).

Plasmid DNA and Tol2 transposase for injection into embryos were prepared as previously described (Thummel et al., 2005; Kassen et al., 2007). The pT2KXIG expression construct and *in vitro* transcribed Tol2 mRNA were combined at a concentration of 25 ng/µl each and injected into 1–4 cell-stage embryos. Founders and independent transgenic lines were identified by out-

crossing F₀ and F₁ adults, respectively, and screening progeny for EGFP expression in the lens. Four independent lines were established for each construct. Expression of EGFP in the differentiating lens fiber cells of transgenic animals was confirmed by epifluorescent illumination of live embryos, whole-mounted embryos or frozen eye sections.

2.6 Morpholino designs and injections

The *lengsin* morpholino oligomers were designed and synthesized by Gene Tools (Corvallis, OR). The translation inhibiting morpholino (morfA, 5'-GGATTCCTCTGATTCTTGCA7GATG-3') anneals to sequence spanning the translational start site, while the splice inhibiting morpholino (morfB, 5'-AGAGATTTTAAGATCTACCGTATCT-3') complements sequence at the exon 1-intron 1 junction of the *lengsin* transcript. A control morpholino (mmC) with 5 mismatches relative to the MorfA sequence was also used: 5'-GGATTgCTCTcATTgTTcCATcATG-3'. The morpholinos were resuspended in nuclease-free water (0.5 mM) and approximately 5 nL (~7 ng) was microinjected into 1–4 cell-stage embryos as previously described (Wei et al., 2004; Shi et al., 2005; Shi et al., 2006). The control and morpholino-injected embryos were raised at 28.5°C and were screened at 24 hpf to remove unfertilized eggs and those embryos exhibiting developmental delays due to morpholino toxicity or non-specific effects. Thus, the *lengsin* morphants used for histological analysis appeared like wild type at the macroscopic level, although the eyes of some morphants were slightly reduced in size compared to the controls (Supplemental Figure 4).

2.7 Histology

The *lengsin* morphant and control embryos were fixed in 2% formaldehyde/2.5% glutaraldehyde/0.1 M cacodylate (pH 7.4) overnight at 4°C, washed in 0.1 M cacodylate, processed, sectioned and stained (0.5% methylene blue/0.5% azure II) as previously described (Vihtelic et al., 2001; Shi et al., 2005).

2.8 Transmission electron microscopy

The *lengsin* morphant and control embryos for transmission electron microscopy (TEM) were processed as previously described (Vihtelic et al., 2001). Prior to TEM, sections were cut and stained to determine the location and histological appearance of the morphant lens lesions. The stained sections were examined using a Hitachi H600 transmission electron microscope (Hitachi High Technologies, Pleasanton, CA).

2.9 Statistical analysis

The *lengsin* morphant lens phenotype, which is characterized by large gaps and separations within the tissue, was quantified by collecting images of eye histological sections and measuring the areas (μm^2) of individual lens tissue separations in controls and morphants using the Spot RT-KE digital camera and accompanying software (Diagnostic Instruments, Sterling Heights, MI). The average areas of the individual separations and the mean total area of separation per lens section were determined for four different groups of fish that included uninjected and mismatch morpholino-injected controls (uiC and mmC, respectively) and morfA- and morfB-injected morphants at 72 hpf. The lenses of 8 uiC, 3 mmC, 6 morfA-injected, and 3 morfB-injected fish were examined in each group. Five optic nerve-containing or immediately adjacent sections were examined for each lens in the different groups. Subsequent to ANOVA, the Fisher's Least Significant Difference test (SPSS 11, Chicago, IL) was used to determine statistical significance in differences between the groups ($p < 0.05$).

3. Results

3.1 Lengsin gene and protein structure

The zebrafish *lengsin* gene consists of 4 exons that span at least 3,423 bp of genomic DNA on chromosome 11 (www.ncbi.nlm.nih.gov/sites/entrez). No *lengsin* duplications were identified in the genome based on database analysis (Ensemble Zv7). In comparison, the mammalian gene for Lengsin consists of 5 exons, although exon 3 is omitted during human transcription (Wyatt et al., 2006). Protein sequence alignments demonstrated 38% and 39% overall amino acid identity between zebrafish Lengsin and the human or mouse Lengsin proteins, respectively (sequences not shown). Like the mammalian and chick protein, zebrafish Lengsin consists of an amino-terminal domain, a Glutamine Synthetase (GS) homology region, and a short carboxyl-terminal domain (Figure 1A). The amino-terminal region accounts for the increased number of amino acids in the zebrafish protein compared to the human and mouse sequences (Figure 1A) and results in the low overall identity between the orthologs.

3.2 Zebrafish lengsin gene expression

The temporal and spatial expression of zebrafish *lengsin* were examined by RT-PCR (Figure 1B). During development, zygotic *lengsin* transcription was first detected at 24 hpf and persisted through the 48 hpf time point (Figure 1B, upper panel). RT-PCR was also used to determine *lengsin* expression in different adult tissues (Figure 1B, lower panel). While *lengsin* transcripts were detected in the adult lens, the other eye tissues, including the retina, cornea and iris, lacked detectable expression. In addition, *lengsin* transcripts were not detected in the brain, caudal fin or internal organs of adult zebrafish (Figure 1B). In contrast to human lengsin, alternative splice products were not identified in zebrafish by RT-PCR of either adult lens or 7 dpf larval eyes (data not shown).

