Vision Research 156 (2019) 66–72

Contents lists available at ScienceDirect

Vision Research

journal homepage: www.elsevier.com/locate/visres

Loss of *foxc1* in zebrafish reduces optic nerve size and cell number in the retinal ganglion cell layer☆

Jurgienne Umali, Alexia Hawkey-Noble, Curtis R. French*

Discipline of Genetics, Faculty of Medicine, Memorial University of Newfoundland, Canada

ARTICLE INFO

Keywords:
foxc1
Zebrafish
CRISPR
Retinal ganglion cells
atoh7
pou4f2
fgf19
Glaucoma

ABSTRACT

Mutation of *FOXC1* causes Axenfeld-Rieger Syndrome (ARS) with early onset or congenital glaucoma. We assessed retinal ganglion cell (RGC) number in zebrafish due to CRISPR-mediated mutation and antisense inhibition of two-forkhead box transcription factors, *foxc1a* and *foxc1b*. These genes represent duplicated homologues of human *FOXC1*. Using a CRISPR induced null mutation in *foxc1b*, in combination with antisense inhibition of *foxc1a*, we demonstrate reduced cell number in the retinal ganglion cell layer of developing zebrafish eyes. As early as 5 days post fertilization (dpf), fewer RGCs are found in *foxc1b* homozygous mutants injected with *foxc1a* morpholinos, and a thinner optic nerve results. Our data illustrates that *foxc1* is required for the expression of *atonal homolog 7* (*atoh7*), a gene that is necessary for RGC differentiation. As markers of differentiated RGCs (*pou4f2)* are downregulated in *foxc1b−/−* mutants injected with *foxc1a* morpholinos and no cell death is observed, our results are consistent with defects in the differentiation of RGCs leading to reduced cell number, as opposed to increased cell death of RGCs or off targets effects of morpholino injection. Our zebrafish model demonstrates that aberrant regulation of RGC number could act in concert with other known glaucoma risk factors to influence the development of congenital and early onset glaucoma due to *FOXC1* mutation.

1. Introduction

The differentiation and survival of retinal ganglion cells (RGCs) is critical for the maintenance of vision, as RGC axons carry information from the eye to visual processing centers in the brain. Progressive damage to these cells or the optic nerve cause glaucoma, one of the most common forms of blindness worldwide (Quigley & Broman, 2006). Inherited forms of glaucoma, such as those caused by loss of function mutations or copy number variations (CNVs) in the forkhead box transcription factor *FOXC1*, can cause an early onset (Ito et al., 2014; Strungaru et al., 2007), or congenital glaucoma (Medina-Trillo et al., 2016; Micheal et al., 2016), suggesting that a proportion of cases could have developmental etiology. As such, we sought to test whether *foxc1* plays a role in the differentiation and maintenance of RGCs early in development using a zebrafish model system.

The differentiation of all retinal neurons is genetically controlled. Under the proposed competency model of retinal neuron generation, proliferating retinal progenitors intrinsically acquire the ability to differentiate into each neuron type through a mechanism that is controlled by the timing of cell cycle exit (Cepko, 1999; Livesey & Cepko, 2001). Retinal progenitors that first exit the cell cycle differentiate in to RGCs, while later exiting progenitors differentiate into interneural cell types such as amacrine, biopolar, and horizontal cells (Rapaport, Wong, Wood, Yasumura, & LaVail, 2004). The exit of progenitors that differentiate into RGCs requires the expression of basic helix loop helix (bHLH) transcription factors such as *atonal homolog 5* (*anth5,* mouse) and *atonal homolog 7* (*atoh7,* zebrafish) (Yang et al., 2003; Kay et al., 2001). Loss of *anth5* in mice or *atoh7* in zebrafish results in a complete lack of retinal ganglion cells with increased numbers of retinal progenitors accumulating in other retinal layers (Yang et al., 2003; Kay et al., 2001). Subsequently, two transcription factors (*pou4f2* and *isl1*) downstream of *atoh7* in zebrafish have been defined that are necessary to drive RCG fate after cell cycle exit (Wu et al., 2015).

It has been demonstrated that sequence variants and copy number variations in human *ATONAL HOMOLOG 7* (*ATOH7*) associate with quantitative traits (endophenotypes of glaucoma) involving the optic nerve such as optic disc area (Macgregor et al., 2010; Venturini et al., 2014), vertical cup to disc ratio (Philomenadin, Asokan, George,

☆ This study was funded with a grant from the Glaucoma Research Society of Canada and from the Faculty of Medicine at Memorial University of Newfoundland.
* Corresponding author at: Discipline of Genetics, Faculty of Medicine, Memorial University of Newfoundland, 5M327 Craig L. Dobbin Genetics Research Centre, 300 Prince Philip Drive, St. John's, A1B 3V6, Canada.
E-mail address: curtis.french@med.mun.ca (C.R. French).

https://doi.org/10.1016/j.visres.2019.01.008
Received 26 July 2018; Received in revised form 17 January 2019; Accepted 17 January 2019
Available online 02 February 2019

