Aqp0a Regulates Suture Stability in the Zebrafish Lens

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Purpose. To investigate the roles of Aquaporin 0a (Aqp0a) and Aqp0b in zebrafish lens development and transparency.

Methods. CRISPR/Cas9 gene editing was used to generate loss-of-function deletions in zebrafish aqp0a and/or aqp0b. Wild type (WT), single mutant, and double mutant lenses were analyzed from embryonic to adult stages. Lens transparency, morphology, and growth were assessed. Immunohistochemistry was used to map protein localization as well as to assess tissue organization and distribution of cell nuclei.

Results. aqp0a−/− and/or aqp0b−/−cause embryonic cataracts with variable penetrance. While lenses of single mutants of either gene recover transparency in juveniles, double mutants consistently form dense cataracts that persist in adults, indicating partially redundant functions. Double mutants also reveal redundant Aqp0 functions in lens growth. The nucleus of WT lenses moves from the anterior pole to the lens center with age. In aqp0a−/−mutants, the nucleus fails to centralize as it does in WT or aqp0b−/−lenses, and in double mutant lenses there is no consistent lens nuclear position. In addition, the anterior sutures of aqp0a−/−, but not aqp0b−/−mutants, are unstable resulting in failure of suture maintenance at older stages and anterior polar opacity.

Conclusions. Zebrafish Aqp0s have partially redundant functions, but only Aqp0a promotes suture stability, which directs the lens nucleus to centralize, failure of which results in anterior polar opacity. These studies support the hypothesis that the two Aqp0s subfunctionalized during fish evolution and that Aqp0-dependent maintenance of the anterior suture is essential for lens transparency.

Keywords: AQP0, lens, zebrafish, Danio rerio, cataract

AQP0 is the most abundant lens membrane protein and is essential for lens development and transparency. Mutations in AQP0 lead to cataract formation in humans, mice, and rats.1 Thus, understanding the normal functions of AQP0 and their disruption in disease could lead to the development of treatments to prevent or delay the leading cause of worldwide blindness.

Mammalian AQP0 exhibits multiple functions in lens fiber cells including water transport (see Ref. 2 for review), anchoring cytoskeletal proteins to the intermediate filament protein filensin,1,5 and regulating connexin 50 function in gap junctions.6,7 Ultimately, these roles all contribute to the generation of the lens refractive index gradient.8

The regulation of AQP0 water permeability has been extensively studied in the Xenopus laevis oocyte expression system by measuring permeability in response to hypotonic stress. Site-directed mutagenesis identified the residues crucial for pH1 and Ca2+ regulation.10,11 Effects of phosphorylation on permeability12 and consequences of the C-terminal truncation that normally occurs in mature lens fiber cells.8,13 In vitro tests have similarly identified amino acids required for adhesion.14,15 Because multiple functions reside in the single mammalian AQP0, it is difficult to separate the requirements for each function experimentally in a mammal. Apparent subfunction alization of two Aqp0s in zebrafish provides an opportunity to overcome this difficulty.

Because of an ancient teleost-lineage genome duplication, zebrafish (Danio rerio) have two orthologs of AQP0, aqp0a, and aqp0b.16 Morpholino (MO) knockdown of expression of either gene leads to cataract formation at 3 days post-fertilization (dpf),17 which can be self-but not cross-rescued.18 Thus, both aqp0a and aqp0b are essential for embryonic lens transparency. They share 85% amino acid identity but diverge in key amino acids crucial for pH regulation of water permeability.17 Aqp0a expressed in Xenopus laevis oocytes shows increased permeability with basic pH, while Aqp0b shows increased permeability with acidic pH.19 Interestingly, it also appears that there are nonwater-permeable variants of Aqp0b (G19S).17,19 The fact that water permeability of Aqp0b is not essential for normal embryonic lens transparency is also supported by DNA-construct rescue of aqp0b knockdown by a mutant version of MIPtfn (N68Q) lacking water permeability (MIPtfn is an AQP0 in killifish).18 These data suggest that Aqp0a and Aqp0b have functionally diverged and that water transport is an essential characteristic of Aqp0a, but not of Aqp0b.