3.3 Temporal and spatial expression of Lengsin protein

To determine Lengsin protein expression patterns, a rabbit polyclonal antiserum was generated to a zebrafish Lengsin bacterial fusion protein encoding the N-terminal amino acids (amino acids 1-89). Immunoblot of whole embryo extracts showed a single 73 kDa protein band corresponding to the full-length protein, while a smaller 38 kDa protein was detected in the adult lens extract (Supplemental Figure 1) suggesting cleavage of the mature protein (Wyatt et al., 2008).

Double label immunohistochemistry of zebrafish embryo whole-mounts was carried out to determine expression of Lengsin in relation to zl-1, a lens-specific antigen (Vihtelic et al., 2001; Vihtelic et al., 2005b). At 24 hpf, zl-1 was detected within the lens, while Lengsin was not detectable at this time (Figure 2A). At 30 hpf, Lengsin expression initiated within central lens fiber cells, while zl-1 was largely restricted to the lens periphery (Figure 2B, arrow and arrowheads, respectively). The majority of lens fiber cells expressed Lengsin, although the most central fiber cells may lack the Lengsin protein at 36 hpf (Figure 2C, arrow and asterisk, respectively). At this time, zl-1 was localized at the lens periphery in a pattern suggestive of the epithelial-fiber cell interface (Figure 2C, arrowheads). Thus, Lengsin was likely expressed in differentiating fiber cells, while zl-1 expression was restricted to the lens periphery at 36 hpf. Between 42–72 hpf, Lengsin became progressively restricted to a subpopulation of cortical fibers, but remained central to the more peripheral zl-1 signal (Figure 2, Panels D–F). Lengsin localization in the central lens at 30 hpf is consistent with primary fiber cell expression.

Lengsin temporal and spatial expression patterns during lens formation were compared to the expression patterns of other lens proteins (Figure 3; Table 2). To begin characterizing the temporal order of zebrafish lens protein expression during development, batches of embryos were whole-mount immunolabeled at 24 or 48 hpf using polyclonal antisera to detect α A-,

α B1-, or β B1-crystallin or Lengsin. At each stage, expression patterns of the three crystallin proteins were compared to Lengsin expression. At 24 hpf, 100% of the embryos (n=19) expressed α B1-crystallin, while 82% (n=17) were positive for β B1-crystallin (Figure 3, Panels B and C, arrows). In comparison, only 19% of the embryos (n=21) possessed detectable α A-crystallin (Figure 3A, arrow) and just a single embryo (n = 20) displayed a Lengsin-positive lens with barely detectable signal (not shown). Similar to β B1-crystallin, 84% of embryos displayed α l-1-positive lenses at 24 hpf (data not shown). Thus, nearly all 24 hpf embryos expressed both α B1- and β B1-crystallin (and the α l-1 antigen), while Lengsin expression was only just beginning at this time. At 48 hpf, 100% of the embryos (n = 15 in all cases) displayed high levels of α A-, α B1- and β B1-crystallin and Lengsin (Figure 3, Panels E–H). Thus, α B1- and β B1-crystallin expression precedes the expression of both α A-crystallin and Lengsin during zebrafish lens formation. Furthermore, α A-crystallin expression likely initiates prior to Lengsin. At 24 and 48 hpf, α A- and β B1-crystallin were restricted to the lens, while α B1-crystallin was also expressed in the retina and other parts of the CNS (Figure 3, compare Panels A, C, E and G to Panels B and F).

Frozen section immunohistochemistry was performed to localize the Lengsin protein in the embryonic and adult zebrafish lens (Figures 4 and 5). At 48 hpf, Lengsin was distributed throughout the lens and became restricted to the lens periphery at 72 hpf (Figure 4, Panels A–C). Based on double label immunolocalization at 48 hpf, Lengsin predominated in the proximal lens fiber mass (Figure 4B, asterisks), while the α l-1 signal was largely concentrated peripherally (Figure 4B, arrow). The rapid and dramatic change in lens morphology that occurs between 48 and 72 hpf resulted in the concentration of Lengsin-positive fibers more distally at 72 hpf (Figure 4, compare Panel C to B). At this time, Lengsin localized along the entire length of most distal fibers, but was restricted to relatively few fibers in the lens transition zone (Figure 4C, arrows and arrowheads, respectively). In addition, Lengsin appeared most concentrated near the lens equator in a zone that may represent an interface between fiber cell ends (Figure 4C, asterisks).

The Lengsin expression pattern at 72 hpf was further defined by staining nuclei in the Lengsin immunolabeled frozen sections (Figure 4, Panels D–F). The lens transition zone was evident proximally where fiber cells began to elongate and lose their nuclei as they differentiate (Figure 4D). Lengsin was identified in one or two nucleated cells at the very base of the lens transition zone, but was not detected in epithelial cells just distal to this zone (Figure 4D, arrow and arrowhead, respectively). In addition, while the most peripheral differentiating nucleated lens fibers were Lengsin-positive, more centrally located (but still nucleated) fiber cells no longer expressed the Lengsin protein (Figure 4D, asterisks). Lengsin-positive fibers were also concentrated adjacent to the central epithelial cells (Figure 4E, arrow). Similar to the epithelial cells near the transition zone, the central epithelial cells also lacked detectable Lengsin (Figure 4E, arrowheads). In comparison, the zebrafish lens central epithelial cells expressed α B1-crystallin at 72 hpf (Figure 4F, arrows). Therefore, during lens growth, Lengsin expression initiates in the lens transition zone and is confined to a narrow layer of cortical fiber cells that extend distally to surround the lens circumference. The restricted fiber cell expression pattern characterizing the Lengsin domain suggests Lengsin expression initiates in the differentiating fiber cells, but the Lengsin protein persists for a relatively short time because the more central cortical fibers lack detectable Lengsin protein.