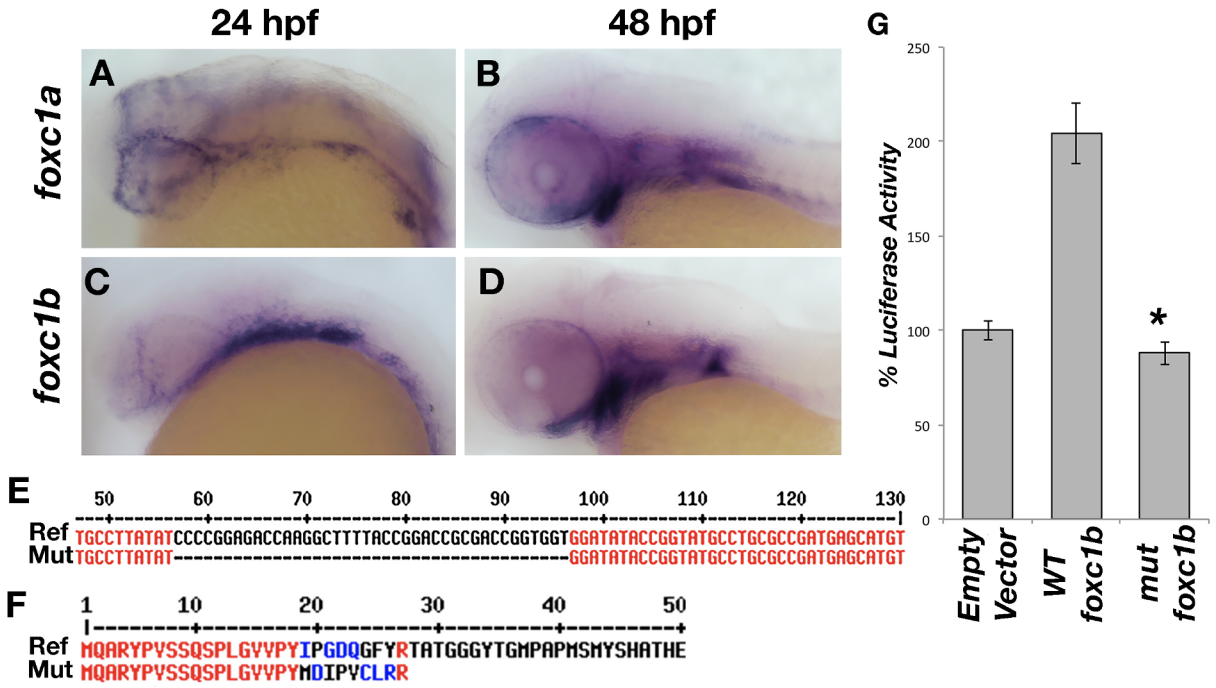

Fig. 1. Zebrafish *foxc1* genes. *foxc1a* is expressed in the neural crest and periocular mesenchyme at 24 hpf (**A**) and in the pharyngeal arches at 48 hpf (**B**). *foxc1b* is also expressed in the periocular mesenchyme and a small number of ocular progenitor cells at 24 hpf (**C**), and in the pharyngeal arches at 48 hpf (**D**). Partial alignment of the reference *foxc1b* coding sequence and the 40 base-pair deletion mutant sequence created by CRISPR genome editing (**E**). The zebrafish mutant strain deleted 40 nucleotides (57–96 of the open reading frame), resulting in a frameshift mutation and predicted premature protein truncation (**F**). Luciferase assays in HEK293 cells using a reporter with 6 copies of the consensus *FOXC1* binding element demonstrates the zebrafish *foxc1b* 40 base pair deletion creates a null mutation, as no luciferase activity (above endogenous levels) is observed (**G**) $p = 9 \times 10^{-5}$.

Lingam, & Sarangapani, 2015), as well as glaucoma risk (Chen et al., 2012), indicating that this transcription factor may play a critical role in the generation of human ocular phenotypes. In this study, we tested whether a gene that causes early onset glaucoma (*FOXC1*) affects the expression of *atoh7* and endophenotypes associated with glaucoma using a zebrafish model system. *foxc1* is expressed in the neural crest cells that contribute to the anterior eye segment and retinal pigmented epithelium (Williams & Bohnsack, 2015) and plays an important role in embryonic cell differentiation and survival. Zebrafish make an ideal model system to study endophenotypes of glaucoma given the conserved retinal laminar structure with humans and overlapping roles of *foxc1* and *atoh7* in eye development with their human counterparts. Here, we show that loss of two zebrafish *FOXC1* homologs (*foxc1a* and *foxc1b*) reduces the expression of genes that drive RGC differentiation and function, with reduced cell number in the RGC layer and reduced optic nerve thickness resulting. Our analysis of critical differentiation factors and cell death in the eye supports a role for *foxc1* in regulating RGC differentiation as opposed to maintenance and survival of RGCs.

2. Materials and methods

2.1. Zebrafish husbandry

All zebrafish strains were reared under standard conditions and embryos staged according to Kimmel, Ballard, Kimmel, Ullmann, and Schilling (1995). All experiments were performed in compliance with the standards set by Memorial University of Newfoundland's Animal Care Committee and by the Canadian Council on Animal Care. Mutants for *foxc1b* were genotyped using polymerase chain reaction (PCR) followed by gel electrophoresis.

Zebrafish *foxc1b* homozygous mutants are viable, as are their progeny. Homozygous embryos were generated from both heterozygous and homozygous incrosses, producing similar data. Wildtype siblings were incrossed to generate control wildtype embryos for experiments utilizing homozygous *foxc1b* incrosses. All embryos were anesthetised using Tricane (0.168 mg/ml) before fixation with 4% PFA, when older than 48 hpf. Embryos used for *in situ* hybridizations were grown past 24 hours post fertilization (hpf) in embryo media treated with 0.003% 1-phenyl 2-thiourea (PTU; Sigma-Aldrich, St. Louis, MO) to prevent pigment formation.

2.2. Generation of foxc1b mutants

To generate *foxc1b* mutants using the CRISPR-CAS9 system, guide RNA sequences targeting the 5 prime region upstream of the forkhead DNA binding domain of *foxc1b* were cloned into and synthesized from pDr274 as per Hwang et al. (2013). Injection mixtures contained 60 ng/µl guide RNA and 300 ng/µl Poly-A tailed CAS9 mRNA. Injected fish were grown to sexual maturity and outcrossed to identify heterozygous founders. The induced INDEL, removing 40 nucleotides of *foxc1b* coding sequence, is predicted to truncate the protein prior to the DNA forkhead-binding domain (Fig. 1). Mutant populations were outcrossed to wildtype (AB) for at least 5 generations to minimize the effect of potential off target mutations. This line is designated *foxc1b*^{ua1018}.

