Zebrafish Optical Development Requires Regulated Water Permeability by Aquaporin 0

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Received: July 15, 2024
Accepted: September 4, 2024
Published: September 27, 2024

Citation: Safrina O, Vorontsova I, Donaldson PJ, Schilling TF. Zebrafish optical development requires regulated water permeability by Aquaporin 0. *Invest Ophthalmol Vis Sci.* 2024;65(11):42. https://doi.org/10.1167/iovs.65.11.42

Purpose. Optical development of the zebrafish eye relies on the movement of the highly refractive lens nucleus from an anterior to a central location in the optical axis during development. We have shown that this mechanism in turn depends on the function of Aquaporin 0a (Aqp0a), a multifunctional and extremely abundant protein in lens fiber cell membranes. Here, we probe the specific cellular functions necessary for rescuing lens nucleus centralization defects in *aqp0a*$^{-/-}$ null mutants by stable overexpression of an Aqp0 orthologue from a killifish, MIPfun.

Methods. We test in vivo requirements for lens transparency and nucleus centralization of MIPfun for auto-adhesion, water permeability (P_f), and P_f sensitivity to regulation by Ca^{2+} or pH by overexpression of MIPfun mutants previously shown to have defects in these functions in vitro or in silico.

Results. Water permeability of MIPfun is essential for rescuing lens transparency and nucleus centralization defects, whereas auto-adhesion is not. Furthermore, water permeability regulation by Ca^{2+} and pH appear residue-dependent, because some Ca^{2+}-insensitive mutants fail to rescue, and pH-insensitive mutants only partially rescue defects. MIPfun lacking P_f sensitivity to both, Ca^{2+} and pH, also fails to rescue lens nucleus centralization.

Conclusion. This study shows that regulation of water permeability by Aqp0 plays a key role in the centralization of the zebrafish lens nucleus, providing the first direct evidence for water transport in this aspect of optical development.

Keywords: Aquaporin 0 (Aqp0a), water permeability, lens development, zebrafish, optical development

Mammalian Aquaporin 0 (AQP0) also known as membrane intrinsic protein (MIP) and formerly known as MIP26, is the most abundant membrane protein in lens fiber cells.[1] The universal importance of AQP0 for lens function is evident by the fact that mutations in AQP0 that disrupt its function result in cataract formation in a variety of species including, humans (summarized in the CATmap[2]), rodents,[3] pandas,[4] and zebrafish.[5] However, determining the exact function of AQP0 in the lens has proven to be difficult because it has emerged that AQP0 is a multifunctional protein, which, in addition to its classical role as a water channel,[6] also plays a role as a junctional protein that mediates cell-to-cell adhesion,[7,8] and as a regulator of other lens proteins, such as Filensin[9] and Connexin 50.[10] This functional complexity is further compounded by the observations that the subcellular distribution of AQP0 in the membrane and its post-translational modification change as a function of lens differentiation and age.[11,12]

In an effort to reduce the complexity associated with studying such a multifunctional protein, we have turned to the zebrafish (*Danio rerio*) as an alternative model system to study the relative contributions of mammalian AQP0 as a water channel and adhesive protein to overall lens transparency and the development of lens power. Similarities between the anatomy and early development of the eye between zebrafish and humans make it a useful model for lens research.[13–18] Furthermore, through an evolutionary whole genome duplication event, the zebrafish lens expresses two *aqp0* genes, *aqp0a* and *aqp0b*. In previous studies, we confirmed the subfunctionalization of the two zebrafish Aqp0s.[5,19,20] Both Aqp0s are necessary for early larval lens clarity,[5,19] but, in adults, only Aqp0a is required for a transparent lens.[5] Aqp0b, but not Aqp0a, has auto-adhesive properties in vitro,[21] which appear dispensable as *aqp0b*$^{-/-}$ null mutants look like wild type from larval stages to adulthood.[5] Both Aqp0a and Aqp0b can permeate water in vitro,[22] but in vivo Aqp0a plays a key role in water influx to maintain water homeostasis in larval lenses, whereas the role of Aqp0b in water efflux is only revealed when Aqp0a is missing.[20] Aqp0a is essential for anterior lens suture stability at older stages, and for normal lens nucleus centralization.[5]

In the wild type zebrafish lens, we have shown that a shift in the position of the high-refractive lens nucleus occurs from an initial anterior location in the optical axis at young larval stages to a central location in older juveniles.[5] This centralization of the nucleus produces a shift of the maximum gradient of refractive index, which results in a change in lens power that ensures light remains correctly focused on

the retina as the zebrafish grows. We hypothesize that this process is driven by a change in water permeability as the loss of Aqp0a, but not Aqp0b, inhibits nucleus centralization. In humans, a similar change in lens power occurs during childhood visual development. After birth, dramatic changes in lens shape,[23] volume, and refractive power are required if the children are to become emmetropic and correctly focus light onto the retina. Hence, the zebrafish lens represents a powerful model to conduct experimental studies into the roles of Aqp0 in not only the maintenance of lens transparency, but also the potential role of regulation of Aqp0-mediated water transport in the process of emmetropization.

Previous studies of the regulation of mammalian Aqp0 function, both in *Xenopus laevis* oocyte water permeability assays as well as molecular models of channel dynamics, have identified key residues thought to be required for the regulation of the water permeability of Aqp0. Auto-adhesion properties of the Aqp0s have also been tested in an in vitro assay.[21] Furthermore, overexpression of full-length Aquaporin 0 (MIPfun) from a killifish (*Fundulus heteroclitus*), which retains the various functions of mammalian Aqp0 and has a higher water permeability[24] similar to the zebrafish Aqp0s, has been shown to be effective in rescuing embryonic cataracts caused by transient knockdown of either Aqp0a or Aqp0b.[19] Subsequent analysis of water homeostasis based on macromolecular crowding in fiber cells suggests that this rescue occurs most likely by restoring water influx in the lens cortex of *aqp0a*$^{-/-}$ mutants and by restoring water influx and efflux in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants.[20] However, how these Aqp0-dependent processes are regulated in fiber cells in vivo to maintain lens transparency and drive nuclear centralization, cannot be determined using such in vitro assays. To achieve this, we performed rescue experiments by stably overexpressed various MIPfun mutants (see Table 1) in zebrafish carrying loss-of-function mutations in *aqp0a* or *aqp0a/aqp0b* and tested

Table 1. Rationale for Design of Rescue Constructs Used in This Study

Wild Type In Vitro Properties

Protein	Test Function	P_f	Auto-Adhesion	Known Properties and Rescue
Aqp0a	N/A	pH 8.5 – high P_f[22]	✗	P_f is higher than in mammalian AQP0 in vitro.[34] Appears to be primary water influx water channel[20] with a particular role at the anterior pole[5]
Aqp0b	N/A	pH 6 – high P_f; pH 8.5 – low P_f[22]	√	P_f is higher than in mammalian AQP0 in vitro (similar levels to Aqp0a[22])
MIPfun	Rescue of Aqp0a and Aqp0b loss	0 Ca^{2+} – high P_f; pH 8.5 – high P_f[32]	√	P_f is higher than in mammalian AQP0 in vitro.[32] Mosaic expression rescued 3 dpf cataract caused by Aqp0a and/or Aqp0b knock-down;[19] or 4 dpf water homeostasis defect in *aqp0a*$^{-/-}$ or partially rescued defects in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ mutants.[20] MIPfun is thought to restore functions of both Aqp0a and Aqp0b

MIPfun Mutant Rescue Constructs

Protein	Test Function	P_f	Auto-Adhesion	Rationale/Phenotype in Previous Rescue Experiments
MIPfun N68Q	P_f	No P_f[19]		N68 is part of the first NPA domain conserved among all AQPs to block protons.[40] MIPfunN68Q results in no P_f, and mosaic expression fails to rescue Aqp0a knockdown 3 dpf cataract[19] or 4 dpf water homeostasis defect in *aqp0a*$^{-/-}$ mutants.[20]
MIPfun N110T	Auto-adhesion	Loss of auto-adhesive function[21]	✗	Zebrafish Aqp0b, like mammalian AQP0[7,8] relies on site 110 for its auto-adhesive functions as shown in adhesive deficient L-type mouse fibroblast cell adhesion assays.[21] Mammals have a P, zebrafish Aqp0b and killifish has an N, and zebrafish Aqp0a has a T in this position.
MIPfun Y75G	P_f Ca^{2+} regulation	0 Ca^{2+} – no P_f change[19]		In mammalian AQP0, the Ca^{2+} sensitive T149 interacts with Y75 to regulate the size of the pore at constriction site 2.[41] MIPfunY75G has no 0 Ca^{2+} P_f sensitivity (position 149 is swapped with 75 in fish), and when injected into zebrafish embryos fails to rescue Aqp0a knockdown cataract.[19]
MIPfun R153A	P_f Ca^{2+} regulation	0 Ca^{2+} – no P_f change (tested for bAQP0)[33]		The cytosolic 150-156 arginine-rich D-loop couples interaction of calmodulin to the constriction site 2. Bovine AQP0 R153A locks the P_f low to abolish increase to 0 Ca^{2+},[33] which is conserved in MIPfun.
MIPfun N40H	P_f pH regulation	Loss of pH regulation by high or low pH (by homology to Salmon Aqp0b2[22])		Tetraploid Atlantic salmon Aqp0b2 which has H at both positions 39 and 40 has no pH sensitivity to permeability. Mutation of either HH to N, restores pH sensitivity for Aqp0b2.[22] Based on these results MIPfunN40H should abolish acidic and alkaline sensitivity.
MIPfun R33L	P_f pH and Ca^{2+} regulation	0 Ca^{2+} – no P_f change; loss of P_f regulation by high or low pH (by homology to bAQP0; see Supplementary Fig. S3)		This study showed that AQP0R33L loses pH (alkaline or acidic) and 0 Ca^{2+} P_f regulation sensitivity. By homology MIPfunR33L is expected to have the same effect.