Zebrafish lens secondary fiber cells elongate, migrate from the developing bow region by 28 hours postfertilization, and extend bidirectionally.20 Their anterior tips migrate toward the center of the lens epithelium, while their posterior tips migrate toward the center of the lens capsule. These fiber cell tips eventually detach from the anterior epithelium or posterior capsule at the poles and interlock end-to-end with opposing fiber cell tips to form the sutures.21
Sutures extend from the embryonic nucleus, comprised of primary fiber cells, to the epithelium at the anterior pole, and to the lens capsule at the posterior pole. The zebrafish lens has a very simple suture architecture, with umbilical or point sutures, formed from crescent-shaped secondary fiber cells that are wider at their centers and taper at their ends to meet at the sutures.22

Suture formation requires a polarity signal in fiber cells that involves asymmetrically localized planar cell polarity (PCP) proteins,25 while the primary cilium is nonessential for mouse anterior suture formation.24 Mutations in the PCP genes Van Gogh-like 2 and Celsr1 perturb suture formation.25 A lens-fiber basal membrane complex, enriched in F-actin, integrinβ1 (Itgβ1) and E-cadherin, rearranges in the final stages of migration and detach from the capsule to form the posterior suture in rat lenses.25 Additionally, migration of elongating fiber cells in zebrafish requires Itgδ5 and fibronectin (Fn1).26

The exact role of AQP0 in the formation or maintenance of lens sutures is unknown. However, SEM analysis in AQP0-deficient heterozygous mouse lenses has shown that posterior fiber cells have abnormally large tips that curve away from the polar axis resulting in defective sutures.27 Homozygous AQP0 knockout (KO) mouse lenses completely fail to form sutures.27 Here we describe the phenotypes of functional genetic knockouts of aqp0a and/or aqp0b in zebrafish, which we call “mipb.”

Mutations overcome the limitations of MO knock-down experiments, which can have off-target effects and are only effective at embryonic and early larval stages. The presence of embryonic cataracts in mutants confirms our original MO results that both Aqp0a and Aqp0b play essential roles in early lens development. While the lenses of single mutants show variably penetrant cataracts with distinct cellular phenotypes, double mutants show highly penetrant, severe cataracts, supporting the conjecture that Aqp0a and Aqp0b have both unique and partially redundant functions.

Large adult aqp0a+/−, but not aqp0b+/−, mutant lenses develop anterior polar opacification, accompanied by anterior sutural instability, disorganization of fiber cell architecture, and a mass of nuclei at the anterior pole. We also found, unexpectedly, that normal development of zebrafish lens optics relies on movement of the lens nucleus from an initial location near the anterior pole in juveniles to the center of the lens in adults. This shift requires Aqp0a. We propose that the instability of the anterior lens suture when Aqp0a is absent leads to failure of nuclear centralization, completely disrupting optics in adult lenses.

**Methods**

**Zebrafish Husbandry**

The animal protocols used in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Zebrafish (AB strain) were raised and maintained at room temperature (RT) and images were used to assess transparency under darkfield and brightfield illumination.