Lengsin was also immunolocalized in the adult zebrafish lens and subcellularly localized in the fiber cells (Figure 5). The adult lens cortical fibers run parallel to the lens surface and converge to single points at the lens poles (Dahm et al. 2007). Within each concentric layer of secondary fibers, the parallel cells interdigitate along their lengths and the cell layers are organized into fiber cell stacks (Dahm et al. 2007). Staining filamentous Actin with fluorophore-conjugated Phalloidin identified the longitudinal fiber cell interfaces and the

overlying epithelial cells (Figure 5A, arrows and arrowheads, respectively). Close inspection of the fiber cell Actin staining patterns revealed the wavy interdigitating lateral interfaces of adjacent cells (Figure 5B, arrows). In the immunolabeled adult lens, cross sectional profiles of the stacked cortical fiber cells revealed that Lengsin is restricted to a zone of peripheral cortical fibers (Figure 5C, arrows). The Lengsin-positive layer was separated from the epithelial cell layer by a zone of fiber cells lacking Lengsin (Figure 5C, asterisks). The linear expression pattern of Lengsin in these peripheral fiber cells suggests Lengsin is concentrated along their lateral edges near the fiber cell interfaces.

To determine the subcellular distribution of Lengsin in the secondary fiber cells, the immunolabeled adult lens sections were stained for Actin (Figure 5, Panels D–I), which defines the fiber cell membranes throughout the lens cortical thickness. In cross sectional profiles, Actin was concentrated at the lateral fiber cell interfaces, which possess the interdigitating processes (Figure 5D, arrows). Within the fiber cells, Lengsin co-localized with Actin at these lateral interfaces as shown by the single channel and merged images (Figure 5, Panel D, actin; Panel E, Lengsin; Panel F, merged image). Lengsin was also observed at the fiber cell lateral margins in longitudinal cell profiles of adult lens sections, although the concentration of Lengsin within these lateral domains was less distinct when observed from this perspective (data not shown). Lengsin expression in adult lens fiber cells differed from α B1-crystallin, which co-localized with Actin at the membrane surrounding the entire fiber cell, giving the α B1-crystallin expression pattern a honeycomb appearance (Figure 5, Panels G–I). These data demonstrate Lengsin is concentrated at the lateral interfaces of a subpopulation of cortical fiber cells in the adult lens.

3.4 The lengsin: EGFP transgenes identify differentiating fiber cells in the zebrafish lens

The temporal and spatial Lengsin expression patterns suggest this lens-specific protein plays a key role during a precise stage of fiber cell differentiation. To take advantage of the *lengsin* expression patterns to mark fiber cell differentiation in zebrafish, stable transgenic lines were created using a 3 kb *lengsin* genomic fragment to regulate fluorescent reporter genes. Two different constructs were used to generate independent lines. In addition to EGFP (*lgs:EGFP*), lines expressing EGFP with a nuclear localization signal were also created (*lgs:EGFP-NLS*). The lines were characterized by analysis of whole-mount embryos and frozen sections (Figure 6). In all lines, transgene expression was restricted to the lens (data not shown). Similar to Lengsin protein, no EGFP was evident at 24 hpf (Figure 6, Panels A and E). At 36 hpf, EGFP was easily detected throughout the central *lgs:EGFP* lens, while only a few EGFP-positive nuclei were observed in the *lgs:EGFP-NLS* lens (Figure 6, compare Panels B and F, arrows). At 48 hpf, the *lgs:EGFP* expression appeared to fill the lens, while multiple nuclei were easily observed in the *lgs:EGFP-NLS* lens (Figure 6, Panels C and G, respectively, arrows). At 72 hpf, the entire *lgs:EGFP* lens appeared fluorescent, while EGFP expression was concentrated in the elongating nuclei of secondary fibers in the *lgs:EGFP-NLS* lens (Figure 6, compare Panel D to H and I, arrows). Transgene expression was not detected in the epithelial cells or epithelial cell nuclei of either the *lgs:EGFP* or *lgs:EGFP-NLS* lines, respectively. Therefore, the reporter gene expression patterns largely recapitulate native *lengsin* gene expression.

3.5 Lengsin functional analysis by antisense gene knockdown

To examine Lengsin function for zebrafish lens formation, an antisense morpholino was injected into 1–4 cell-stage embryos to specifically inhibit Lengsin translation (morfA). In addition, a second *lengsin* morpholino, designed to inhibit *lengsin* mRNA splicing (morfB), was used in independent knockdown experiments. As a control for phenotypic specificity, a five base mismatched morpholino relative to morfA (mmC) was also injected independently. Whole-mount immunolabeling using the polyclonal anti-Lengsin serum verified the

elimination of detectable Lengsin protein in the *lengsin* morphants at 48 hpf (Supplemental Figure 3).