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the relative contributions of water transport and adhesion to the maintenance of lens transparency and lens nucleus centralization. Using this approach, we show that the regulation of Aqp0 water permeability is the key parameter associated with driving nuclear centralization during visual development during larval stages and for the maintenance of lens transparency in adult fish.

Methods

Zebrafish Husbandry

Animal protocols used in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and have been approved by the Institutional Animal Care and Use Committee of University of California, Irvine protocol no. AUP-23-099. Zebrafish lines including the wildtype AB strain and mutants made in that background were maintained under standard laboratory conditions. Zebrafish were euthanized in tricaine (MS-222; Sigma-Aldrich, St. Louis, MO, USA) before lens dissection.

Mutants and Transgenic Lines

Previously characterized maternal-zygotic (MZ) homozygous single null *MZaqp0a*$^{-/-}$, and double *MZaqp0a*$^{-/-}$/*MZaqp0b*$^{-/-}$ mutants were used[5] for overexpression of rescue transgenes. For rescue of mutant phenotypes, the Tol2 transposable element system[25] was utilized to create *Tg(HuβB1cry:MIPfun-IRES-mCherry)* constructs with a 200–base pair fragment of the human βB1 crystallin promoter[26] to drive expression of *Fundulus heteroclitus* Aqp0 (MIPfun) specifically in the lens. The Tol2 constructs expressing wildtype MIPfun are referred to in this paper as "MIPfun" or MIPfun with point mutations (Table 2), synthesized by PCR mutagenesis (Quik Change II XL Site-Directed Mutagenesis Kit; Agilent Technologies), were injected into single *aqp0a*$^{-/-}$ or double *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ mutants. Stably expressing transgenic (F2) were generated to reduce phenotypic variability. This was achieved by screening of injected F0s for positive germline integration using the mCherry marker and outcrossing with *aqp0a*$^{-/-}$ or *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ correspondingly. F1s carrying the transgene were outcrossed again and F2 stable mutants were raised for comparison of phenotypes with control mutant lines. The various F2s had similar levels of mCherry expression suggesting similar levels of plasmid expression. Because zebrafish grow at different rates depending on food availability and animal density, we used standard length (SL) to determine developmental stage.[27,28] We have previously shown a strong correlation between SL and zebrafish lens growth,[5] as well as lens maximum refractive index.[29] In this study, 7 developmental stages were assessed starting from SL = 3 to 5 mm (5 dpf) to SL > 20 mm (up to ~14 months of age). This encompassed the process of centralization of the lens nucleus, and allowed us to assess the effects of rescue construct overexpression on larval, juvenile, and adult lens phenotypes. At least six zebrafish (12 lenses) per genotype were assessed at each developmental stage.

Lens Dissection and Transparency Analysis

Lenses were dissected as previously described[5,30] and imaged using a stereo-microscope (Olympus SZX12F; Olympus Corp., Tokyo, Japan) with a dynamic positioning controller (Olympus DP70 with a model 2.1.1.183 controller; Olympus Corp., Tokyo, Japan). To analyze cataract or transparency defects, lenses were oriented equatorially (through the optical axis) and illuminated by bright field or dark field microscopy. To test focusing ability, a microgrid field finder (Lovins Field Finder; Electron Microscopy Sciences, Hatfield, PA, USA) was focused through the optical axis of a lens. Images were compiled using ImageJ, version 1.51n (http://imagej.nih.gov/ij/; National Institutes of Health [NIH], Bethesda, MD, USA) and a raster graphics editor (Adobe Photoshop CS5, version 12.0; Adobe Systems, San

Table 2. Construct/Mutant Summary

		Ability to Rescue Phenotypes (Y = Yes, N = No, – Data Were Not Collected)			
Protein	**In Vitro Properties**	**3 dpf Cataract Caused by Aqp0a Knockdown[19]**	**Water Homeostasis Defect in 4 dpf *aqp0a*$^{-/-}$ Null Mutant[20]**	**Adult Lens Transparency Defects in *aqp0a*$^{-/-}$ Null Mutants (This Study)**	**Adult Lens Nucleus Centralization Defects in *aqp0a*$^{-/-}$ Null Mutants (This Study)**
Aqp0a	pH 8.5 – high P_f[22]	N/A	N/A	N/A	N/A
MIPfun	0 Ca^{2+} – high P_f; pH 8.5 – high P_f[32]	Y	Y	Y	Partial, almost to wild type levels
MIPfun N68Q	No water permeability in vitro[19]	N	N	N (worse)	N
MIPfun N110T	Loss of auto-adhesive function[21]	—	—	Y	Partial
MIPfun Y75G	0 Ca^{2+} – no P_f change[19]	—	—	N (worse)	N
MIPfun R153A	0 Ca^{2+} – no P_f change (by homology to bAQP0)[33]	—	—	Y	Mid-old stage rescued
MIPfun N40H	Loss of pH regulation by high or low pH (by homology from Salmon Aqp0b2[22]	—	—	Partial	Young and old stages rescued
MIPfun R33L	0 Ca^{2+} – no P_f change; loss of P_f regulation by high or low pH (by homology to bAQP0; see Supplementary Fig. S3)	—	—	N (worse)	N

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Jose, CA, USA). Lens transparency was assessed subjectively and categorized as normal, mild, or severe cataract. Minor transparency defects were classified separately, including ring-like cataract phenotypes or loss of transparency in the lens cortex. Phenotype frequency analyses of the lenses by developmental stage are presented in Supplementary Figure S1.

Lens Nucleus Centralization

We used previously established methods to determine relative localization of the lens nucleus in the optical axis.[5,30] Briefly, dissected lenses were oriented perpendicular to the optical axis with poles and sutures parallel to the plane of focus (axial orientation). The distance of the center of the lens nucleus to the center of the lens (r) was normalized as a function of the lens radius (a) (Fig. 1E). The normalized center of the lens nucleus localization (r/a) was graphed as a function of zebrafish standard length, where r/a 0.0 indicates the center of the lens and 1.0 is the lens surface.

Xenopus Laevis Oocyte Permeability Assays

Previously established protocols were used[31] to measure permeability in *Xenopus laevis* oocytes. Wildtype MIPfun was cloned into a transcription vector (pXBG) driven by the T3 transcription promoter. Oocytes from *Xenopus laevis* were obtained from Ecocyte (Austin, TX, USA) and injected typically with 10 ng of RNA encoding wild type MIPfun or MIPfun with a point mutation of R33L generated using the mMessage mMachine T3 kit (Ambion/Life Technologies), as described previously.[32] The oocytes were incubated in 100% ND96 with either control 1.8 mM Ca^{2+} (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and pH 7.5) or with pH adjusted to 6.5 or 8.5 to test effects of pH or 0 Ca^{2+} ND96 (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, and pH 7.5). Oocytes were incubated in the test 100% ND96 for 5 minutes before the swelling assay, which were performed at room temperature (20–21°C) by transferring oocytes from a 200 mOsm to a 70 mOsm (30% (v/v) ND96) solution adjusted to the desired Ca^{2+} concentration or pH. Water permeability, P_f, was calculated from optical measurements of the increase in cross-sectional area of the oocyte with time in response to diluted ND96 using: $P_f = ((d(V/V_0)/dt)(V_0/S_0))/(\Delta_{osm}V_w)$ where V is the volume as a function of time, V_0 is the initial volume, S_0 is the geometric surface area at time 0, Δ_{osm} is the osmotic gradient, and V_w is the molar volume of water.