**Lens Tissue Processing**

**Gross Lens Analysis and Lens Dissection**

PTU-treated 2 to 4 dpf zebrafish were euthanized and lenses transparency assessed using a microscope (Zeiss Axioplan 2; Oberkochen, Germany) with differential interference contrast (DIC) optics, which highlighted refractive abnormalities. Images were captured using a digital camera (ORCA-ER; Hamamatsu Photonics KK, Hamamatsu City, Japan) and acquired using commercial software (Velocity 5, version 5.2.1; PerkinElmer, Akron, OH, USA). Larval or adult zebrafish were euthanized and transparency of dissected lenses was visualized with a microscope and digital camera with a dynamic positioning controller (Olympus SZX12 microscope and Olympus DP70 with a model 2.1.1.183 controller; Olympus Corp., Tokyo, Japan). For in situ transparency assessment, a fiber optic light source (Schott KL 1500; Schott AG, Mainz, Germany) was used for oblique illumination from two sides to visualize opacities. Standard length and eye diameter were measured following previously established guidelines.20 Lenses were dissected in phosphate buffered saline (PBS) (Fisher BioReagents, Pittsburgh, PA, USA) at room temperature (RT) and images were used to assess transparency under darkfield and brightfield illumination. Optical properties were assessed by the ability of lenses to focus a field finder grid (Lovins Field Finder; Electron Microscopy Sciences, Hatfield, PA, USA). Lenses were oriented equatorially, with poles and sutures perpendicular to the plane of focus, or axially, with poles and sutures parallel to the plane of focus. Zebrafish lenses are not completely rotationally symmetrical, so to determine lens size, diameters were measured in an equatorial orientation. The position of the lens nucleus was measured to the center of the lens by expressing the position of the nucleus as the normalized coordinate, r/a, where a is the radius of the lens and r is the distance from the center of the lens to the center of the nucleus. The value of (a − r) is the distance of the center of the nucleus from the anterior pole, which is the number we actually measured, since it is difficult to precisely locate the center of the lens. We calculated the value of r/a as [1 − (a − r)/a], where a and (a − r) were the actual measured values, for experimental convenience. For a nucleus at the center of the lens (r = 0), the value of the normalized coordinate r/a is 0. For a nucleus centered at the surface of the lens (possible only for a point nucleus) the value of the normalized coordinate r/a is 1. In fact, we never observed a nucleus center closer to the surface than an r/a value of about 0.5. r/a was graphed as a function of standard length (SL). The positions of centers of adult nuclei were measured when the presumptive embryonic nucleus was evident.
overnight. After PBS washes, embryos or lenses were cryoprotected by placing into 10% sucrose (wt/vol), 20% sucrose for 1 hour at RT and overnight in 30% sucrose at 4°C. We collected 12 to 14 μm cryosections containing the lenses onto gelatin-coated slides. Adult equatorial lens sections at 12 to 14 μm were collected onto microscope slides (Superfrost/Plus; Fisher Scientific, Hampton, NH, USA). For whole lens analysis, after fixation, lenses were permeabilized in PBS + 10% Triton + 1% dimethyl sulfoxide (DMSO) overnight at 4°C. Lens membrane protein was extracted from decapsulated adult zebrafish lenses (ProteoExtract Native Membrane; Calbiochem, La Jolla, CA, USA).

**Antibodies**

Polyclonal rabbit anti-sera were raised against C-terminal peptides of zebrafish Aqp0a (CVRGLSERAFLGKGNK) or Aqp0b (CFSERALTSLGSRPPE, Covance, Inc., Princeton, NJ, USA) as per Froger et al.,17 and purified (Ecoron-Pac Serum IgG; Bio-Rad Laboratories, Hercules, CA, USA). Aqp0a and Aqp0b peptides for competition studies were also generated (Thermo Fisher Scientific, Rockford, IL, USA). Monoclonal mouse anti-alpha Tubulin (GT114) was purchased from Genetex (Irvine, CA, USA).

**Immunohistochemistry**

Embryonic head sections or adult lens sections were labeled with anti-Aqp0a or anti-Aqp0b antibodies (1:500) overnight at 4°C. Following PBS washes, AlexaFluor 488 goat anti-rabbit secondary antibody (1:500; Thermo Fisher Scientific) was applied for 2 hours at RT. Lens plasma membranes were labeled with wheat germ agglutinin AlexaFluor 594 (1:50; Life Technologies, Grand Island, NY, USA) and DAPI (1:1000) for 2 hours at RT or overnight at 4°C. Whole permeabilized lenses were incubated in AlexaFluor Phalloidin-546 (1:200; Thermo Fisher Scientific) and DAPI (1:1000). Images were acquired with a confocal microscope and imaging software (Nikon Eclipse Ti-E with NIS-Elements AR; Nikon Corp., Tokyo, Japan). Images were viewed and compiled using a commercial viewer (NIS-Elements, version 4.2; Nikon Instruments, Melville, NY, USA) and ImageJ, version 1.51n (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and a raster graphics editor (Adobe Photoshop CS5, version 12.0; Adobe Systems, San Jose, CA, USA).

**Western Blot Analysis**

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were labeled by incubation with rabbit anti-Aqp0a or anti-Aqp0b antibodies (1:500) overnight at 4°C, and goat anti-rabbit horse radish peroxidase (HRP) (1858415, 1:10,000; Pierce Biotechnology, Waltham, MA, USA) for 1 hour at RT and visualized by ECL Prime (GE Healthcare Life Sciences, Pittsburgh, PA, USA) using the Fujifilm LAS 4000 imager (Tokyo, Japan). To ensure equal protein loading, blots were stripped (2% SDS, 0.7% b-mercaptoethanol, 0.775% Tris-HCl [wt/vol] in TBS-T) for 30 minutes at 50°C, blocked, and incubated with mouse anti-alpha Tubulin (1:500) overnight at 4°C, followed by goat anti-mouse HRP (1858413, 1:8000; Pierce Biotechnology) for 1 hour at RT and visualized as above.