2.3. Cell transfections and luciferase assays.

HEK293 cells were plated and grown to at least 80% confluency using DMEM media supplemented with 10% fetal calf serum. Lipofectamine transfection reagent (ThermoFisher) was used for all transfections. 0.5 µg of plasmid containing the wildtype or mutant zebrafish *foxc1b* open reading frame (ORF) sequences were co-transfected with a 0.25 µg *FOXC1* Reporter plasmid (Saleem, Banerjee-Basu, Berry, Baxevanis, & Walter, 2001) that drives luciferase expression from the thymidine kinase minimal reporter and 6 copies of the FOXC1 consensus binding sequence (Pierrou, Hellqvist, Samuelsson, Enerback, & Carlsson, 1994). 0.1 µg of constitutively active Renilla luciferase reporter plasmid was co-transfected in every well to account for

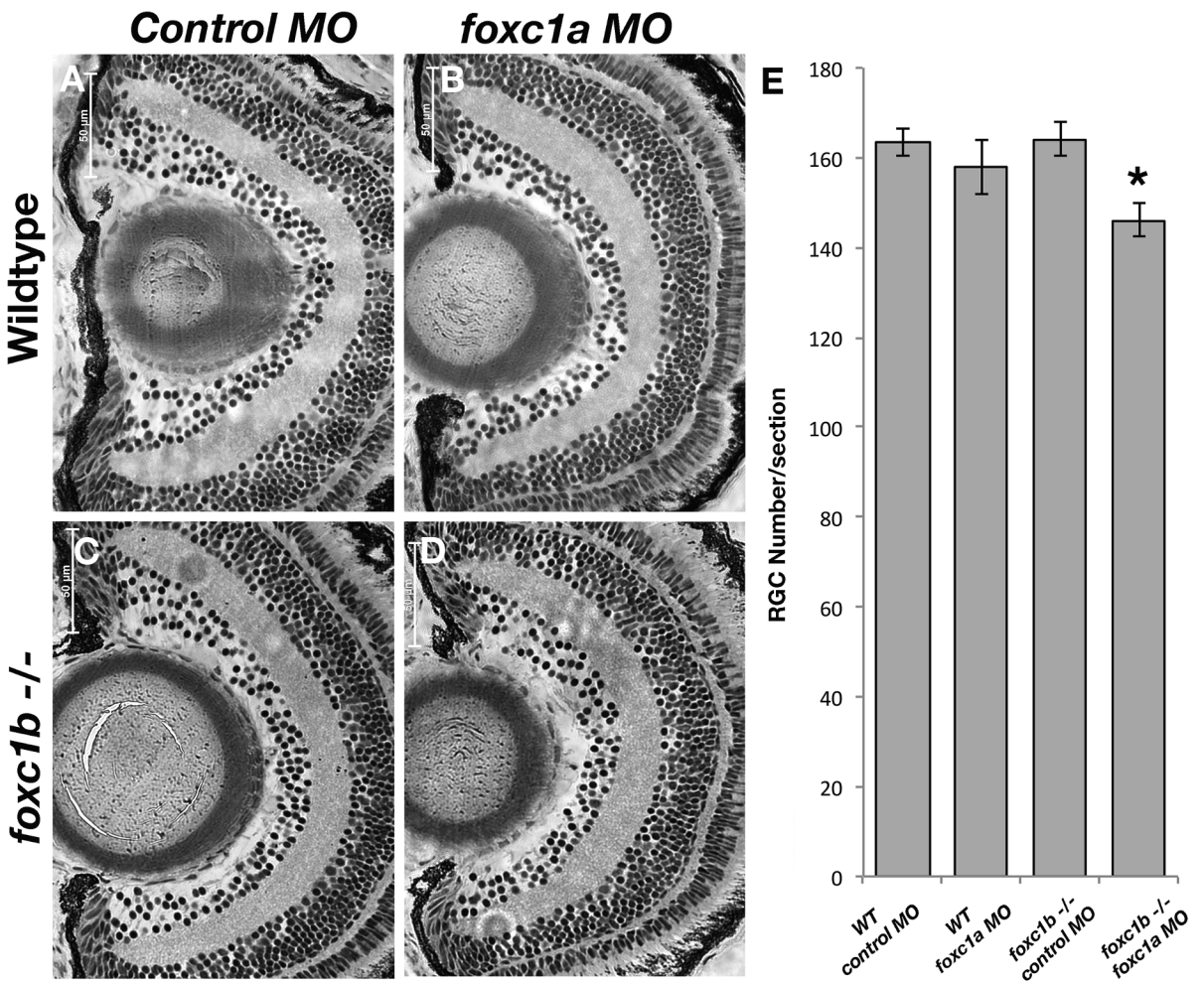

Fig. 2. Loss of *foxc1* function causes reduced cell number in the RGC layer. *foxc1a* morpholino injection into a *foxc1b* −/− background (**D**) reduced RGC number when compared to wildtype embryos injected with standard negative control morpholinos (**A**), wildtype embryos injected with *foxc1a* morpholinos (**B**), or *foxc1b* mutants injected with control morpholinos (**C**). This is statistically significant p = 0.013 (**E**, comparison of **A-D**). Data presented as mean ± SEM.

transfection efficiency and protein synthesis levels. Luciferase detection was accomplished using the dual luciferase assay kit from Promega.

2.4. Morpholino injections

Morpholino antisense oligonucleotides targeting the *foxc1a* translation site (CCTGCATGACTGCTCTCCAAAACGG) (Skarie & Link, 2009) were used to knockdown *foxc1a* function in 1 to 4 cell-stage in *foxc1b*$^{-/-}$, *foxc1b*$^{+/-}$, and AB strain of wild-type embryos. For comparison, morpholinos directed against p53, which show no overt phenotype in early embryos (GCGCCATTGCTTTGCAAGAATTG) (Robu et al., 2007) or a standard negative control morpholino having no specific target in the zebrafish genome (CCTCTTACCTCAGTTACAATTTATA) were utilized, with no differences observed between the two control injections.

2.5. Whole mount in situ hybridization

The protocol for whole mount *in situ* hybridizations as presented by Thisse and Thisse (2008) was used in the current study. Probes were generated from total RNA with subsequent cloning of cDNA sequences into pCR4topo (ThermoFisher). Probes were labelled with DIG, and alkaline phosphatase coupled anti-DIG FAB fragments (Roche) were used for probe detection. Embryos were permeabilized with proteinase k for 5 min (24 hpf), 15 min (32 hpf), 20 min (35 hpf) or 25 min (48 hpf). BM purple (Roche) was used for coloration.

2.6. Sectioning, RGC and optic nerve quantification

For resin sectioning, fixed embryos were dehydrated in a series of ethanol washes, subsequently washed into 50%/50% ethanol/Methachrylate, and finally into Methachrylate to polymerize at 4 °C for at least 12 hours. 3 µm sections were cut using a Leica RM2165 automated microtome. Eye sections were stained with Celestine-Blue and Eosin-Phloxine, and photographed using a Zeiss AxioImager Z1 compound microscope.