Statistical Analysis

To test for statistical differences in lens nucleus localization between genotypes at different stages of development, a Generalized Estimating Equation model on the mean log Normalized Axial Nucleus assuming an exchangeable correlation structure was applied with one to two observations on each animal. Data were graphed as the mean normalized relative localization of the lens nucleus (r/a), with values closer to 0.0 representing the lens nucleus being exactly centrally placed in the optical axis of the lens, whereas values increased further from 0.0 indicate that the lens nucleus is closer to the anterior pole. Loess polynomial smoothers (and corresponding 95% confidence intervals) were used to indicate trends in data. Two methods were used to test for differences between localization of the lens nucleus. First, pairwise comparisons between slopes and for three cross-sectional groups by standard length. The P values > 0.05 were deemed statistically significantly different. False discovery rate (FDR) was applied to correct for multiple comparisons (full analysis is presented in Supplementary Table S1). Second, cross-sectional comparisons of the mean log normalized axial nucleus at 3 different cross sections of standard length 1.96 mm, 12.87 mm, and 23.77 mm ± 1.5 standard deviation (SD; Supplementary Fig. S2) was carried out and FDR applied to correct for multiple comparisons. Statistical analyses are shown in Supplementary Tables S2 to S4. To test the probability that means of the *Xenopus laevis* oocyte permeability assays are different between different groups, a pairwise student *t*-tests on all of the data in each data set using the R (R-project) function was performed. We show P values for appropriate conditions in the legends of Supplementary Figure S3.

Results

Our experimental design involved determining if stable transgenic overexpression of either wild type MIPfun or various MIPfun mutants (see Table 1) carrying loss-of-function mutations in Aqp0a or Aqp0b can maintain lens transparency in older zebrafish and/or restore lens nucleus centralization in larval stages. Throughout the results section, we present lens transparency phenotypes first, followed by lens nucleus centralization results. *aqp0a*$^{-/-}$ and *aqp0a*$^{-/-}$*MIPfun* from Figure 2 will serve as negative and positive controls, respectively, for ease of comparison with transgenic rescue phenotypes in Figures 3 to 6.

Stable Misexpression of Wild Type MIPfun Partially Rescues Aqp0a and Aqp0a/Aqp0b Loss of Function Opacity and Lens Nucleus Centralization Defects in Zebrafish

We previously showed that transient misexpression of a full length *Fundulus heteroclitis* Aquaporin 0 (MIPfun) can rescue early larval cataract in zebrafish caused by knockdown of Aqp0a or Aqp0b.[19] Mosaic overexpression of MIPfun in loss-of-function *aqp0a*$^{-/-}$ or *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ mutants also partially rescued early larval water homeostasis defects.[20] To test if expression of a full length MIPfun can rescue loss-of-function mutant phenotypes at older developmental stages, we analyzed lens transparency and lens nucleus localization of *aqp0a*$^{-/-}$ or *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double null mutants stably expressing *Tg(HuβB1cry:MIPfun-IRES-mCherry)*, hereafter referred to as "MIPfun". Transgenic expression of MIPfun partially rescued lens transparency and minor transparency defects in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants, which typically included dense nuclear cataract and loss of lens transparency from larval stages to adulthood (see the juvenile stage shown in Fig. 1B). The *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants overexpressing MIPfun showed no obvious cataracts, only mild defects, and poor ability to focus a microgrid (see Fig. 1C).

Lens nucleus centralization is part of normal zebrafish lens development, and this process fails in *aqp0a*$^{-/-}$ single or *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants, where the lens nucleus remains localized closer to the anterior pole in the optical axis.[5] MIPfun overexpression improved lens nuclear centralization in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants between SL = 9 and 17 mm (see Figs. 1D–1G), albeit not to

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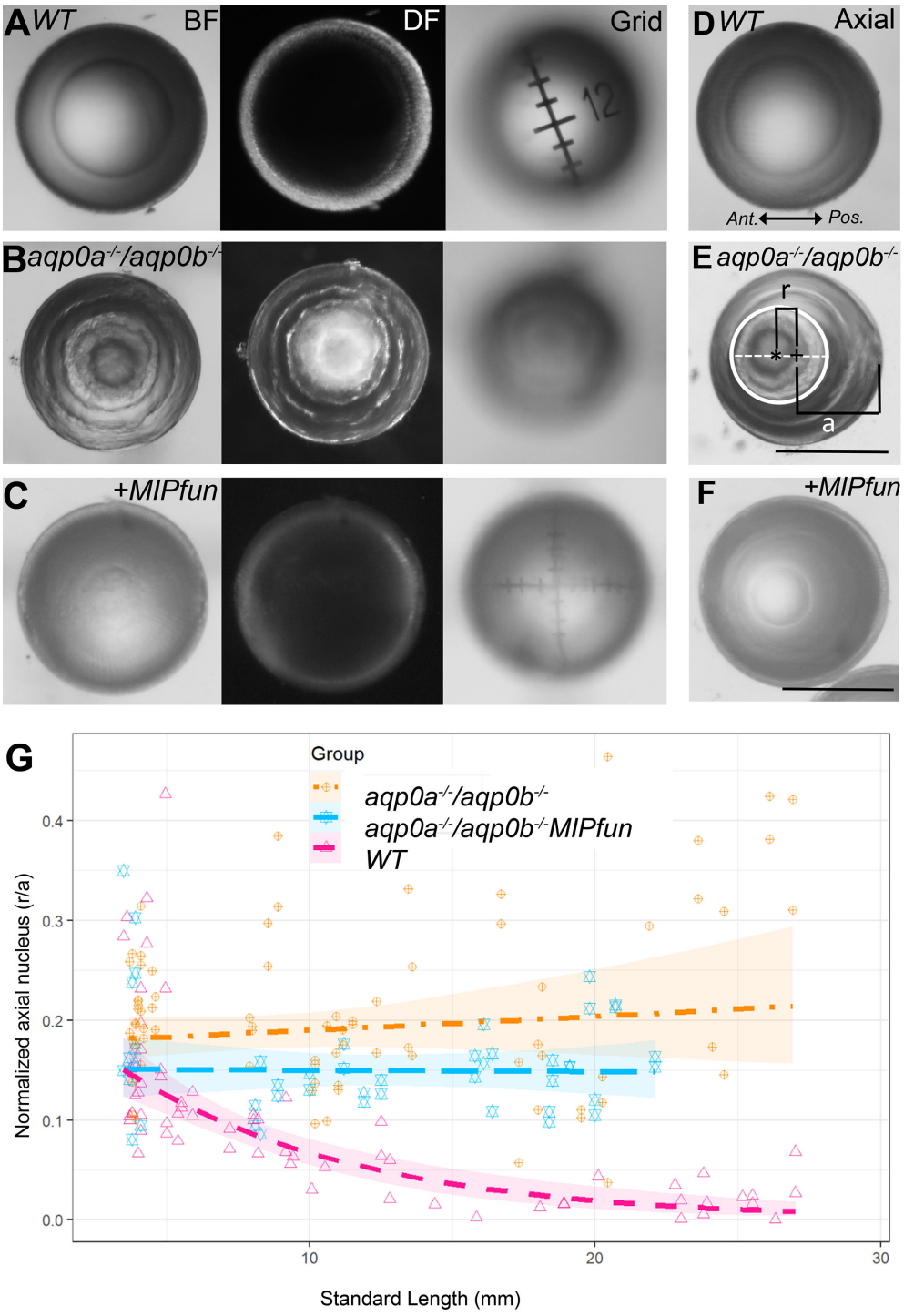

Figure 1. MIPfun partially rescues lens transparency, optics and lens nucleus centralization in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants. Representative juvenile lenses dissected from wild type (**A, D**) (standard length (SL) 9.2 mm), *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ (**B, E**) (SL 11 mm) or transgenic *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ zebrafish stably overexpressing MIPfun (**C, F**) (SL 11.2 mm) are shown. Wild type lens imaged through the optical axis under brightfield (BF) or darkfield (DF) illumination are clear (**A**), whereas *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ double mutants lens (**B**) reveal severe nuclear cataract with cortical transparency defects and inability to focus a microgrid which is reduced with overexpression of MIPfun due improved refraction and focal ability (**C**). The same lenses from **A** to **C** were imaged perpendicular to the optical axis and used to display the relative localization of the lens nucleus (**D–F**). The measurement of the localization of the lens nucleus center in relation to the lens center is shown (**E**), where * = lens nucleus center, + = lens center, a = lens radius, and r = distance of center of lens nucleus from lens center. (**G**) Overexpression of MIPfun in *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ resulted in a more centrally localized lens nucleus compared with *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ at mid-age (SL = 9–17 mm), with no differences at shorter SL (<9 mm) or longer SL (>17 mm) stages. The mean axial nucleus localization at a mean SL = 12.87 mm ± 1.5 SD of normalized lens nucleus was statistically significantly different ($P = 0.001$) between the 2 groups, whereas there was no difference in the 2 slopes ($P = 0.589$). Scale bars = 250 µm.