**Statistical Analysis**

R commander version 3.3.0 (from 2016-05-03) was used for statistical analysis.31 Standard linear regression was used to assess the relationships between lens diameter and SL, eye diameter and SL, and lens diameter and eye diameter. A statistical test of proportions was used to estimate the difference in incidence rate of cataracts between each pair of genotype groups. The Holm method was used to calculate adjusted P values that account for multiple testing (six pairs of genotype groups necessitated six tests) of each data set (2, 3, and 4 dpf).32 The log of the normalized axial nucleus measurement (r/a) had an approximately linear relationship with SL (Supplementary Fig. S5), so linear regression was used to assess the relationship between the log normalized axial nucleus measurement and SL, controlling for genotype group. All computations and graphics were completed in the statistical language and programming environment R.

**RESULTS**

**Embryonic and Adult Lens Transparency Require Both Aqp0a and Aqp0b**

To study the role of Aqp0a or Aqp0b in lens development, transparency and function, we created loss-of-function aqp0a, aqp0b, and aqp0a/b mutants using CRISPR-Cas9-mediated gene editing (Fig. 1A). Loss of either Aqp0a or Aqp0b protein in mutants was confirmed by Western blot analysis (Fig. 1B). The effects of loss of Aqp0a and/or Aqp0b function on lens transparency were analyzed in living embryos using DIC imaging. At 2 to 3 dpf, lenses in a subset of embryos displayed obvious opacities, which became less evident by 4 dpf (Fig. 2A). Cataract frequency at 2 dpf was 8% in aqp0a−/−, 9% in aqp0b−/−, and 36% in double mutants, compared to 1% in WT (Figs. 2B and C) and by 5 dpf was ~12.5% in both single and double mutant fish (0% in WT; Supplementary Fig. S1). Morphologic defects were restricted to the lens nucleus at both 2 and 3 dpf, while the lens cortex and epithelium appeared unaffected (Fig. 2A). Unexpectedly, cataracts in double mutants typically appeared less severe than in single mutants (Fig. 2A, right column). While the obvious cataracts seen at earlier stages in the mutants disappeared by 4 dpf (Figs. 2A, 2B), refractive defects remained. These manifested as marked differences in refraction at the cortex-nucleus interface in contrast to the consistently clear, smoother refractive gradient at the cortex-nucleus boundary of WT lenses (Fig. 2A arrows, bottom row).

Transparency in anesthetized older fish lenses was analyzed in vivo using an oblique light to highlight opacity under brightfield illumination (Figs. 3A, 3B). To localize regions of lens opacity in mutants, dissected adult lens transparency was assessed by darkfield illumination at both poles, as well as the nuclear and equatorial regions (Fig. 3C). In juvenile fish at 16 to 18 mm SL, only double mutant lenses showed obvious opacity (Fig. 3A). In adults larger than 23 mm SL, aqp0a−/− mutants also exhibited opacity (Fig. 3B) localized to the anterior pole (Fig. 3C; anterior pole is outlined with a dotted line). This was not evident in aqp0b−/− mutants (Figs. 3B, 3C). Double mutant lenses had similar anterior polar opacities as well as obvious nuclear cataracts (Fig. 3C). Although the severity and extent of the opacity from the center of double mutant lenses varied (Supplementary Fig. S2), cataracts were consistently present in embryonic nuclei, which are comprised of primary fiber cells.33

To analyze the consequences of the opacity observed in vivo on lens transparency and optics, adult dissected lenses were imaged through their optical axis (equatorial orientation). Both brightfield and darkfield illumination revealed mild refractive problems in aqp0a−/− mutants at the cortex-nucleus interface and at the lens periphery. But with the same illumination, double mutants showed obvious nuclear opacity
and more widespread cortical fiber-to-fiber membrane stacking defects (for more examples of double mutant phenotypes, see Figs. 4A, 4B, Supplementary Fig. S2).