Cell counts encompassing one section from each eye (prior to the optic nerve) were utilized for quantification (WT + control MO n = 13, WT + *foxc1a MO* n = 8, *foxc1b* −/− + Control MO n = 12, *foxc1b* −/− + *foxc1a MO* n = 14). Researchers were blinded to genotype in order to perform counts using ImageJ. Optic nerve thickness was quantified using ImageJ in one section per eye at the point where inner plexiform and photoreceptor layers meet (WT + control MO n = 8, WT + *foxc1a MO* n = 5, *foxc1b* −/− + Control MO n = 5, *foxc1b* −/− + *foxc1a MO* n = 8). Data is presented as means ± standard error of the mean (SEM). Significance testing was accomplished using a one factor ANOVA with post-hoc Turkey HSD Test.

2.7. Acridine orange staining

Acridine orange staining was performed as previously described (Beckman, 2017). Briefly, live zebrafish were immersed in 10 µg/ml acridine orange (Sigma) in embryo media for 30 min, and washed twice

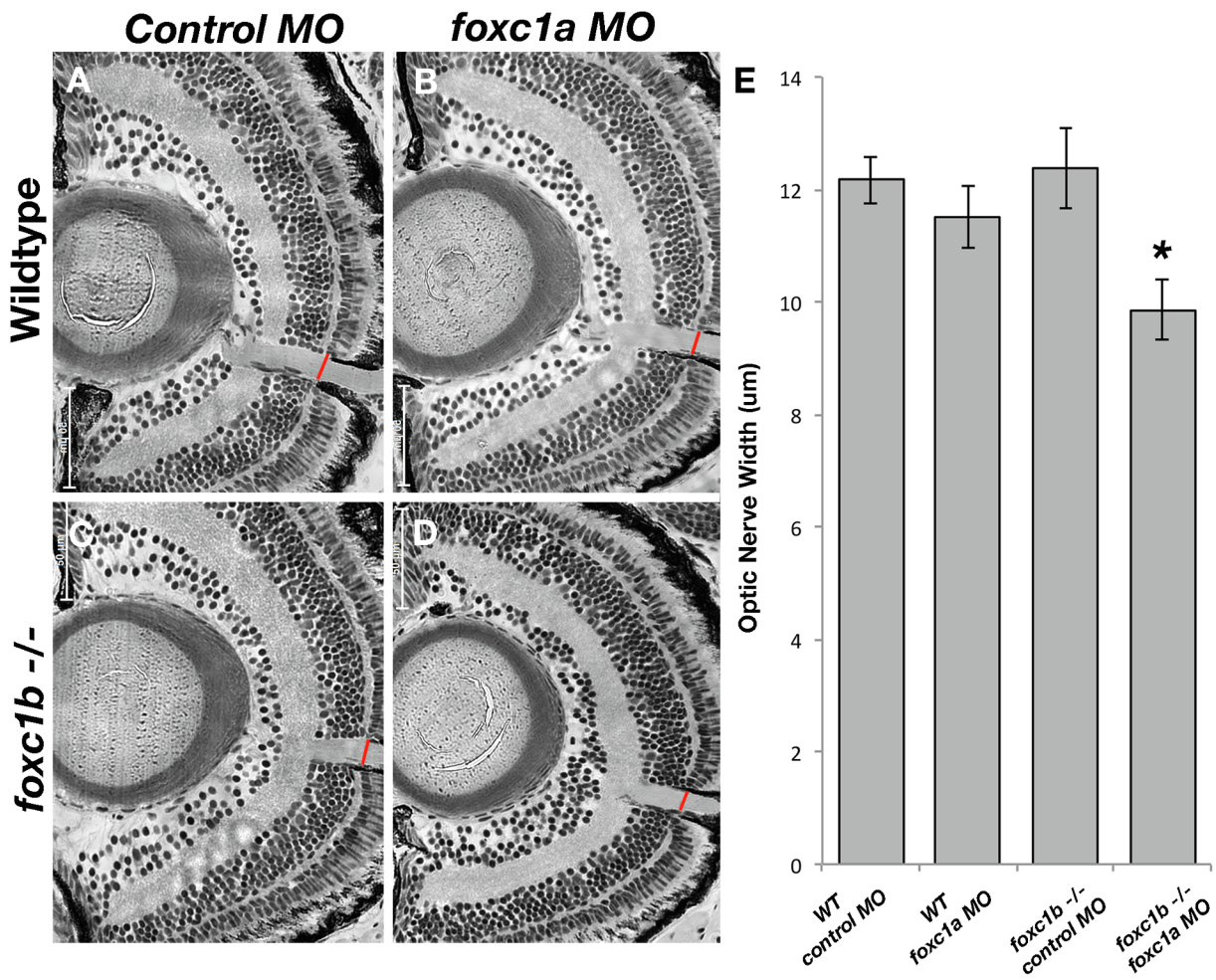

Fig. 3. Loss of *foxc1* function reduced optic nerve thickness. *foxc1a* morpholino injection into a *foxc1b* −/− background reduced optic nerve thickness (**D**) when compared to wildtype embryos injected with standard negative control morpholinos (**A**), wildtype embryos injected with *foxc1a* morpholinos (**B**), *foxc1b* mutants injected with control morpholinos (**C**) This is statistically significant (**E**, comparison of **A–D**, p = 0.03 (**C**). Data presented as mean ± SEM. Red line indicates area of measurement. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in embryo media before fluorescent microscopy (Nikon SMZ18).

3. Results

3.1. Zebrafish foxc1 genes are expressed in the neural crest derived periocular mesenchyme

foxc1a is expressed in the neural crest cells and periocular mesenchyme at 24 hpf (Fig. 1A). By 48 hpf, *foxc1a* expression in found in the most anterior pharyngeal arches (Fig. 1B). The expression of *foxc1b* partially overlaps with *foxc1a* in the neural crest and periocular mesenchyme, however unlike *foxc1a*, the expression of *foxc1b* is confined to the periocular mesenchyme located in the nasal most domain. A small number of cells in the eye proper also express *foxc1b* at 24 hpf (Fig. 1C). By 48 hpf *foxc1b* expression is limited to the dorsal most pharyngeal arches (Fig. 1 D).