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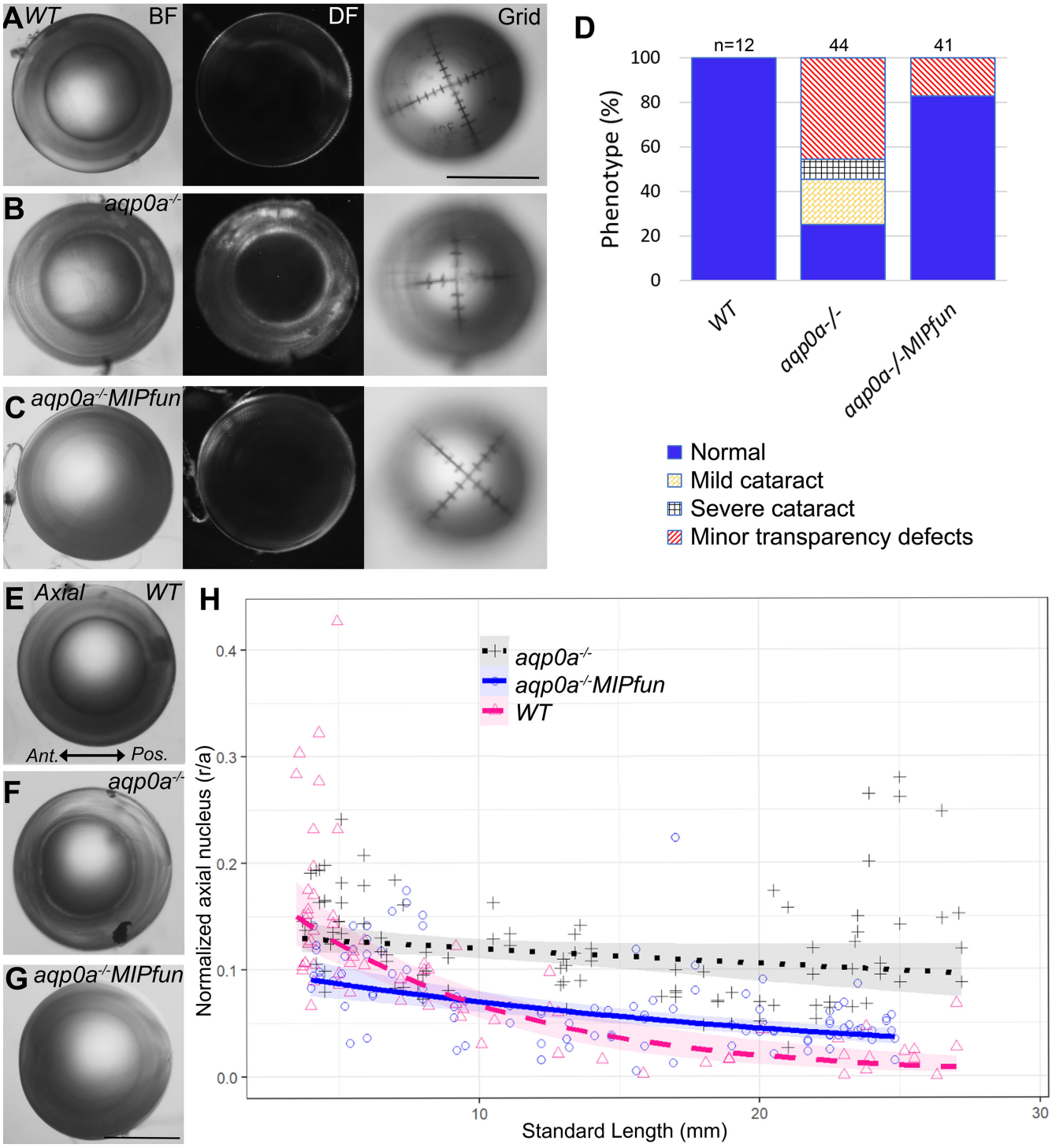

Figure 2. MIPfun partially restores lens transparency, optics, and nucleus centralization due to loss of Aqp0a. Representative lenses dissected from adult wild type (**A**) (SL = 20.1 mm), aqp0a$^{-/-}$ (**B**) (SL = 23.8 mm), and aqp0a$^{-/-}$MIPfun transgenic zebrafish (**C**) (SL = 24.7 mm) imaged in equatorial orientation (through the optical axis) under brightfield (BF) or darkfield (DF) illumination reveal improved transparency in lenses expressing MIPfun (**C**) compared to aqp0a$^{-/-}$ (**B**). Similar improvement was seen in the ability of lenses to focus a grid. (**D**) Frequency of abnormal phenotypes in lenses from adult zebrafish (SL > 20 mm) was reduced in aqp0a$^{-/-}$ overexpressed MIPfun, with a complete loss of cataract. Refer to Supplementary Figure S1 for phenotype frequency by developmental stage. The same lenses from **A** to **C** were imaged perpendicular to the optical axis displayed the relative localization of the lens nucleus (**E–G**). (**H**) Overexpression of MIPfun results in the lens nucleus centralizing (closer to 0.0) more similar to wild type, compared to aqp0a$^{-/-}$ mutants, where the lens nucleus fails to centralize during development. The mean crossectional lens nucleus localization is statistically different at longer SLs between aqp0a$^{-/-}$ and transgenics overexpressing MIPfun ($P = 0.000$) as well as a difference in slopes (0.003). There is also a statistically significant difference between wild type and aqp0a$^{-/-}$MIPfun slopes ($P = 0.001$) and at the shortest ($P = 0.003$) and longest ($P = 0.002$) SL cross-sectional means indicating partial rescue. Scale bars = 500 µm.

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wild type levels indicating partial recovery of the function required for this process.

Microscopic observations of dissected lenses from *aqp0a*$^{-/-}$ mutants revealed mostly normal lens transparency in zebrafish of SL < 20 mm (see Supplementary Fig. S1), consistent with our previous studies.[5] We observed transparency defects in approximately 40% of mutants at SL = 20 to 23 mm that included obvious ring-like cataract at the nucleus-cortex interface and opacity at the lens periphery, with cataract frequency increasing to 40% in fish SL > 23 mm (see Supplementary Fig. S1, Figs. 2B, 2F, Supplementary Fig. S5). In contrast, wild type and MIPfun-expressing lenses had no visible cataract at SL > 20 mm (see Supplementary Fig. S1, Figs.2A, 2C, 2D). These improvements in lens clarity and optics validate the use of MIPfun to study molecular functions that are essential for lens nucleus centralization in *aqp0a*$^{-/-}$ mutants.

The *aqp0a*$^{-/-}$ mutant lens nuclei remained localized closer to the anterior pole at all stages, compared with wild type in which the lens nucleus gradually shifted from an initial anterior to a central location (see Figs. 2E–H). These data are consistent with previous results showing a failure of the lens nucleus to centralize in Aqp0a-deficient lenses.[5] MIPfun overexpression partially rescued the defect in lens nucleus centralization of *aqp0a*$^{-/-}$ shifting the lens nucleus to be more central in the optical axis at older stages (see Figs. 2G, 2H). Thus, stable expression of MIPfun rescued function/s of Aqp0a required for normal transparency and optics by restoring the position of the lens nucleus, providing a useful tool for deciphering specific Aqp0a cellular functions required for these processes. Previous studies of bovine AQP0, zebrafish and Atlantic salmon Aqp0s have pinpointed key residues required for specific functions of Aqp0, which we systematically tested for requirements in rescuing *aqp0a*$^{-/-}$ mutant lenses.

Auto-Adhesion by MIPfun is Not Required to Rescue Lens Defects in *aqp0a*$^{-/-}$ Mutants

Previous in vitro studies of zebrafish Aqp0s have shown an arginine at position 110 in Aqp0b is critical for its role in cell adhesion, whereas Aqp0a T110 has poor auto-adhesive function.[21] To test if auto-adhesive functions of MIPfun are required to rescue *aqp0a*$^{-/-}$ mutant defects, MIPfun altered in this conserved residue (N110T)[21] was stably (F2) overexpressed in *aqp0a*$^{-/-}$ mutants. *aqp0a*$^{-/-}$*MIPfunN110T* genetically modified lenses were clear and transparent, similar to WT or *aqp0a*$^{-/-}$*MIPfun* transgenics, in contrast to *aqp0a*$^{-/-}$ lenses (see Figs. 3A–D). Lens nuclei centralized in *aqp0a*$^{-/-}$*MIPfunN110T* overexpressing lenses similar to wild type (see Figs. 3E–H). These results suggest that Aqp0-mediated auto-adhesion is not important for lens transparency and lens nucleus centralization.

Water Transport by MIPfun is Required to Rescue Aqp0a-Deficient Lens Defects

To test if water transport via MIPfun is required for lens development and nuclear centralization in zebrafish, we expressed MIPfunN68Q, previously shown to eliminate water permeability in *Xenopus laevis* oocyte assays.[19] Stable overexpression of MIPfunN68Q in zebrafish *aqp0a*$^{-/-}$ mutants increased the frequency and severity of cataract compared to *aqp0a*$^{-/-}$ mutants at all stages, resulting in 100% abnormal lens phenotypes in zebrafish from SL > 11 mm (see Figs. 4A, 4C, 4D, Supplementary Fig. S1). The lenses overexpressing MIPfunN68Q also failed to focus light through a microgrid, similar to *aqp0a*$^{-/-}$ lenses (see Figs. 4A, 4C, right panels). These results support previous findings where MIPfun required water permeability to rescue cataract at 3 dpf resulting from Aqp0a knockdown[19] as well as being required to rescue water homeostasis defects in *aqp0a*$^{-/-}$ mutants.[16]

MIPfunN68Q overexpression in *aqp0a*$^{-/-}$ failed to rescue the lens nucleus centralization defect, with the nucleus even more anteriorly placed at younger stages than in *aqp0a*$^{-/-}$ (see Figs. 4E–H). This suggest that water permeability is an essential function of MIPfun for lens nucleus centralization during development.