Histology was performed to examine the morphology of the *lengsin* morphant eye tissues (Figure 7). At 48 hpf, the morphant eyes appeared similar to wild type. At 72 hpf, the *lengsin* morphant retinas were reduced in size compared to the controls, although the retinal structural organization including the photoreceptor, inner nuclear and ganglion cell layers appeared equivalent to wild type (Figure 7, compare Panels C and D to A and B). In contrast, the 72 hpf *lengsin* morphants exhibited severe lens defects, which were not observed in the controls. At this age, the control lenses (Figure 7, Panels E and F) were characterized by a single layer of epithelial cells covering the distal and lateral aspects of the lens, while nuclei of differentiating secondary fiber cells were evident in the transition zone near the lens proximal pole (Figure 7, Panels E and F, arrowheads and arrows, respectively). In the wild-type lens, the elongating secondary fibers appeared to reach towards the lens equator so the fiber cell distal tips came to lie in close apposition to the overlying epithelium. In contrast, the *lengsin* morphant lenses exhibited large gaps and separations within the lens cortex, distal to the elongating secondary fiber nuclei of the lens transition zone (Figure 7, Panels C, D, G and H, asterisks). The translation inhibiting morpholino (Figure 7, Panels C and G; morfA) and splice inhibiting morpholino (Figure 7, Panels D and H; morfB) resulted in nearly identical phenotypes, although the morfB-induced changes were less severe (Figure 7, compare Panels D and H to C and G). The tissue separations near the morphant lens equator were usually located just proximal to the developing iris, which lies on the lens exterior. The location of the lens defects supports the idea that Lengsin knockdown affected the newly differentiating secondary fiber cells. In addition, the *lengsin* morphant lenses were smaller and lacked the regular concentric arrangement of central differentiated fiber cells displayed by the control lenses (Figure 7, compare Panels G and H to E and F). Therefore, Lengsin knockdown resulted in lens tissue defects whose locations correlated with the Lengsin protein expression pattern and the restriction of Lengsin to the most peripheral differentiating secondary fiber cells. Furthermore, the locations of the lens tissue gaps observed in the morphants were in the area of Lengsin protein that was concentrated near the lens equator as demonstrated by immunohistochemistry (see Figure 4C).

To verify the biological significance of the *lengsin* morphant lens phenotype, the total areas of the tissue separations per lens were quantified in both morfA- and morfB-injected embryos and compared to controls. During the course of our analysis, small separations in the dorsal lens periphery adjacent to the epithelial cell layer in the region proximal to the iris were observed in the controls, including both the uninjected wild-type and mismatch control morpholino-injected embryos (Figure 7, Panels A, B, E and F, small arrows). The frequency and nature of these lens structural aberrations were further examined by careful histological analysis of the 72 hpf wild-type zebrafish lens. In 81% of the wild-type lenses examined (n=21), at least one small separation was observed in the dorsal lens adjacent to the epithelial cell layer. The lens tissue separations in these controls were neither as frequent nor as large as those in the *lengsin* morpholino-injected embryos. In addition, the *lengsin* morphant lens tissue gaps were located both dorsally and ventrally and extended towards the lens interior, which was not observed in the controls. Finally, a quantitative analysis of control and morphant lenses demonstrated significant differences in the average total separation areas per lens. Uninjected wild-type and mismatch morpholino-injected embryo lenses exhibited average total separation areas of 257 and 108 μm^2 , respectively, while the morfA and morfB morpholino-injected embryos displayed average total separation areas of 1,175 and 667 μm^2 , respectively. The differences in the average total separation areas per lens between the two experimental groups and the control groups were statistically significant ($p < 0.05$) and further confirmed the specificity of the Lengsin knockdown phenotype.

3.6 Transmission electron microscopy reveals secondary fiber cell defects in the *lengsin* morphant lens

To examine the *lengsin* morphant lens phenotype in more detail, transmission electron microscopy (TEM) was performed on morpholino-injected (*morfA*) and non-injected wild-type embryos at 72 hpf (Figure 8). The control lens exhibited regularly arranged elongating nuclei of tightly packed differentiating fiber cells in the transition zone (Figure 8A, asterisks). In addition, the central epithelial cells and underlying fiber cells in the control were closely adhered and the fiber cells evenly and uniformly aligned (Figure 8B, asterisk and arrows, respectively). In contrast, the *lengsin* morphant lens cortical fibers displayed several abnormalities compared to the control (Figure 8, Panels C–F). First, the morphant fiber cells appeared to lack the extreme length characterizing the wild-type fiber cells. Instead, the morphant fiber cell ends remained in close proximity to the cell nucleus (Figure 8C, arrow and double arrowheads, respectively). The fiber cells appeared to end at, or border, the large acellular gaps within the morphant lens (Figure 8C, arrow and asterisk, respectively). In addition, some morphant fiber cells were shriveled along their lengths and had pulled away from the adjacent fiber cells with only a few membranous strands of attachment to the neighboring fiber cells (Figure 8D, arrows). Large electron-dense vacuoles were also observed in some *lengsin* morphant lens cortical fibers (Figure 8E, asterisks) and the fiber-fiber interfaces in the morphant lenses were less distinct than the controls (Figure 8, compare Panels F to B, arrows). The TEM demonstrates secondary fiber cell pathology in the *lengsin* morphant lens and suggests the *Lengsin* knockdown results in a failure of these cells to properly differentiate.