3.2. The *foxc1b*^{ua1018} allele contains a null mutation.

Zebrafish contain two *FOXC1* homologues, located on chromosome 2 (*foxc1a*) and 20 (*foxc1b*). Although *foxc1a* has been reported to be the zebrafish homolog of human *FOXC1*, and *foxc1b* to the homolog of human *FOXC2* (Skarie and Link, 2009; Topczewska et al., 2001), both copies of zebrafish *foxc1* display significant synteny with human chromosome 6p25 where *FOXC1* is located. CRISPR based mutagenesis of *foxc1b* resulted in a 40 base-pair deletion in the *foxc1b* open reading frame, leading to a predicted frameshift and truncation prior to the Forkhead DNA binding domain (Fig. 1E and F). Luciferase assays were performed in cell culture, utilizing a reporter construct that drives luciferase from the *FOXC1* consensus binding sequence and demonstrates this mutation likely produced a null allele, as similar luciferase readings are obtained for the empty expression vector and the vector containing the open reading frame for the *foxc1b*^{ua1018} allele (Fig. 1G).

3.3. Loss of foxc1a and foxc1b results in reduced cell number in the RGC layer and thinner optic nerves.

foxc1b homozygous mutants are viable and are produced in the proper Mendelian ratios. Loss of *foxc1b* alone had no effect on cell number in the RGC layer (Fig. 2C) or the thickness of the optic nerve (Fig. 3C), nor did inhibition of *foxc1a* on its own (Fig. 2B, Fig. 3B) when compared to wildtype embryos injected with negative control morpholinos (Fig. 2A, Fig. 3A). When *foxc1a* morpholinos are injected into *foxc1b* −/− embryos, a statistically significant reduction of cells in the RGC layer is observed in ocular sections at 5 dpf (Fig. 2D, E, WT 163.5 ± 3.2 vs *foxc1b* −/− + *foxc1a MO* 146.1 ± 3.6 p = 0.013). Similarly, inhibition of *foxc1a* in a *foxc1b* −/− mutant background reduced the thickness of the optic nerve (Fig. 3D, E, WT 12.2 µm ± 0.42 vs *foxc1b* −/− + *foxc1a* MO 9.9 µm ± 0.55, p = 0.03). Homozygous mutants do not display increased cell death as a result of *foxc1a*

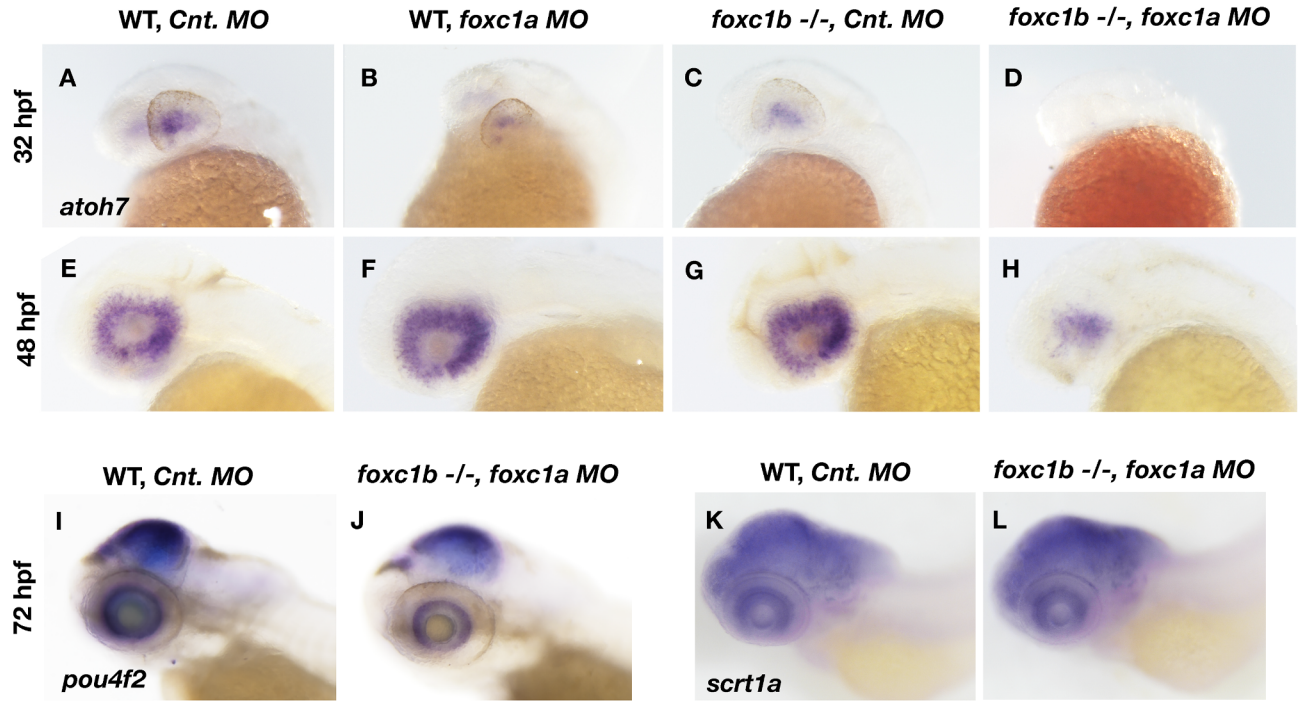

Fig. 4. Loss of *foxc1* affects markers of RGC differentiation. Expression of *atoh7* is induced in the nasal-ventral domain of the retina at 32 hpf (A), and encompasses the entire retina by 48 hpf (E) in wildtype embryos injected with a control morpholino. Loss of *foxc1a* on its own through morpholino inhibition (B and F) had no effect on *atoh7* initiation at 32 hpf, nor its expression throughout the retina at 48 hpf. Similarly, no change in *atoh7* expression is observed in *foxc1* −/− injected with control morpholinos at either 32 hpf (C) or 48 hpf (G). Injection of *foxc1a* morpholinos into a *foxc1b* −/− background impaired the initiation of *atoh7* expression at 32 hpf (D), and reduced expression throughout the retina at 48 hpf (H). *pou4f2* is expressed throughout the RGC layer at 3 dpf (I), and is reduced in *foxc1b* -/- injected with *foxc1a* morpholinos (J). A marker of Amacrine cells, *scrta1,* is not affected (K and L).

morpholino injection (Supplementary Fig. 1), thus indicating that cell death or morpholino off target effects (Robu et al., 2007) are not likely responsible for the reduction of cell number and the reduction of optic nerve thickness.