Residues Involved in Water Permeability Regulation are Essential for MIPfun to Rescue Lens Defects *in aqp0a*$^{-/-}$ Mutants

Studies of regulation of AQP0/MIPfun water permeability, both in oocyte assays and molecular simulations, have identified key residues that sense changes to pH or Ca^{2+} that alter AQP0 permeability.[6] To gain insights into how such regulation of Aqp0a water permeability contributes to lens transparency and nucleus centralization, we stably overexpressed MIPfun with mutations predicted to perturb regulation of water permeability by external Ca^{2+} and/or pH. First, we tested MIPfun mutated at either Y75G or R153A. Mutation Y75G has previously been shown to abolish sensitivity to zero external Ca^{2+} in *Xenopus* oocyte experiments.[19] R153A has been predicted from bovine AQP0 experiments to have the same response in MIPfun.[33]

Similar to MIPfunN68Q, MIPfunY75G failed to rescue lens defects in the *aqp0a*$^{-/-}$ mutants and increased the frequency of cataract with 100% abnormal lens phenotypes in zebrafish > 11 mm SL (see Figs. 5C, 5E; Supplementary Fig. S1). In contrast, stable transgenic expression of a MIPfunR153A construct in *aqp0a*$^{-/-}$ mutants rescued the lens phenotypes to the same frequency as MIPfun, with approximately 80% normal lenses in zebrafish > 20 mm SL, suggesting that this residue is less important in water regulation.

Similar to the lens transparency defect, MIPfunY75G failed to rescue the nuclear centralization defect in *aqp0a*$^{-/-}$ mutants, whereas MIPfunR153A rescued lens nucleus centralization, but only at mid and older stages (approximately SL > 12 mm). At SL < 12 mm, the 2 rescues have similar values of r/a for lens nucleus centralization to *aqp0a*$^{-/-}$. These data support the hypothesis that regulation of Aqp0's water permeability is required for lens development and maintenance in vivo, however, the exact role of Ca^{2+} in regulating is nuanced.

In order to test requirements for residues implicated in pH regulation of water permeability, we expressed MIPfun with an N40H mutation expected to abolish acidic and alkaline sensitivity of zebrafish Aqp0a based on studies of Atlantic salmon Aqp0b2, which has no sensitivity to external acidic or alkaline pH.[22] Wild type zebrafish Aqp0a permeability increases in response to pH 8.5.[22] Another mutation, MIPfunR33L, has been suggested to abolish both Ca^{2+} and pH regulation based on homology to bovine AQP0 and oocyte swelling assays (see Supplementary Fig. S3.)

Although a subset of lenses in which we overexpressed MIPfunN40H showed defects at all ages (see Fig. 6C,

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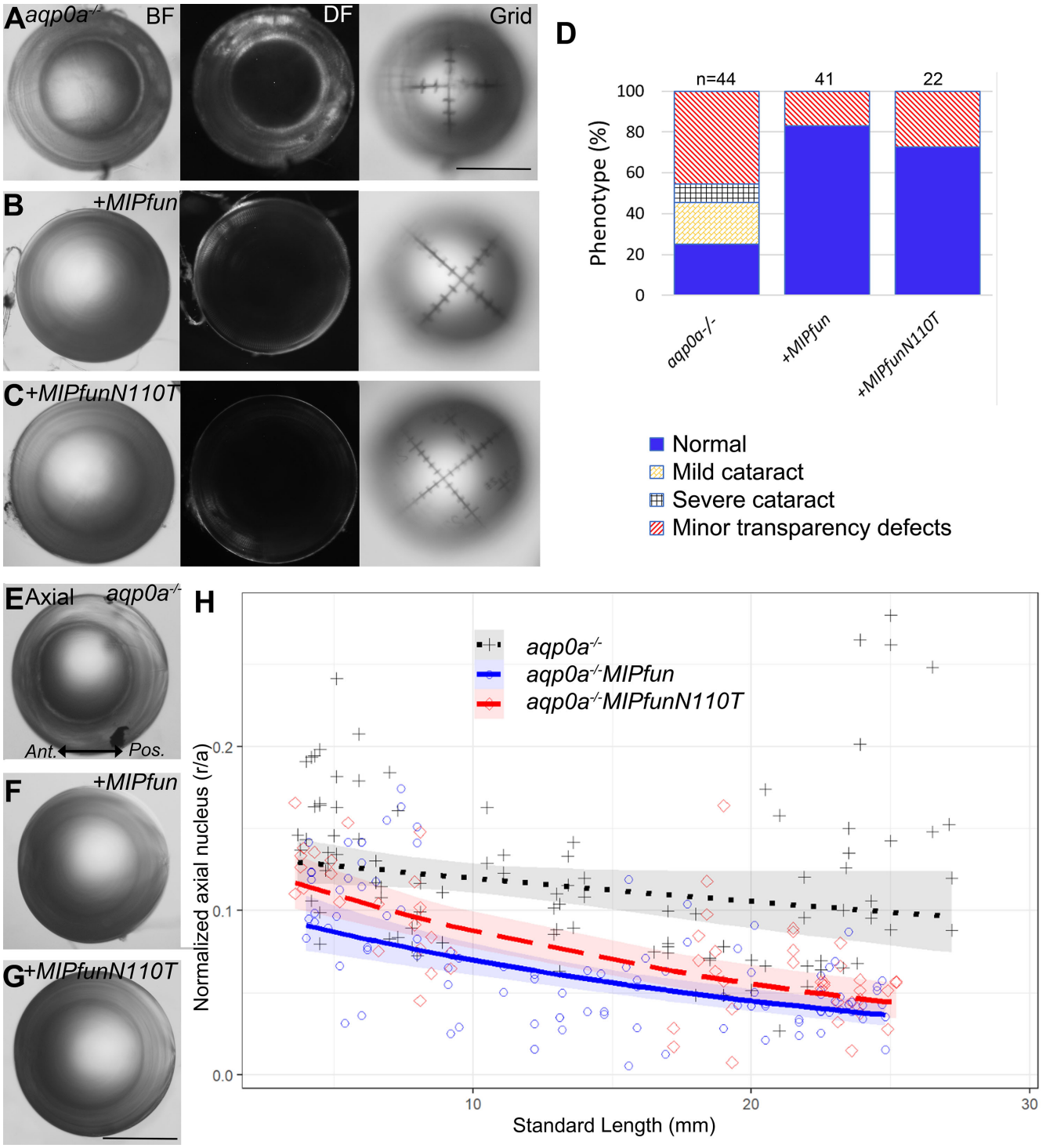

Figure 3. Auto-adhesion by MIPfun is not required for lens transparency, optics, and lens nucleus centralization. Representative lenses from adult *aqp0a*$^{-/-}$ mutants (**A**) (SL = 23.8 mm), *aqp0a*$^{-/-}$ mutants overexpressing MIPfun (**B**), (SL = 24.7 mm) or *aqp0a*$^{-/-}$ mutants overexpressing an auto-adhesive defective MIPfunN110T (**C**) (SL = 23.9 mm) were imaged through the optical axis under bright field (BF) or dark field (DF) illumination. Lens defects evident in *aqp0a*$^{-/-}$ mutants (**A**), as well as the optical defects when focusing a grid. The optical properties were improved by overexpression of MIPfun (**B**) or MIPfunN110T (**C**). (**D**) Both MIPfun or MIPfunN110T increased the frequency of normal phenotypes in adult fish SL > 20 mm, with no cataract observed. Refer to Supplementary Figure S1 for phenotype frequency by developmental stage. The same lenses from **A** to **C** were imaged perpendicular to the optical axis displayed the relative localization of the lens nucleus (**E–G**). (**H**) Overexpression of MIPfunN110T results in the lens nucleus centralizing closer to 0.0) like seen in MIPfun, compared to *aqp0a*$^{-/-}$ mutants, where the lens nucleus fails to centralize. The mean crossectional lens nucleus localization is statistically different at mid and longer SL between *aqp0a*$^{-/-}$ and in transgenics overexpressing MIPfunN110T ($P = 0.000$), as is the slope of the trends ($P = 0.001$). At shorter SL, the mean nucleus localization is similar between *aqp0a*$^{-/-}$ and MIPfunN110T overexpressing mutants. Scale bars = 500 µm.