4. Discussion

Zebrafish *lengsin* was identified by EST analysis of adult zebrafish lens (Vihtelic et al., 2005a). To understand *Lengsin* function in the vertebrate lens, a polyclonal antiserum was generated to immunolocalize zebrafish *Lengsin* and antisense *lengsin* morpholinos were utilized to knockdown *Lengsin* protein during early lens formation. Reduction of *Lengsin* during development caused lens dysmorphogenesis characterized by large tissue separations in the cortex. These results demonstrate *Lengsin* specifically marks terminally differentiating lens fiber cells and they suggest *Lengsin* plays a role in fiber elongation or the establishment of fiber cell interactions.

4.1 *Lengsin* expression patterns during zebrafish lens formation and growth

During lens development, zebrafish *Lengsin* is expressed in both primary and secondary fiber cells. The zebrafish lens cells appear relatively homogenous at 24 hpf with nucleated cells forming a solid tissue mass (Li et al., 2000; Soules and Link, 2005; Dahm et al., 2007). At 36 hpf, lens epithelial cells are morphologically apparent as a single layer on the distal lens surface and the nuclei of the elongating secondary fiber cells located near the lens proximal pole (Soules and Link, 2005; Dahm et al., 2007). Thus, primary fiber cell differentiation in zebrafish commences prior to 36 hpf. *Lengsin* is detected in elongated central lens cells at 30 hpf and expression expands to include peripheral lens fibers at 36 hpf. This suggests *Lengsin* expression initiates in the differentiating primary fiber cells at approximately 30 hpf and includes differentiating secondary fiber cells at 36 hpf. This is in contrast to mouse, where *Lengsin* is not detected in the primary fiber cells, but only in the secondary fibers (Wyatt et al., 2008). Similar to mouse, zebrafish *Lengsin* is not expressed in the epithelial cells and central lens fibers lack detectable *Lengsin* at 72 hpf, suggesting the half-life of *Lengsin* protein, or at least the epitope recognized by the antiserum, must be short.

The appearance of *Lengsin* in the differentiating lens fiber cells is preceded by the expression of several crystallin proteins including α A-, α B1- and β B1-crystallin. In adults, *α B1-crystallin* transcripts were largely restricted to the lens tissue, while *α B2-crystallin* transcription

was identified in brain, heart, liver and skeletal muscle, in addition to the lens (Posner et al., 1999; Smith et al., 2006). This suggests the expression patterns of the zebrafish α B-crystallins may change between development and the adult, although more detailed analysis of the gene and protein expression patterns will be necessary to fully examine this possibility. In contrast to the Alpha-crystallins, Lengsin is a late developmental marker for lens fiber cell differentiation and identifies differentiating secondary fibers in the growing lens.

Lengsin localized to the fiber cell plasma membranes. This suggests the protein may interact with lens fiber cytoskeletal elements. The lens fiber cytoskeleton consists of Actin filaments and associated regulatory proteins, microtubules, and two different intermediate filament networks. In mammals, a Vimentin-based lens network is found in both epithelial and fiber cells, while Filensin (BFSP1) and CP49 (BFSP2) intermediate filament proteins are confined to the lens fiber cells (Sandilands et al., 1995; Perng et al., 2007). During fiber differentiation, these cytoskeletal complexes undergo marked organizational changes independent of one another. Thus, Vimentin levels in the maturing fiber cells fall sharply, as evidenced by a well-demarcated decline in signal intensity correlating with the zone of fiber cell nuclear loss (Sandilands et al. 1995). The sharp decline in Lengsin protein in the peripheral fiber cell population is reminiscent of the dramatic reduction in Vimentin that occurs during fiber cell maturation. Indeed, zebrafish Lengsin may interact with Vimentin because yeast two-hybrid and recombinant protein experiments demonstrated full-length mouse Lengsin binds the 2B filament region of Vimentin and the equivalent region of CP49 (Wyatt et al., 2008). Therefore, the zebrafish Lengsin expression domain coincides with a specific stage of fiber elongation. The relatively transient expression of Lengsin may be necessary for cytoskeleton reorganization or formation of the fiber cell lateral protrusions leading to the initial stabilization of cell-cell interactions.

4.2 Lengsin transgenes enable visualization of differentiating lens fibers

The lens-specific and spatially restricted protein expression patterns demonstrate Lengsin is an excellent marker for a critical time period in the dynamics of differentiating fiber cells in the zebrafish lens. To take advantage of this expression pattern and visualize the differentiating fiber cell population in developing zebrafish embryos, stable transgenic lines were created using a *lengsin* promoter fragment to regulate EGFP expression. Based on the temporal and spatial expression patterns of fluorescent reporter protein expression in the transgenic lenses, the transgenes are expressed in patterns that largely mimic the native *lengsin* gene, with EGFP detected in the lens fiber cells after 24 hpf. The localization of EGFP in the transgenic lenses indicates the reporter protein diffuses throughout the fiber cell mass. This expression pattern suggests the zebrafish lens possesses syncytial properties similar to that demonstrated for chick and mouse (Kaiser and Maurice, 1964; Goodenough et al., 1980; Goodenough, 1992). Alternatively, the GFP may simply persist in the lens center and increase in concentration over time as fiber cells mature and lose water. The stable *lgs:EGFP* (and *lgs:EGFP-NLS*) transgenic lines represent useful reagents for the further characterization of lens development and growth in zebrafish.