3.4. Loss of both foxc1 homologues reduces markers of RGC differentiation

As *foxc1* is expressed in the early embryo prior to the differentiation of retinal ganglion cells, we hypothesized that loss of *foxc1* function may affect the expression of *atonal homolog 7* (*atoh7*), a key regulator of retinal ganglion cell differentiation. At 32 hpf, the expression of *atoh7* is initiated in the ventral-nasal domain of the eye in wildtype embryos (Fig. 4A). In *foxc1b* mutants injected with *foxc1a* morpholinos, this initiation is delayed or absent (Fig. 4D). Initiation occurs normally in wildtype embryos injected with *foxc1a* morpholinos (Fig. 4B) and in *foxc1b* mutants injected with a control morpholino (Fig. 4C), indicating that the initiation of *atoh7* expression is not altered when only one *foxc1* paralog is manipulated. By 48 hpf, expression of *atoh7* is found throughout the retina in wildtype embryos (Fig. 4E). Loss of both *foxc1* homologues via injection of *foxc1a* morpholinos into a *foxc1b* homozygous mutant background results in a reduction of *atoh7* expression (Fig. 4H). As with 32 hpf, there is no difference in the expression of *atoh7* at 48 hpf in wildtype embryos injected with *foxc1a* morpholinos (Fig. 4F), or in homozygous *foxc1b* mutants injected with control morpholinos (Fig. 4G), again indicating loss of both *foxc1* homologues is required to negatively affect *atoh7* expression. Expression of *pou4f2*, one of two genes needed to drive RGC fate downstream of *atoh7* (Wu et al., 2015), is also reduced upon loss of both *foxc1* genes (Fig. 4I, J). Finally, as loss of retinal ganglion cell fate is often observed in conjunction with increased numbers of amacrine cells, we tested the expression of *scrt1a*, a marker of amacrine cell fate (Cherry, Trimarchi, Stadler, & Cepko, 2009). We do not observe any difference in the expression of *scrt1a* in *foxc1* loss of function embryos when compared to wildtype embryos injected with control morpholinos (Fig. 4K, L).

3.5. Loss of foxc1a and foxc1b has little effect on early eye specification and oxidative stress response genes

FGF signalling plays a key role in early eye development (Nakayama et al., 2008; Tamimi et al., 2006; Francisco-Morcillo et al., 2005) and is down regulated throughout the embryo, including the eye and pectoral fin bud in *foxc1b* mutants injected with *foxc1a* morpholinos (Fig. 5A and B). However markers of early eye field specification, such as *retinal homeobox gene 2* and *3* (*rx2* and *rx3*) remain unaffected (Fig. 5C–F). Given the association of *FOXC1* with ARS and glaucoma, and its role in regulating oxidative stress response in tissue culture (Ito et al., 2014; Berry et al., 2008), we also tested the expression of genes involved in the response to oxidative stress. The expression of platelet derived growth factor alpha (*pdgfra*) is found in the lens of wildtype embryos at 24 hpf (Supplementary Fig. 2A), is mildly down-regulated due to loss of *foxc1a* and *foxc1b* (Supplementary Fig. 2B). This down- regulation becomes more pronounced at 48 hpf, when *pdgfra* is confined to the neural crest cells of the pharyngeal arches (Supplementary Fig. 2C,D). The expression of two members of the heat shock 70 class of proteins that are expressed in the zebrafish eye (*hspa5* and *hspa9*) is not altered due to loss of *foxc1* function (Supplementary Fig. 2E–H).

4. Discussion

In this study, we developed a zebrafish model of *foxc1* loss of function and demonstrate reduced cell number in the RGC layer and reduced optic nerve thickness early in development. In our model, fewer RGCs are observed at 5 days post fertilization, approximately three days after their differentiation. Given the many roles of *FOXC1* in early development, and the recent reports of congenital glaucoma due to homozygous loss of *FOXC1* (Micheal et al., 2016), we tested whether

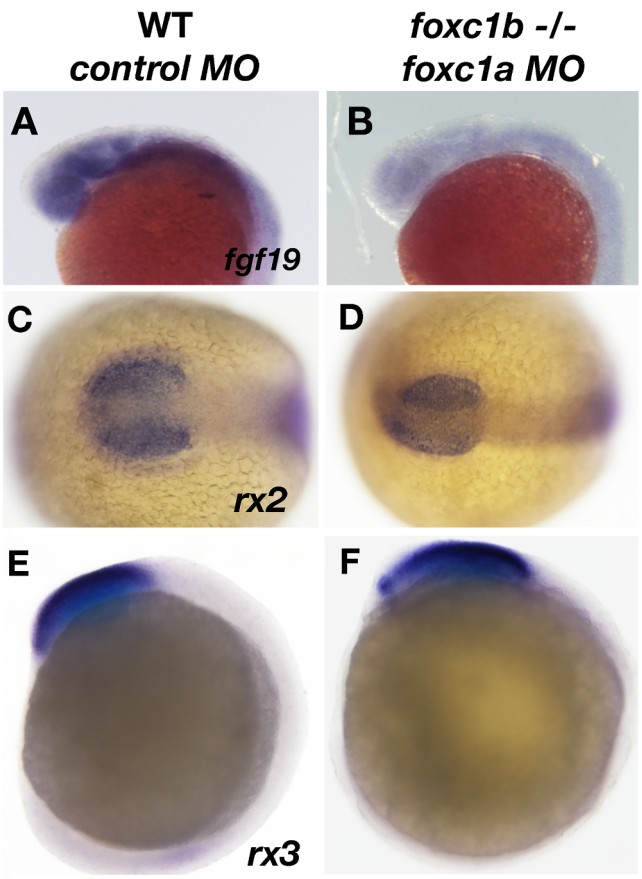

Fig. 5. Expression of *fgf19* is disrupted due to loss of *foxc1* function. *fgf19* expression is found ubiquitously in the head at 20 hpf (A), and is reduced in *foxc1b* −/− injected with *foxc1a* morpholinos (B). The expression of two early *retinal homeobox* genes (*rx2* and *rx3*) is not altered in these embryos (*rx2*, 16 somites C, D, *rx3,* 10 somites, E, F). Lateral views (A, B, E, F) and dorsal views (C, D).