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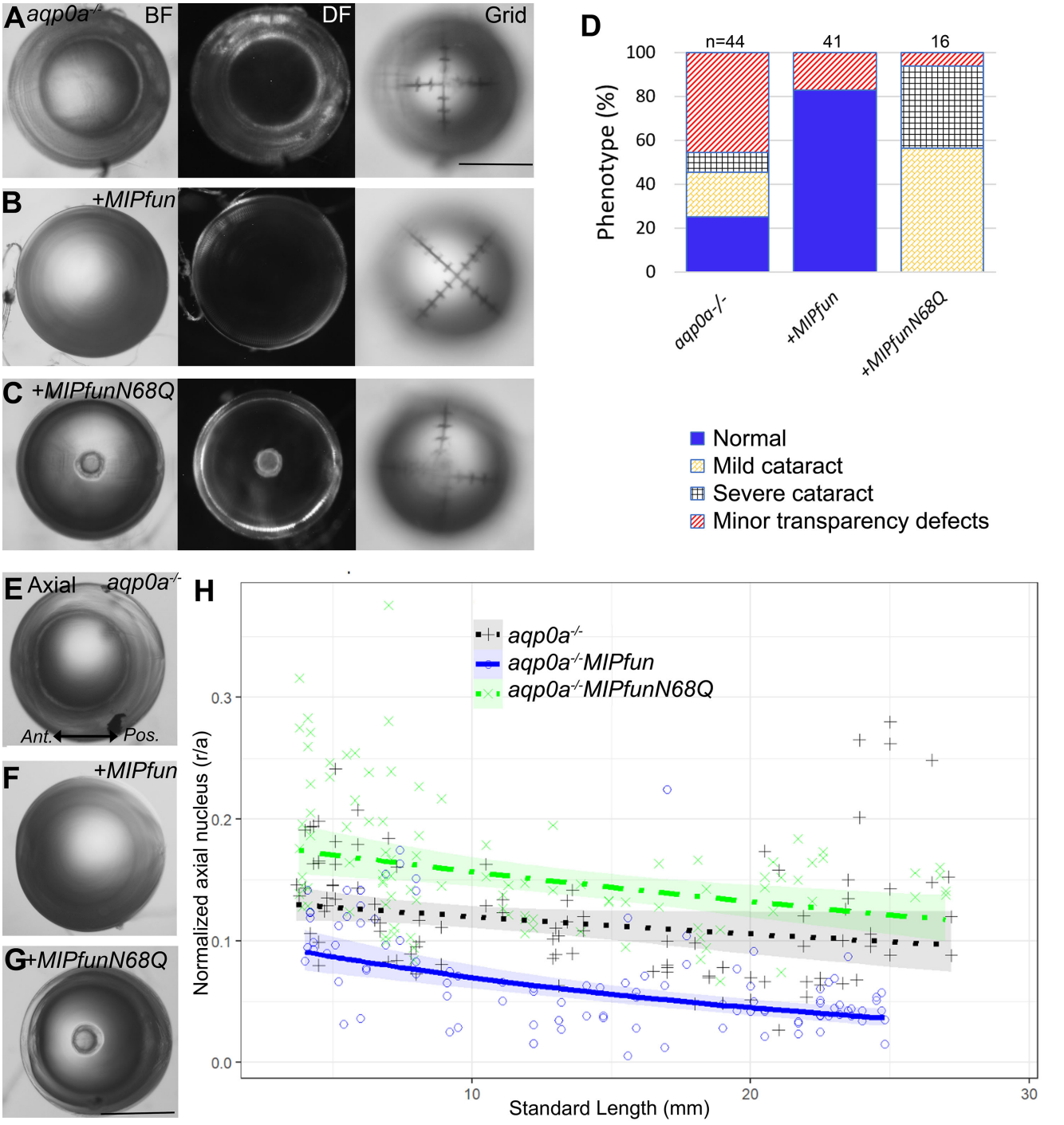

Figure 4. Water transport of MIPfun is required for lens transparency, optics, and lens nucleus centralization. Representative lenses from adult *aqp0a*$^{-/-}$ mutants (**A**) (SL = 23.8 mm), *aqp0a*$^{-/-}$ mutants overexpressing MIPfun (**B**) (SL = 24.7 mm) or *aqp0a*$^{-/-}$ mutants overexpressing a water transport defective MIPfunN68Q (**C**) (SL = 25.9 mm) were imaged through the optical axis under bright field (BF) or dark field (DF) illumination. The ability to focus was tested by focusing through the lens onto a grid. A dense nuclear cataract, poor transparency in the center of the lenses, and inability to focus a grid clearly were observed in aqp0a$^{-/-}$MIPfunN68Q lenses. (**D**) The frequency of cataract in adult fish SL > 20 mm was higher in +MIPfunN68Q compared to *aqp0a*$^{-/-}$ mutants. Refer to Supplementary Figure S1 for phenotype frequency by developmental stage. The same lenses from **A** to **C** were imaged perpendicular to the optical axis displayed the relative localization of the lens nucleus (**E–G**). (**H**) Overexpression of MIPfunN68Q in *aqp0a*$^{-/-}$ resulted in failure of the lens nucleus centralization, and the nucleus is more anteriorly localized than in *aqp0a*$^{-/-}$ at shorter SLs ($P = 007$ at cross sectional mean nucleus r/a at SL = 1.96 mm ± 1.5 SD) and mid SLs ($P = 0.000$ at cross sectional mean nucleus r/a at SL = 12.87 mm ± 1.5 SD. The slope of graph is statistically different between *aqp0a*$^{-/-}$ and mutants expressing MIPfunN68Q ($P = 0.003$). Scale bars = 500 µm.

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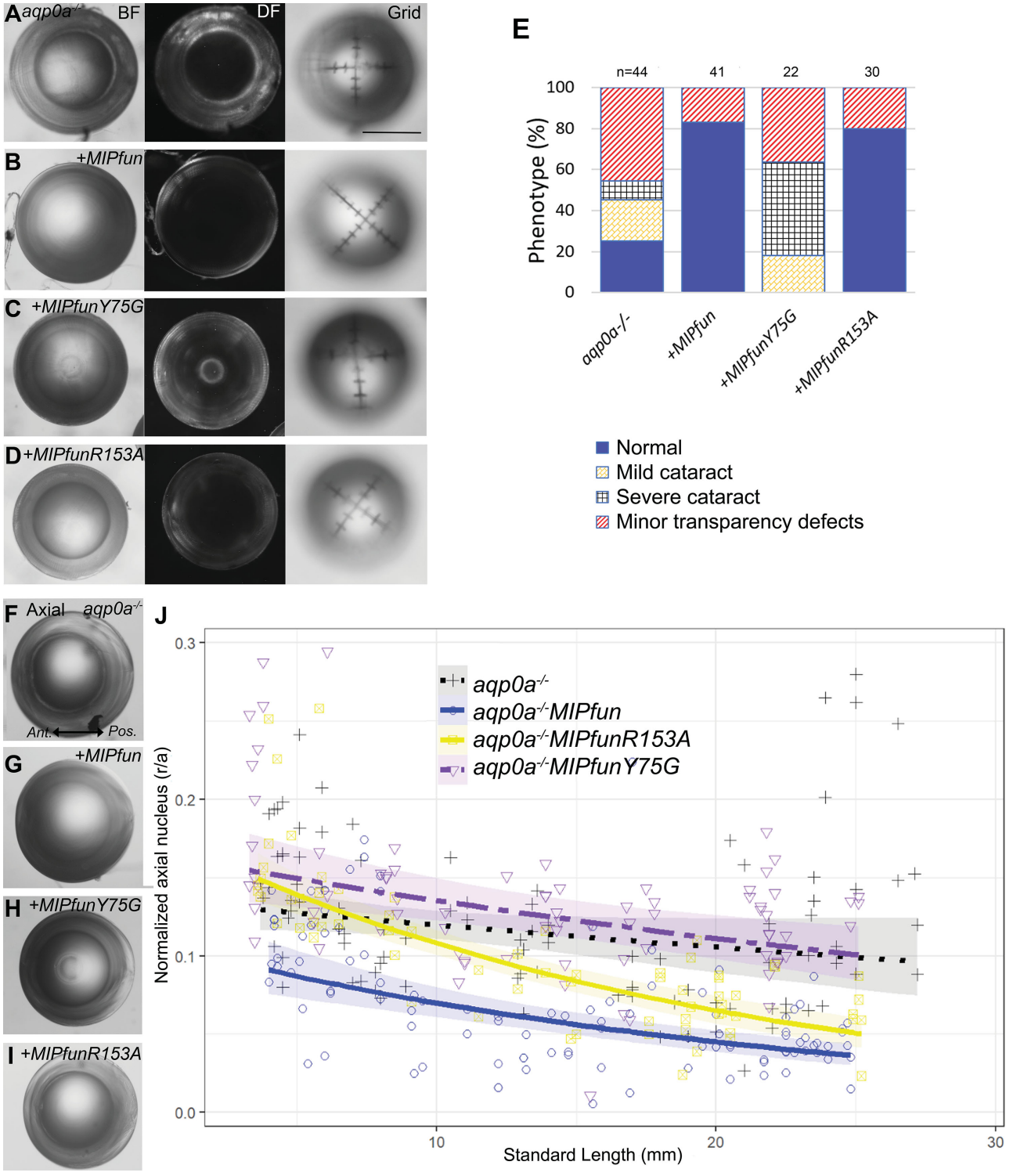