4.3 Lengsin knockdown during development disrupts lens cortical fiber organization

Several strategies were employed to verify the specificity of the Lengsin knockdown phenotype. First, two different morpholinos were utilized independently (Nasevicius and Ekker, 2000; Ekker and Larson, 2001). One morpholino (morfA) was designed to prevent Lengsin translation by annealing to sequences surrounding the mRNA translation initiation codon. A second morpholino (morfB) was designed to inhibit RNA splicing by annealing at the *lengsin* exon 1-intron 1 junction. Immunolabeling of whole-mount morphant embryos (morfA) demonstrated the elimination of detectable Lengsin protein at 48 hpf. The lens defects in both morphant classes were nearly identical, although the morfA-induced pathology

generally appeared more severe than the *morfB*-induced lens abnormalities, which may be due to greater efficiency of the *morfA*-mediated translation inhibition compared to the *morfB*-dependent splice inhibition. In addition to employing two different morpholinos, a control morpholino with five mismatched bases relative to *morfA* (mmC) failed to cause any significant phenotype as determined by stereomicroscopy and histology. Finally, the morphometric analysis confirmed there were significant differences in the lens fiber defects between the control groups (uiC and mmC-injected embryos) and experimental groups (*morfA*- and *morfB*-injected).

Transmission electron microscopy of control and morphant lenses demonstrated defects in the secondary fiber cells near the lens transition zone. In the control lens, single healthy elongating secondary fibers could be traced through many microscopic fields indicative of the dramatic lengths achieved as they mature and extend towards the lens poles. In contrast, two types of pathological change characterized the *lengsin* morphant secondary fibers. First, some fiber ends were observed adjacent to, or passing near, the lens cortical spaces visualized by histology. This suggests the fiber cells were truncated. Alternatively, the cells may still be very long, but disorganized such that they exit the plane of the sections. Three dimensional reconstructions are necessary to fully evaluate the morphant fiber cell morphology. Second, elongating fibers were shrunk and retracted from their neighbors. In these cases, a few membranous strands attached the adjacent fiber cells. Thus, some *lengsin* morphant fibers may fail to elongate, while others may be unable to maintain their shape and interconnections to adjacent cells. In addition, the *lengsin* morphant lens defects were confined to the peripheral fiber cells corresponding to the *Lengsin* protein expression domain in age-matched fish and further confirmed the *Lengsin* knockdown phenotype specificity.

The appearance of lens cortical separations in the *lengsin* morphants was most severe at 72 hpf. At 5 dpf, the morpholino-injected embryos possessed a near wild-type-appearing lens (data not shown). The apparent resolution of the morphant lens phenotype is likely due to several factors. Upon injection into early embryos, the morpholinos are distributed throughout the developing embryo as cells divide. Consequently, the morpholino concentration within individual cells is reduced as cells continue to multiply during tissue formation. In the lens, germinative zone epithelial cells continue to divide subsequent to differentiation of the epithelial and primary fiber cells. This lens epithelial progenitor population continues to provide cells throughout life that differentiate and form new secondary fibers. Thus, the morpholinos within the lens germinative and thus, transition zone, will be exhausted as lens growth proceeds and may account for recovery of the wild-type lens fiber structure and organization as new fiber cells are generated with wild-type *Lengsin* levels. On the other hand, the defective morphant fiber cells may become packed tighter in the deeper lens fiber layers and merely appear more organized as the result of their compression. Central fiber cell organization appeared disrupted in the 72 hpf morphant lens. The genetic elimination of *Lengsin* will be required to definitively determine the role *Lengsin* plays for lens development and growth.

4.4 Summary

Lengsin is a late marker required for vertebrate lens fiber cell differentiation. In zebrafish, *Lengsin* expression in the differentiating fiber cells was preceded by αA -, $\alpha B1$ - and $\beta B1$ -crystallin. The tight temporal and spatial restriction of the *Lengsin* protein during fiber cell differentiation suggests *Lengsin* defines a precise stage of fiber cell maturation. *Lengsin* expression near the base of the lens proximal pole defines differentiating fibers prior to cell morphological changes such as elongation or nuclear degradation. Thus, *Lengsin* serves to molecularly demarcate the zebrafish lens transition zone. By identifying *Lengsin* interacting proteins, additional insights into lens fiber cytoskeletal organization and the regulation of

cytoskeletal rearrangements during fiber cell differentiation may be obtained. In addition, the polyclonal antisera and stable transgenic lines generated for these studies will be useful for future analyses to determine the molecular basis for zebrafish lens formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Lengsin domain organization and gene expression. (A) The zebrafish, human, mouse and chick Lengsin proteins include a GS (Glutamine Synthetase) homology region composed of the conserved β -GRASP (zebrafish amino acids 248-335) and GS catalytic domains (zebrafish amino acids 346-593). The amino acid sequence within the zebrafish GS homology region is 55%, 54% and 61% identical to this region of sequence in the human, mouse and chick proteins, respectively. In addition to a carboxyl-terminal domain (C-ter) of approximately 75 amino acids, Lengsin proteins possess an amino-terminal domain (N-terminal) whose length varies between species. The numbers of amino acids within the N-terminal domains are shown within the black bars. The calculated molecular weights (MW, in Daltons) for Lengsin from each