zebrafish *foxc1* may regulate embryonic retinal ganglion cell differentiation. Using our zebrafish model system, we demonstrate that *foxc1* is required for expression of *atoh7*, a key regulator of retinal ganglion cell differentiation (Yang et al., 2003; Kay et al., 2001; Brzezinski et al., 2012). Our results support a hypothesis whereby *foxc1* controls RGC differentiation through regulation of *atoh7* expression during retinogenesis and suggests that fewer differentiating RGCs may be one facet of congenital glaucoma development. We also observed decreased expression of *pou4f2* (formally *brn3b*), a RGC marker downstream of *atoh7* (Wu et al., 2015) in agreement with deregulated RGC differentiation. There is no evidence of increased cell death in the eyes of *foxc1b* −/− mutants injected with *foxc1a* morpholinos, arguing that at increased cell death does not contribute to the reduced RGC number by 5 dpf. As neither *foxc1a* or *foxc1b* are expressed in retina progenitor cells, it is likely that the effects of loss of *foxc1* function are non-cell autonomous with respect to the control of *atoh7* expression. It is possible that signals emanating from the anterior segment or ocular vasculature, both affected by loss of *foxc1* function (Skarie and Link, 2009; Smith et al., 2000; French et al., 2014), could affect the expression of *atoh7* and subsequent RGC differentiation.

Recent GWAS studies have identified genetic variation near *ATOH7* as associated with endophenotypes concerning glaucoma, such as optic disc diameter (Philomenadin and Asokan, 2015; Nannini et al., 2017), highlighting the role of this gene in etiology of in the development and/or maintenance of structures relevant to glaucoma. In addition to its role in RGC differentiation, mutations in *ATOH7* can cause ocular vascular defects, such as persistent hyperplasia of the primary vitreous (PHPV), a condition that involves persistence of embryonic ocular vasculature in juvenile and adult eyes, and often leads to glaucoma (Prasov et al., 2012). Mutation or CNV of *FOXC1* also causes a host of ocular vasculature defects (Skarie and Link, 2009; Seo et al., 2012), and given the lack of *foxc1* expression in RGCs or retinal precursors, it is possible that defects in retinal ganglion cell differentiation are secondary to defects in ocular vasculature.

Zebrafish contain duplicated *foxc1* genes (*foxc1a* and *foxc1b*), with both displaying synteny with human chromosome 6p25 where *FOXC1* is located. No statistically significant change in cell number in the RGC layer and optic nerve thickness were observed in *foxc1b-/-* alone, or in wildtype embryos injected with *foxc1a* morpholinos, while manipulation of both *foxc1* genes reduces cell number and optic nerve thickness. Together, these data indicate overlapping functions with respect to *foxc1a* and *foxc1b* dependent ocular development in zebrafish. In support of this, no difference was observed in the expression of *atoh7* at any time point tested in *foxc1b* mutants alone, or in wildtype embryos injected with *foxc1a* morpholinos, while the combination of *foxc1a* morpholino inhibition in a *foxc1b* homozygous mutant background reduced the initiation of *atoh7* at 32 hpf, as well as at later time points when *atoh7* is expressed throughout the eye. This not only highlights the redundant roles of both *foxc1a* and *foxc1b* in zebrafish RGC generation, but also demonstrates the specificity of the *foxc1a* morpholino. The induction of phenotypes in in *foxc1b* homozygous mutants via use of the *foxc1a* morpholino, but not wildtype embryos argues against the possibility of off target effects, as these would likely be observed in the wildtype embryos injected with *foxc1a* morpholinos.

Reduced cell number in the eye could result from defects in early specification of ocular progenitor cells, as opposed to specific defects in RGC differentiation. We note no change in the expression of genes required for early ocular development such as *retinal homeobox 2* and *3* (*rx2* and *rx3*) in our *foxc1* loss of function model, indicating that early specification of ocular progenitors from presumptive forebrain tissue is unaffected. In agreement with cell culture models (Tamimi et al., 2006), we detect reduced expression of *fgf19*, a gene that regulates eye growth and patterning (Nakayama et al., 2008; Hernandez-Bejarano et al., 2015; Vinothkumar et al., 2008). FGF signalling has been demonstrated to influence neurogenesis in the retina (Willardsen et al., 2014; Chen et al., 2013), and regulate the expression of *atonal homolog* genes in the eye (Willardsen et al., 2009). Thus it likely that *fgf19* expression is required downstream of *foxc1* and upstream of *atoh7* to regulate cell number in the RGC layer, however we have not directly tested this in the current study.

Oxidative stress could also influence ocular cell survival (Ster et al., 2014; Chrysostomou et al., 2013) as inhibition of *FOXC1* in cultured ocular cells results in increased oxidative stress (Ito et al., 2014; Berry et al., 2008). Platelet derived growth factor receptor alpha (*pdgfra*) has been demonstrated to regulate the response to oxidative stress (Kanamoto, Rimayanti, & Kiuchi, 2011) lies downstream of *foxc1* (French et al., 2014), and provides a protective effect on RGCs (Johnson et al., 2014). At early stages when *pdgfra* is expressed in the lens and periocular mesenchyme, we observe only a subtle down-regulation of expression in *foxc1b* homozygous mutants injected with *foxc1a* morpholinos. We do however observe a more pronounced down regulation of *pdgfra* at later stages when expressed in the pharyngeal arches. The regulation of *pdgfra* by *foxc1* in the pharyngeal arches may contribute to craniofacial defects often observed in ARS patients, but likely has little effect on eye development. Lastly, we do not detect a difference in the expression of heat shock proteins in the eye that are required to respond to stressors that could affect eye health, as evidenced by the expression of *hspa5* and *hspa9*. Thus in our zebrafish model of congenital glaucoma, we do not attribute the lack of stress response as a key factor in regulating RGC number, based on gene expression patterns. We have not, however, directly tested the response of *foxc1b* mutation alone or in combination with *foxc1a* morpholinos to oxidative stress challenges that could provide conclusive data for such a hypothesis.

5. Conclusions

The main finding of this paper is that loss of *foxc1a* and *foxc1b* in zebrafish causes endophenotypes of glaucoma such as reduced cell number in the RGC layer and reduced optic nerve thickness. In these animals, expression of key regulators of RGC differentiation (*atoh7* and *pou4f2)* is disrupted. Our data supports a model whereby defective RGC differentiation comprises one facet of the etiology of congenital glaucoma.