Figure 5. Ca^{2+} regulation of MIPfun water permeability is required for lens transparency, optics, and lens nucleus centralization. (**A**) Representative lenses from adult *aqp0a*$^{-/-}$ mutants (**A**) (SL = 23.8 mm), *aqp0a*$^{-/-}$ mutants overexpressing MIPfun (**B**) (SL = 24.7 mm), *aqp0a*$^{-/-}$ mutants overexpressing MIPfunY75G (**C**) (SL = 24.8 mm) or MIPfunR153A (**D**) (SL = 25.1 mm) were imaged through the optical axis under bright field (BF) or dark field (DF) illumination. The ability to focus was tested by focusing through the lens onto a grid. The lenses misexpressing MIPfunY75G revealed severe nuclear cataract and poor focusing through a grid, whereas there are no obvious transparency defects in the lenses with R153A mutation. (**E**) The frequency of severe cataract in adult zebrafish SL > 20 mm was higher in +MIPfunY75G compared to aqp0a$^{-/-}$ mutants, whereas mutants overexpressing MIPfunR153A only had transparency defects at the same frequency as MIPfun. Refer to Supplementary Figure S1 for phenotype frequency by developmental stage. The same lenses from **A** to **D** were imaged perpendicular to the optical axis displayed the relative localization of the lens nucleus (**F–I, J**). Overexpression of MIPfunY75G in aqp0a$^{-/-}$ resulted in failure of the lens nucleus centralizing at all developmental stages with the relative nucleus localization similar to aqp0a$^{-/-}$ at all stages. Overexpression of MIPfunR153A resulted in more centralized lens nuclei in mid and longer SLs compared to aqp0a$^{-/-}$ ($P = 0.001$ and $P = 0.000$) indicating partial rescue of centralization. At shorter SLs, MIPfunR153A overexpressing lenses had more anteriorly placed lens nuclei, like aqp0a$^{-/-}$. Scale bars = 500 µm.

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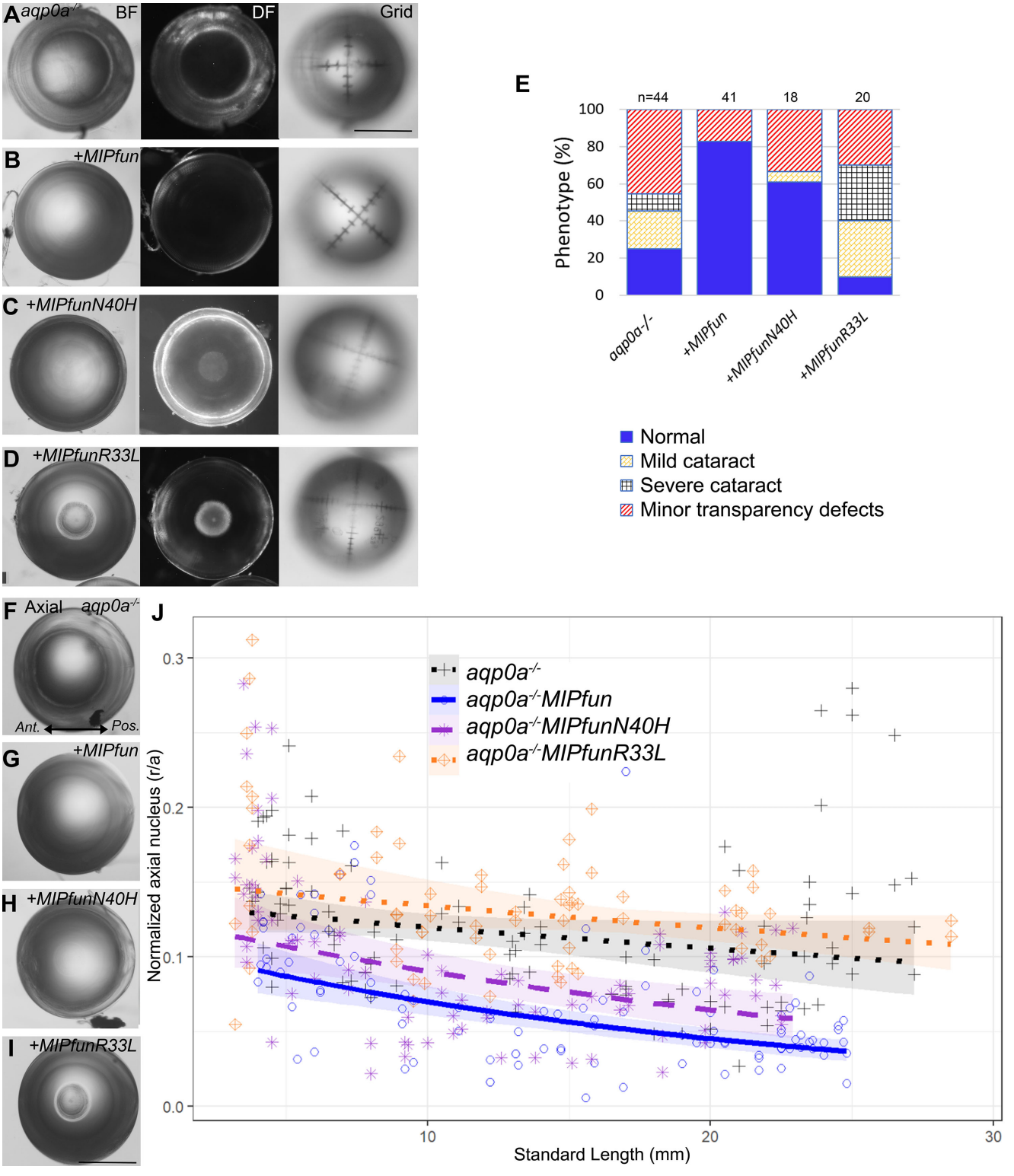

Figure 6. MIPfun water permeability regulation via R33L and less via N40H is required for lens clarity, optics, and lens nucleus centralization. Representative dissected lenses from adult *aqp0a*$^{-/-}$ (**A**) (SL = 23.8 mm), *aqp0a*$^{-/-}$*MIPfun* (**B**) (SL 24.7 mm), *aqp0a*$^{-/-}$*MIPfunN40H* (**C**) (SL = 20 mm) or *aqp0a*$^{-/-}$*MIPfunR33L* (**D**) SL = 21.5 mm) were imaged through the optical axis under bright field (BF) or dark field (DF) illumination. The ability to focus was tested by focusing through the lens onto a grid. Whereas most *aqp0a*$^{-/-}$ mutant lenses misexpressing N40H were transparent, some as shown had cataract and inability to focus a grid clearly, as did the R33L expressing lenses. (**E**) In adult zebrafish of SL > 20 mm, the frequency of optical defects was similar between *aqp0a*$^{-/-}$ and *aqp0a*$^{-/-}$*MIPfunN40H*, while *aqp0a*$^{-/-}$*MIPfunR33L* had higher frequency of cataract than *aqp0a*$^{-/-}$. Refer to Supplementary Figure S1 for phenotype frequency by developmental stage. The same lenses from **A** to **D** were imaged perpendicular to the optical axis displayed the relative localization of the lens nucleus (**F–I**). (**J**) Overexpression of R33L in *aqp0a*$^{-/-}$ did not rescue the failure to centralize the lens nucleus, whereas overexpression of MIPfunN40H lead to a more centralized lens nucleus. Mean cross-sectional lens nucleus localization between aqp0a$^{-/-}$ and mutants overexpressing MIPfunN40H were different at mid and longer SL ($P = 0.000$ and $P = 0.001$). Scale bars = 500 µm.

Supplementary Fig. S1), this mutation reduced the numbers with cataract and the frequency of minor transparency defects in lenses from zebrafish > 20 mm SL, compared with *aqp0a*$^{-/-}$ (see Fig. 6E). Unexpectedly, however, at 15 to 20 mm SL approximately 40% of animals had a mild opacity (see Supplementary Fig. S4), which appeared to recover in animals > 20 mm SL. In contrast, *aqp0a*$^{-/-}$*MIPfunR33L* transgenics had frequencies of cataract and minor transparency defects that were similar to *aqp0a*$^{-/-}$ mutants (see Figs. 6D, 6E). Grids focused through the lenses looked clear in most transgenics overexpressing MIPfunN40H (see Supplementary Fig. S4) but were blurry in MIPfunR33L (see Fig. 6D).

Overexpressing MIPfunN40H rescued the lens nucleus centralization defect of *aqp0a*$^{-/-}$, except at juvenile stages of development, whereas MIPfunR33L did not (see Figs. 6F–J). These data show that pH regulation of MIPfun permeability has less effect on transparency and lens nucleus centralization, whereas Ca^{2+} regulation is more important.