The ability of lenses to focus a grid image provided a rough assessment of their optical properties. Grids behind WT and \textit{aqp0b} \textsuperscript{-/-} mutant lenses were visible and well-focused (Fig. 4C). But grids behind \textit{aqp0a} \textsuperscript{-/-} and double mutant lenses were almost invisible and blurred (Fig. 4C).

To determine Aqp0 effects on growth, lens size was compared with eye size and standard length (SL) in \textit{aqp0a} \textsuperscript{-/-}, \textit{aqp0b} \textsuperscript{-/-}, and \textit{aqp0a/b} mutants using brightfield imaging. As zebrafish grow at different rates depending on food availability, anterior eye segment size correlates better with SL than with age,\textsuperscript{34} so SL was used for staging past 4 dpf.\textsuperscript{30} Lenses in the double mutants grew slower than in WT or single mutants, and this was lens-specific, not a result of delayed eye growth (Supplementary Figs. S3, S4). Thus, in addition to their roles in lens transparency, Aqp0a and Aqp0b have partially redundant roles in lens growth.

\textbf{Aqp0a and Aqp0b Show Similar Spatial and Temporal Expression Patterns}

Phenotypic differences between \textit{aqp0a} \textsuperscript{-/-} and \textit{aqp0b} \textsuperscript{-/-} mutants could reflect spatially and temporally specific patterns of protein expression. To test this, Aqp0a and Aqp0b expression was examined by immunohistochemistry in sections. Purified antibodies directed against the C-terminus of Aqp0a or Aqp0b detected a punctate signal for both at 1 dpf (Figs. 5A, 5B, green). At 2 dpf, the labeling strongly correlated with lens cell membranes (WGA, red), and became restricted to the cortical fiber cell membranes from 5 dpf onward. Antibodies were also tested on mutant lenses, confirming specificity for each ortholog (Figs. 5C, 5D). As is well documented in mammalian lens fiber cells,\textsuperscript{35} C-terminal immunoreactivity of Aqp0a and Aqp0b was lost in mature lens fiber cells. This coincided with degradation of nuclei (blue) in the lens center, as well as loss of WGA signal, indicating the tight compaction of fiber cells to form the lens nucleus.

Anti-Aqp0a (Fig. 5E) or anti-Aqp0b (Fig. 5F) antibodies strongly labeled all cortical fiber cell membranes in adult zebrafish lenses. Immunoreactivity was abruptly lost at the boundary between the inner cortex and lens nucleus, where it was completely absent. The normalized fluorescence intensity of Aqp0a and Aqp0b as a function of normalized distance from the lens center (0.0 is the lens center, and 1.0 is the epithelium) showed that the C-terminal signals for both, Aqp0a and Aqp0b, was lost at an r/a of about 0.65. Thus, Aqp0a and Aqp0b show very similar patterns of when they are first detected, how they insert into the membrane, and how they localize to the periphery.

\textbf{Aqp0a Is Required for Nuclear Centralization and Anterior Suture Stability}

To probe the mechanisms leading to the anterior polar opacification seen in \textit{aqp0a} \textsuperscript{-/-} mutants but not in \textit{aqp0b} \textsuperscript{-/-} mutants, lenses were dissected at four developmental stages and imaged perpendicular to the optical axis (axial orientation; Fig. 6A). Surprisingly, in young WT lenses (3.4–3.7 mm SL) the lens nucleus was initially displaced toward the anterior pole (r/a = 0.2) and later centralized in full-grown adults (r/a = 0.6; Fig. 6B). \textit{aqp0a} \textsuperscript{-/-} and double mutant lens nuclei also started off closer to the anterior pole in young fish; but in \textit{aqp0a} \textsuperscript{-/-} adult mutant lenses, the nucleus failed to centralize and remained shifted toward the anterior even in older fish (r/a = 0.1 at 25 mm SL). In double mutants, nuclear centralization was completely disrupted, and nuclear position varied greatly, with a mean estimated axial nucleus center location of r/a = 0.2 at all stages (Figs. 6A, 6B; Supplementary Figure S5).