species are shown in the column on the right. (B) RT-PCR of whole embryos and adult tissues was used to determine the temporal and spatial *lengsin* expression patterns. Zygotic *lengsin* transcription is first detected at 24 hpf (591 bp product). In adult zebrafish tissues (lower panel), *lengsin* is restricted to the lens, while transcripts are not detected in the other eye tissues, the brain, caudal fin or internal organs, β -actin transcripts (lower rows in each panel) were amplified from each sample as a control. Abbreviations: ant seg, anterior segment minus lens; post seg, posterior segment minus retina; int org, internal organs.

Figure 2.

Lensesin protein expression during development. Whole-mount embryos were immunolabeled with polyclonal anti-Lensesin (green signal) and monoclonal antibody zl-1 (red signal), a lens-specific marker in zebrafish. At 24 hpf (Panel A), only zl-1 is detected in the differentiating lens cells. At 30 hpf (Panel B), Lensesin expression initiates in the central lens (arrow), while zl-1 is localized at the lens periphery (arrowheads). At 36 hpf (Panel C), Lensesin is expressed in many fiber cells (arrow). The central fibers lack Lensesin protein (asterisk) and zl-1 remains concentrated at the lens periphery near the epithelial cell bases (arrowheads) at this time. Lensesin becomes progressively restricted to a subpopulation of elongated peripheral lens cells between 42–72 hpf (Panels D-F; arrows in Panels E and F). Scale bar in Panel A (25 μ m) applies to all panels.

Figure 3.

Crystallin protein expression precedes Lingsin during zebrafish lens formation. Polyclonal antisera were used to detect zebrafish α A-, α B1- and β B1-crystallin in whole-mount embryos at 24 and 48 hpf (Panels A–D and E–H, respectively). At 24 hpf, α A-crystallin is barely detectable (Panel A, arrow), while α B1- and β B1-crystallin are strongly expressed (arrows in Panels B and C, respectively) and Lingsin (Panel D) is not detected. At 48 hpf, these three crystallin proteins (Panels E–G) and Lingsin (Panel H) are detected at high levels in the lens (arrows). At 24 and 48 hpf, α A-crystallin, β B1-crystallin and Lingsin are restricted to the lens, while α B1-crystallin is also expressed in the retina and other parts of the CNS (compare Panels A, C, D, E, G and H to B and F). The lenses in some of the immunolabeled embryos appear yellow because the red channel gain was increased to highlight the extralenticular embryonic tissues. Scale bars represent 50 μ m (Panels A–D) and 200 μ m (Panels E–H).

Figure 4.

Lentsin immunolocalization in the embryonic lens. Frozen eye sections from 48 hpf (Panels A and B) and 72 hpf (Panels C–F) embryos were immunolabeled using the Lentsin antiserum. Lentsin is restricted to the lens and is not detected in the retina or other eye tissues at 48 hpf (Panel A, arrow). The Lentsin immunolocalization pattern changes dramatically between 48 hpf (Panel B) and 72 hpf (Panel C) with a shift from the proximal lens fibers (Panel B, asterisks) to the distal lens fibers and lens equator (Panel C, arrows and asterisks, respectively). Lentsin is also detected in the lens transition zone at 72 hpf (Panel C, arrowheads). In the growing lens at 72 hpf, Lentsin expression initiates at the base of the lens transition zone (Panel D, arrow), but is not detected in either the lateral or central epithelial cells (arrowheads in Panels D and E, respectively). Lentsin is not detected in the more central nuclei-containing fibers (Panel D, asterisks). Compared to Lentsin, α B1-crystallin is expressed in both fiber and epithelial cells at 72 hpf (Panel F, arrows). Scale bars represent 50 μ m (Panel A) or 25 μ m (Panels B–F).

Figure 5.

Lensin localization in the adult lens. Phalloidin-AF488 staining demonstrates filamentous Actin is concentrated at the lateral fiber cell membranes and membranes of the epithelial cells (Panel A, arrows and arrowheads, respectively). The lateral processes of the fiber cells are characterized by high levels of Actin (Panel B, arrows). Cross sectional profiles of the adult lens cortical fibers, immunolabeled using the Lensin antiserum (Panel C), reveal Lensin (green signal, arrows) is localized within a subpopulation of cortical fibers separated from the epithelial cell layer (nuclei stained blue, arrowheads) by fibers lacking detectable Lensin (asterisks). Double-immunolabeled lens sections reveal Lensin (green) is co-localized with Actin (red) at the lateral margins of the fibers (Panel D, Actin; Panel E, Lensin; Panel F, Merge). In contrast to Lensin, α B1-crystallin localizes around the entire fiber cell membrane (Panel G, Actin; Panel H, α B1-crystallin; Panel I, Merge). Scale bars represent 20 μ m (Panels A and B), 50 μ m (Panel C), 30 μ m (Panels D–F) or 15 μ m (Panels G–I).

Figure 6.