Discussion

In our previous studies, we have shown that the loss-of-function of Aqp0a, but not Aqp0b, impairs not only lens transparency but also the process of nuclear centralization.[5] In this current study, we have used the killifish Aqp0 orthologue, MIPfun, which retains the water permeability[19,32,34] and adhesive properties[21] of the two zebrafish Aqp0 orthologues, to not only confirm the relative contributions of Aqp0a and Aqp0b to the maintenance of lens transparency and nuclear centralization but also the molecular mechanisms by which these processes are regulated. The key findings from this study are summarized in Table 2. Briefly, our results demonstrate that transgenic overexpression of MIPfun in vivo partially restores Aqp0 function in the absence of both Aqp0a and Aqp0b (see Fig. 1) or only Aqp0a (see Fig. 2) confirming the results of our previous experiments that utilized transient expression of MIPfun at larval stages.[19,20] In addition, by using point mutants of MIPfun that have been shown in vitro to lack specific cellular functions of MIPfun we show that the auto-adhesive function of MIPfun is not required to rescue the *aqp0a*$^{-/-}$ mutant phenotype (see Fig. 3), but its water transport function is essential (see Fig. 4). Finally, we have tested if the regulation of MIPfun permeability by Ca^{2+} and pH affects its ability to rescue the *aqp0a*$^{-/-}$ mutant phenotype and show that two of three MIPfun mutant constructs with a loss of sensitivity to 0 Ca^{2+} fail to rescue *aqp0a*$^{-/-}$ phenotypes (see Figs. 5, 6). In contrast, MIPfun mutants (N40H) lacking pH sensitive permeability regulation only partially rescue transparency and lens nucleus centralization (see Fig. 6). However, when combined with loss of Ca^{2+} sensitivity (R33L), MIPfun fails to rescue transparency or nucleus centralization defects (see Fig. 6). These results using stable MIPfun transgenics deficient in Ca^{2+} regulation throughout development are consistent with our previous transient expression experiments using Ca^{2+} insensitive MIPfun to rescue *aqp0a*$^{-/-}$ knockdown cataract phenotypes in larval (3 dpf) fish.[19] Taken together, the ability of specific MIPfun mutants to rescue the *aqp0a*$^{-/-}$ mutant phenotype suggest that the regulation of Aqp0a water permeability is important for the maintenance of lens transparency and is required for nucleus centralization in the zebrafish lens.

Whereas Aqp0a has essential functions as a water channel throughout lens development, Aqp0b, which has adhesive properties, is only essential for maintenance of lens transparency and water homeostasis if Aqp0a is also missing,[5,20] as seen in double *aqp0a*$^{-/-}$/*aqp0b*$^{-/-}$ null mutant lenses, which form early larval cataracts and then nuclear cataract from 6 dpf, show transparency defects as well as an anterior polar opacity (see Fig. 1). Thus auto-adhesion mediated by Aqp0b does not seem to contribute to the maintenance of lens transparency and is not required for nucleus centralization. Similarly, MIPfun mutants lacking adhesive properties effectively rescue the centralization defect (see Fig. 3). In contrast, the overexpression of a water channel dead MIPfun results in a more severe lens transparency defect and less centralized lens nucleus than *aqp0a*$^{-/-}$ null mutants (see Fig. 4). This could be due to the nonfunctional Aqp0 disrupting membrane structure and membrane protein interactions. AQP0 monomers have been shown to cooperate in tetramers,[35] thus introduction of a nonfunctional Aqp0 may have a dominant-negative affect on permeability. Therefore, overexpression of MIPfunN68Q may disrupt the normal function of existing aquaporins in the lens causing more severe phenotypes.

The nuclear region of the zebrafish lens, which exhibits the highest refractive index, moves from an anterior to a central location in the optical axis during larval and juvenile development of the zebrafish eye, and this process is inhibited in *aqp0a*$^{-/-}$ mutants.[5] This centralization of the lens nucleus serves to alter the profile of the gradient of refractive index (GRIN), which together with the shape of the lens sets its refractive power. Based on this observation we have hypothesized that nuclear centralization functions allow the lens to establish a functional optical system in zebrafish as the eye grows during larval stages.

Our results suggest that regulation of water permeability properties of Aqp0a help drive the process of nuclear centralization to mediate these crucial changes in lens function. However, it is important to stress that we have not directly tested the requirement of Aqp0a regulated water permeability in lens nucleus centralization, but instead tested the ability of MIPfun mutants to rescue the *aqp0a*$^{-/-}$ mutant phenotype. Due to sequence homology to MIPfun and previous studies showing its importance for stability of the anterior pole[5] and water influx,[20] it is likely that Aqp0a similarly exhibits a regulated water permeability that drives nucleus centralization. If so, we envisage that during the centralization process cell signaling pathways alter the water permeability of Aqp0a to drive changes in the volume of the tips of fiber cells located at the anterior pole of the zebrafish lens and that this localized increase in cell volume causes the observed displacement of the nucleus toward the center of the lens.

Such a localized subcellular change in Aqp channel function is not without precedent in the lens. In the rat lens, the trafficking of Aqp5 to and from the membrane alters water permeability.[36] Furthermore, membrane trafficking of Aqp5 is regulated by pharmacological modulation of contractility of the ciliary muscle,[37] which alters the tension applied to the lens via their zonules, activating mechanosensitive TRPV1 and TRPV4 channels that regulate lens water transport.[38] Interestingly, activation of TRVP1 by a decrease in zonular tension causes removal of Aqp5 from the apical tips of fiber cells at the anterior pole of the lens but not the basal tips of fibers at the posterior pole.[39] Based on these observations, changes in Aqp5-mediated water permeability at the anterior pole of the rat lens have been hypothesized to alter the anterior surface curvature therefore the refractive power of the aspheric rat lens.

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Hence, based on analogy to Aqp5 in the mammalian lens it is interesting to speculate that Aqp0a in the zebrafish lens also acts at the anterior suture as a regulated water channel to increase water permeability that drives nuclear centralization. Consistent with this view, water is more constricted (e.g. due to high macromolecular crowding) in larval lenses of *aqp0a*$^{-/-}$ mutants, at the anterior suture compared to wild type lenses,[20] confirming its localized role in water influx in this area. We speculate that Aqp0a may use two mechanisms to regulate water permeability at the anterior suture of the zebrafish lens. Like Aqp5, Aqp0a could traffic into and out of the membrane at the anterior suture. Alternatively, the probability of channels that are already in the membrane being open could be regulated by changes in Ca^{2+} levels, as suggested by the inability of MIPfun mutants lacking Ca^{2+}-sensitivity to rescue the nuclear centralization (see Figs. 5, 6). Future work is required to distinguish between these two possible mechanisms of changes in Ca^{2+} levels and/or Aqp0a membrane trafficking and their roles in fiber cell volume at the anterior suture. Regardless of the actual mechanism our data suggest that the process of nucleus centralization is driven by a localized change in water permeability of the tips of fiber cells at the anterior pole.

In conclusion, by using a variety of MIPfun mutants to rescue the *aqp0a*$^{-/-}$ mutant phenotype we have shown that the regulation of Aqp0a water permeability is involved in the process of lens nuclear centralization. Because we hypothesize that nuclear centralization changes the refractive power of the lens during a specific period of development and growth of the zebrafish eye future experiments will focus on studying the processes that regulate nuclear centralization as a model to understand the role of the lens in optical development.

Acknowledgments

The authors thank James E. Hall for the initiation of the zebrafish Aquaporin line of research and for his wisdom, knowledge, and support. Thank you to Karin L. Németh-Cahalan for providing the AQP0R33L *Xenopus laevis* oocyte data, and to Jaylen Michael Lee from the Biostatistics, Epidemiology & Research Design Unit at the University of California, Irvine for designing and carrying out statistical analysis on the nucleus centralization data. We would also like to thank Daniel B. Dranow for helpful advice and discussions on molecular biology for the rescue experiments.

Supported by the National Institutes of Health grant R01EY031587 (TFS, OS, IV, and PJD), National Institutes of Health grant R01DE13828 (TFS), National Institutes of Health grant R01DE30565 (TFS), National Science Foundation MCB2028424 (TFS and OS), Auckland Medical Research Foundation Sir Harcourt Caughey Award 3725794 (IV), Aotearoa New Zealand National Eye Centre Research Fellowship by John Phillips (IV).

Author Contributions: Conceptualization: O. Safrina, I. Vorontsova, and T. F. Schilling. Methodology: O. Safrina and I. Vorontsova. Investigation: O. Safrina and I. Vorontsova. Visualization: O. Safrina and I. Vorontsova. Supervision: T. F. Schilling, and P. J. Donaldson. Writing—original draft: O. Safrina, I. Vorontsova, T. F. Schilling, and P. J. Donaldson. Writing—review & editing: O. Safrina, I. Vorontsova, T. F. Schilling, and P. J. Donaldson.

Data Availability: All data can be made available upon request to the corresponding author.

Disclosure: **O. Safrina**, None; **I. Vorontsova**, None; **P.J. Donaldson**, None; **T.F. Schilling**, None

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