Anterior polar opacification and anterior displacement of the lens nucleus could result, at least in part, from defects in the stability of the anterior suture. To assess suture integrity, whole fixed lenses were examined for fiber cell morphology using F-actin immunohistochemistry and cell nuclear position using DAPI staining (Fig. 7). Z-projections of the anterior pole
(Fig. 7A) as well as analysis of optical slices taken 46 μm from the anterior pole (Fig. 7B) revealed that while WT and aqp0b−/− fiber cell morphology was highly ordered, with fiber cell rows meeting in the center to form the anterior suture, aqp0a−/− and double mutant anterior poles displayed a disorganized mass of F-actin labeling as well as ectopic fiber cell nuclei often occupying the position of the anterior suture (Figs. 7A, 7B). Furthermore, deeper in the lens cortex, 76 μm from the anterior pole where WT lenses had no nuclei in the fiber cells, aqp0a−/− and double mutants had many ectopic fiber cell nuclei, while aqp0b−/− mutants also showed the occasional ectopic fiber-cell nucleus (Fig. 7C). Thus, cataracts and nuclear displacement in Aqp0-deficient lenses correlated with disruption of the anterior suture.

**DISCUSSION**

Water transport, adhesion, and cytoskeletal protein anchoring are essential functions provided by AQP0 that ultimately determine lens physiology and optics. It has been impossible to determine how each individual function contributes to lens transparency in mammalian systems, but such a distinction is facilitated in zebrafish by subfunctionalization of duplicate Aqp0s in teleosts.17,18 Here we show by functional knock out of the two zebrafish AQP0 orthologs that Aqp0a, but not Aqp0b, is required for anterior suture stability, consistent with a role for water transport at the suture. By analyzing the time course of the appearance of these suture defects, we also discovered that the zebrafish lens nucleus shifts from an anterior position at younger stages, to a central location in the adult lens and that this shift fails in Aqp0a mutants. We propose that this early lens...
placement anteriorly is required for emmetropia at larval stages and its centralization depends on a stable anterior suture (Fig. 8). These results further support our previous evidence for overlapping but distinct functions for Aqp0a and Aqp0b in the lens and set the stage for future experiments testing the ability of mutant constructs lacking water transport, cytoskeletal regulation, or adhesion to rescue mutant phenotypes to test these hypotheses.

Overlapping Roles for Aqp0a and Aqp0b

A lens still forms in aqp0a/b double mutants, indicating that Aqp0 function is not required for lens specification or

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**FIGURE 3.** Cataracts form in double mutants and large aqp0a<sup>−/−</sup> mutants. Lens opacity assays were performed on live, anesthetized fish at a range of juvenile and adult stages. WT fish had no cataracts at any stage analyzed, even at very large adult stages. Double mutants had cataracts at all stages analyzed: (A) SL 8 to 11 mm (100%, n = 4 fish), 11 to 15 mm (100%, n = 6), 15 to 20 mm (100%, n = 11); (B) 20 to 23 mm (100%, n = 2), > 23 mm (100%, n = 5). At young stages, aqp0a<sup>−/−</sup> or aqp0b<sup>−/−</sup> mutants had no opacity ([A] shown at 15–20 mm SL), while large adult aqp0a<sup>−/−</sup> (> 23 mm) had consistent cataract (100%, n = 8) compared to much lower frequency in aqp0b<sup>−/−</sup> (25%, n = 8, lenses were typically clear as shown in [B]). Excised lenses from aqp0a<sup>−/−</sup> or double mutant large adults had anterior polar opacities (anterior pole is outlined with a yellow dotted line) while double mutants also had nuclear cataracts (C). Please see Supplementary Figure S2 for more examples of double mutant phenotypes.

**FIGURE 4.** Aqp0a is required for normal lens optics. Representative lenses dissected from WT, aqp0a<sup>−/−</sup>, aqp0b<sup>−/−</sup>, or double mutant zebrafish of SL >23 mm imaged through their optical axis (equatorial orientation). Lenses imaged under brightfield (BF; [A]) and darkfield (DF; [B]) illumination reveal a clear lens for WT and aqp0b<sup>−/−</sup> mutants, while aqp0a<sup>−/−</sup> mutants have mild refractive changes between the nucleus and lens periphery. Double mutant lenses have a severe nuclear cataract with cortical refractive problems. (C) Optical properties of lenses were tested by focusing through the lens onto micrometer grids, which revealed poor optical properties of the aqp0a<sup>−/−</sup> and double mutant lenses.
differentiation. However, double mutant lenses grow slower and severe morphologic and optic defects arise earlier than in single-mutant lenses, providing evidence for at least some functional redundancy between the two orthologs, including a conserved role in lens growth. Similarly, mouse lenses lacking AQP0 function have reduced lens diameters and wet weight.36–38 Taken together, the zebrafish and mouse results suggest that Aqp0a and Aqp0b share a common lens development property with mammalian AQP0, possibly water permeability. Reduced size could be a result of decreased water transport into the lens via Aqp0, which in mice accounts for 80% of water permeability of lens fiber cell membranes.36 Thus, mammalian and zebrafish Aqp0s have conserved roles in lens development, likely due to their functions in water permeability.