Lensin transgene expression during zebrafish lens formation. Two different stable transgenic lines were generated using *lensin* 3 kb genomic sequences to regulate expression of either EGFP [*Tg(lgs:EGFP)nt20*, Panels A–D] or EGFP with a nuclear localization signal [*Tg(lgs:EGFP-NLS)nt21*, Panels E–I]. Embryo whole-mounts (Panels A–H) and a frozen tissue section (Panel I) are shown. EGFP expression was not detected from either construct by epifluorescent illumination of whole embryos at 24 hpf (Panels A and E). However, EGFP is clearly detected and restricted to the lens at 36 hpf (Panel B, arrow). Between 48–72 hpf, the lens EGFP intensity increases as the fiber cells fill with the fluorescent protein (Panels C and D, arrows). EGFP-NLS is detected in a few nuclei at 36 hpf (Panel F, arrows) with the number of EGFP-positive nuclei increasing between 48–72 hpf (Panels G and H, arrows). The frozen section (Panel I) demonstrates the elongating fiber cell nuclei are EGFP-positive in the *Tg(lgs:EGFP-NLS)nt21* fish at 72 hpf (arrows). Scale bars represent 50 μ m (Panels A, D, E, G–I) and 25 μ m (Panels B, C and F).

Figure 7.

Histology of *lengsin* morphant lens. Plastic resin-embedded eye sections from 72 hpf uninjected control (uiC; Panels A and E), mismatch morpholino-injected control (mmC; Panels B and F) and *lengsin* morphant embryos (morfA, Panels C and G; morfB, Panels D and H) were stained with methylene blue and azure II. The control lenses display a single layer of epithelial cells and nuclei of the differentiating secondary fibers can be visualized in the transition zone (Panels E and F, arrowheads and arrows, respectively). Small cortical separations (short arrows) are present in some control lenses (Panels A, B, E and F). The *lengsin* morphant lenses (Panels C, D, G and H) exhibit large separations (asterisks) between cortical fibers and the overlying epithelial cells in the region near the lens equator and distal to the differentiating secondary fiber nuclei. The morphant lens is also reduced in size and lacks the concentric central fiber cell arrangement displayed by the controls (compare Panels G and H to Panels E and F). The lenses shown in Panels C, D, G and H are from different *lengsin* morphants. Abbreviations: PL, photoreceptor layer; INL, inner nuclear layer; GCL, ganglion cell layer; uiC, uninjected control; mmC, mismatch control morpholino. The scale bars in Panels A and E (50 μ m) also apply to Panels B–D and F–H, respectively.

Figure 8.

Transmission electron microscopy of *lengsin* morphant lens. Serial sections of uninjected control and *lengsin* morphant (*morfA*) lens were stained with uranyl acetate and lead citrate. Control lens section images (Panels A and B) were taken in the lens transition zone (Panel A, asterisks denote nuclei) and at the interface between the central epithelial cells and underlying fiber cells (Panel B, asterisk and arrows, respectively). Panels C and D show the *lengsin* morphant lens transition zone of two different fish. In Panel C, a large gap (asterisk) is located just distal to the secondary fiber nucleus (double arrowheads). The fiber cell end appears to curve around the perimeter of the tissue gap (arrow). Panel D demonstrates morphant lens secondary fibers are shrunken and retracted from the adjacent fiber cells (arrows). Electron-

dense vacuoles are evident in some morphant lens fibers (Panel E, asterisks). The *lengsin* morphant fiber cell separations in the area adjacent to the central epithelial cells are less distinct compared to the control (compare Panel F to B, arrows). Scale bars represent 2 μm (Panels A, D and F), 1 μm (Panel B) and 3 μm (Panels C and E).

Table 1

RT-PCR primers

Designation	Sequence	Nucleotides	Exon	Paired with	T _m (°C)
F1	5'-TTTGGTTGGGAGAAAGGGAGC-3'	30-51	1	B4	52°C
F3	5'-GGTGGATGGAGGAAGTATGAGTGC-3'	201-224	2	B7, B21	52°C, 56°C
F15	5'-TTTCAGGCATCCGATTGGGTG-3'	766-787	2	B20	58°C
F19	5'-TTGGAATGGATGCCGCTGAC-3'	1466-1485	4	B21	58°C
F21	5'-CATTGGCCACCTTTTTCACCTACG-3'	1539-1561	4	B21	58°C
B4	5'-TCTGAATAGGGCTTGAAGGTGC-3'	641-662	2	F1	52°C
B7	5'-TTGCTGACTGGGATGTGCCAACCC-3'	789-812	3	F3	52°C
B20	5'-TACGCTTGAATCCGTCACAGTCC-3'	1918-1939	4	F15	58°C
B21	5'-GCACCCACAAATCACACTGTCCTC-3'	2035-2057	4	F3, F19, F21	56°C, 58°C, 58°C

Table 2

Lens protein temporal expression patterns

	α A-crystallin	α B1-crystallin	β B1-crystallin	zl-1	Lensin
24hpf	19% (21)	100% (19)	82% (17)	84% (19)	5% (20)
48hpf	100% (15)	100% (15)	100% (15)	100% (15)	100% (15)

Percentages of embryos at each age expressing the indicated protein in the lens. Numbers in parentheses indicate the total numbers of embryos.