aqp0a/b double mutants also form cataracts at a higher frequency at 2 dpf than either single mutant (Fig. 2A), suggesting an additive effect of losing both Aqp0s on a common water transport function. Cataracts persist at 3 dpf, when the zebrafish lens nucleus compacts and loses both fiber cell nuclei and organelles (Fig. 5A), and likely becomes emmetropic in WT.39 Strikingly, however, obvious cataracts are no longer visible at 4 dpf in single or double mutants (Fig. 2A). Cataracts induced by morpholino knockdown of aqp0 expression also recover lens transparency at similar stages,17,18 but this recovery was attributed to a gradual loss of morpholino effectiveness. The present results suggest instead that the lens can compensate for the loss of Aqp0 function, at least temporarily, to restore transparency. We speculate that early embryonic cataracts in zebrafish likely reflect transient loss of cell volume regulation—either cell swelling or accumulation of extracellular fluid between fiber cells. In the absence of either or both Aqp0 proteins, this volume disruption is corrected at 4 dpf, likely by another osmoregulatory mechanism. AQP5, the other AQP present in mammalian lenses35,40 would be a logical osmoregulatory candidate. Zebrafish, however, lack a clear AQP5 homolog,41 which has been suggested to have arisen as a terrestrial adaptation associated with hydration of the cornea and nictitating membrane (see Ref. 42 for review).

We observed a lower frequency of cataracts at 3 dpf (~12%) for all mutants compared to previous MO knockdowns of either Aqp0 (~80%).18 This could reflect differences in genetic compensation, off-target effects,43 or MO toxicity and upreg-

**Figure 5.** Expression of Aqp0a and Aqp0b in WT zebrafish lenses; 1 to 3 dpf eye cryosections labeled with (A) anti-Aqp0a or (B) anti-Aqp0b antibodies (green), plasma membrane label wheat germ agglutinin (WGA, red), and DAPI (blue). High power images of Aqp0a or Aqp0b labeling only were taken from regions outlined by yellow-dashed boxes. aqp0a+/− lenses labeled for F-actin (phalloidin, red) and DAPI (blue) as well as with (C) anti-Aqp0a antibody, and aqp0b−/− labeled with (D) anti-Aqp0b antibody serve as negative controls. Adult lens cryosections were labeled with (E) anti-Aqp0a or (F) anti-Aqp0b antibodies (green), and WGA (red). Equatorial lens section is shown with antibody labeling only detected in the cortex. High power images from the outer cortex (o.c.), inner cortex (i.c.), and nucleus (nuc.) are taken from regions outlined by yellow-dashed boxes. (G) Normalized intensity of either Aqp0a (gray) or Aqp0b (yellow) antibody labeling in adult lenses as a function of distance from lens center. Each experiment is representative of at least six independent lenses analyzed.
p53, which can cause stress-induced defects in lens morphology. Rescue of MO-induced cataracts by injection of DNA constructs encoding Aqp0 supports genetic specificity of the knockdowns.

Despite the lack of obvious cataracts at 4 dpf, aqp0a/b double mutant lenses, and to a lesser extent the single mutants, show refractive problems at this stage. This is characteristic of fiber stacking defects (Fig. 2A), similar to that seen in fn1/− zebralsh lenses. An oblique light-source reveals that nuclear cataracts reappear in double mutants beginning at 6 dpf (Supplementary Fig. S2A). In contrast, nuclear cataracts do not form or reappear in single mutants (Figs. 3, 4, 6). Our results suggest overlapping functions for Aqp0a and Aqp0b in early lens development, specifically for stacking and maintenance of the embryonic lens nucleus.

Based on our immunohistochemical analyses, it seems unlikely that the distinct phenotypes of aqp0a/− and aqp0b/− are due to spatial, temporal, or subcellular differences in Aqp0a and Aqp0b protein expression and localization. Furthermore, both show C-terminal signal loss in mature fiber cells. This could be due to inaccessibility of the epitope due to the compaction of fiber cells, but more likely results from proteolytic cleavage and is well-documented for AQP0 in mammalian fiber cells. This may indeed be a common feature of aquatic species, particularly those with free-swimming, feeding larvae that need to develop a functional visual system early in life. Interestingly, sea bream (Acanthopagrus butcheri), like zebralsh, rely on vision for hunting and evading predators as early as 5 to 6 dpf. Due to the short lens-to-retina distance at these early stages, we propose that positioning the high-refractive-index lens nucleus closer to the anterior pole facilitates focusing light onto the retina for emmetropization (Fig. 8A). As the lens grows, the lens-to-retina distance increases, and a centrally located lens nucleus now provides a better focus.

A Novel Role for Aqp0 in Lens Nucleus Centralization

Zebrafish lens development at embryonic and early larval stages, as well as in adults, has previously been well described in the literature. However, developmental changes in the lens between 6 dpf and adulthood have not been systematically documented. We discovered that the lens nucleus first forms at the anterior pole and progressively centralizes with age, a phenomenon not described in the lenses of other species. Centralization requires Aqp0a, but not Aqp0b, confirming subfunctionalization between the two orthologs.

What is the functional significance of this nuclear displacement? Larval zebralsh rely on vision for hunting and evading predators as early as 5 to 6 dpf. Due to the short lens-to-retina distance at these early stages, we propose that positioning the high-refractive-index lens nucleus closer to the anterior pole facilitates focusing light onto the retina for emmetropization (Fig. 8A). As the lens grows, the lens-to-retina distance increases, and a centrally located lens nucleus now provides a better focus.
**Aqp0a Regulates Anterior Suture Stability**

Anterior polar opacity and failure of the lens nucleus to centralize in aqp0a−/− mutants correlates with a defect in anterior pole integrity as assessed by F-actin immunohistochemistry. aqp0a−/− and double mutants exhibit epithelial and fiber cell disruptions that extend into the anterior cortex, while the aqp0b−/− lens anterior pole is unaffected (Fig. 7). This correlates with reduced and disorganized F-actin labeling. Multilayered epithelial cell nuclei also appear to fuse with a mass of underlying fiber cell nuclei in place of the anterior suture. Since younger aqp0a−/− lenses do not display anterior polar opacity, or obvious sutural defects, these results suggest a defect in suture stability as the lens grows.

Why is an AQP0 so critical for anterior suture stability? This is a conserved feature of AQPO since lens sutures fail to form in mouse AQPO−/- lenses, and multilayered anterior epithelia result in anterior polar cataracts in Cat^0^ homozygous lenses (AQPO with a long tandem repeat sequence insertion). Our previous studies suggest that in zebrafish, Aqp0a is primarily a water channel, unlike Aqp0b, suggesting that water transport may be particularly critical at the anterior suture. The rapid elongation of differentiating fiber cell tips is achieved by increases in cytoplasmic volume. The anterior tips of fiber cells may rely on Aqp0a for this influx. Interestingly, swelling of the anterior tips of differentiating lens fiber cells has also been described in Cat^0^ lenses, hinting at similar dysregulation of water transport by Aqp0a at these tips. Future studies using lens vesicles derived from Aqp0 mutant zebrafish lenses will address this issue.

Sutural instability may arise by different mechanisms in fish and mice. In Cat^0^ lenses, sutural disruptions are evident early (postnatal days 1-14), whereas zebrafish lens anterior opacities and anterior pole disruptions only appear at adult stages. AQPO may function as a cytoskeletal anchor shaping sutural structure. Mammalian AQPO interacts with filensin, and such a role is consistent with the defects we see in the actin filament cytoskeleton of aqp0a−/− lenses. Moreover, the actin cytoskeleton is central to lens development and transparency. Future experiments testing the ability of injected mutant constructs that eliminate water transport, cytoskeletal regulation, or adhesion to rescue the phenotypes of aqp0a−/− and double mutants will allow us to test these hypotheses.
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