Conflict of interest statement

The authors have no conflict of interest to declare

Acknowledgments

We would like to thank Dr. Jacqueline Vanderluit for the use of her microscope and camera, and Liana Dimitrova for histology services. Critical comments on this manuscript were provided by Dr. Paul Chrystal (University of Alberta) and Danielle French (Memorial University). We thank Dr. Fred Berry and Dr. Michael Walter (University of Alberta) for providing the FOXC1 reporter luciferase construct. The authors would also like to thank Gerissa Fowler (Memorial University) for masking all data prior to quantification. This work was funded with a grant from the Canadian Glaucoma Research Society. Data contained in this manuscript was presented at the 2018 Mid-Atlantic SDB Regional Meeting.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.visres.2019.01.008.

References

Beckman, S. (2017). Using Acridine Orange to Measure Cell Death in Ethanol Treated Zebrafish Embryos. Biotek Application Note, Cell Imaging.

Berry, F. B., Skarie, J. M., Mirzayans, F., Fortin, Y., Hudson, T. J., Raymond, V., et al. (2008). FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Human Molecular Genetics, 17*(4), 490–505.

Brzezinski, JAt, Prasov, L., & Glaser, T. (2012). Math5 defines the ganglion cell competence state in a subpopulation of retinal progenitor cells exiting the cell cycle. *Developmental Biology, 365*(2), 395–413.

Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Current Opinion in Neurobiology, 9*(1), 37–46.

Chen, S., Li, H., Gaudenz, K., Paulson, A., Guo, F., Trimble, R., et al. (2013). Defective FGF signaling causes coloboma formation and disrupts retinal neurogenesis. *Cell Research, 23*(2), 254–273.

Chen, J. H., Wang, D., Huang, C., Zheng, Y., Chen, H., Pang, C. P., et al. (2012). Interactive effects of ATOH7 and RFTN1 in association with adult-onset primary open-angle glaucoma. *Investigative Ophthalmology & Visual Science, 53*(2), 779–785.

Cherry, T. J., Trimarchi, J. M., Stadler, M. B., & Cepko, C. L. (2009). Development and diversification of retinal amacrine interneurons at single cell resolution. *Proceedings of the National Academy of Sciences of the United States of America, 106*(23), 9495–9500.

Chrysostomou, V., Rezania, F., Trounce, I. A., & Crowston, J. G. (2013). Oxidative stress and mitochondrial dysfunction in glaucoma. *Current Opinion in Pharmacology, 13*(1), 12–15.

Francisco-Morcillo, J., Sanchez-Calderon, H., Kawakami, Y., Izpisua Belmonte, J. C., Hidalgo-Sanchez, M., & Martin-Partido, G. (2005). Expression of Fgf19 in the developing chick eye. *Brain Research. Developmental Brain Research, 156*(1), 104–109.

French, C. R., Seshadri, S., Destefano, A. L., Fornage, M., Arnold, C. R., Gage, P. J., et al. (2014). Mutation of FOXC1 and PITX2 induces cerebral small-vessel disease. *J Clin Invest, 124*(11), 4877–4881.

Hernandez-Bejarano, M., Gestri, G., Spawls, L., Nieto-Lopez, F., Picker, A., Tada, M., et al. (2015). Opposing Shh and Fgf signals initiate nasotemporal patterning of the zebrafish retina. *Development, 142*(22), 3933–3942.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., et al. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology, 31*(3), 227–229.

Ito, Y. A., Goping, I. S., Berry, F., & Walter, M. A. (2014). Dysfunction of the stress-responsive FOXC1 transcription factor contributes to the earlier-onset glaucoma observed in Axenfeld-Rieger syndrome patients. *Cell Death and Disease, 5*, e1069.

Johnson, T. V., DeKorver, N. W., Levasseur, V. A., Osborne, A., Tassoni, A., Lorber, B., et al. (2014). Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. *Brain, 137*(Pt 2), 503–519.

Kanamoto, T., Rimayanti, U. H. O., & Kiuchi, Y. (2011). Platelet-derived growth factor receptor alpha is associated with oxidative stress-induced retinal cell death. *Current Eye Research, 36*(4), 336–340.

Kay, J. N., Finger-Baier, K. C., Roeser, T., Staub, W., & Baier, H. (2001). Retinal ganglion cell genesis requires lakritz, a Zebrafish atonal Homolog. *Neuron, 30*(3), 725–736.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics, 203*(3), 253–310.

Livesey, F. J., & Cepko, C. L. (2001). Vertebrate neural cell-fate determination: Lessons from the retina. *Nature Reviews Neuroscience, 2*(2), 109–118.

Macgregor, S., Hewitt, A. W., Hysi, P. G., Ruddle, J. B., Medland, S. E., Henders, A. K., et al. (2010). Genome-wide association identifies ATOH7 as a major gene determining human optic disc size. *Human Molecular Genetics, 19*(13), 2716–2724.

Medina-Trillo, C., Aroca-Aguilar, J. D., Mendez-Hernandez, C. D., Morales, L., Garcia-Anton, M., Garcia-Feijoo, J., et al. (2016). Rare FOXC1 variants in congenital glaucoma: Identification of translation regulatory sequences. *European Journal of Human Genetics, 24*(5), 672–680.

Micheal, S., Siddiqui, S. N., Zafar, S. N., Villanueva-Mendoza, C., Cortes-Gonzalez, V., Khan, M. I., et al. (2016). A Novel Homozygous Mutation in FOXC1 Causes Axenfeld Rieger Syndrome with Congenital Glaucoma. *PLoS ONE, 11*(7), e0160016.

Nakayama, Y., Miyake, A., Nakagawa, Y., Mido, T., Yoshikawa, M., Konishi, M., et al. (2008). Fgf19 is required for zebrafish lens and retina development. *Developmental Biology, 313*(2), 752–766.

Nannini, D. R., Torres, M., Chen, Y. I., Taylor, K. D., Rotter, J. I., Varma, R., et al. (2017). A genome-wide association study of vertical cup-disc ratio in a Latino population. *Investigative Ophthalmology & Visual Science, 58*(1), 87–95.

Philomenadin, F. S., Asokan, R. N. V., George, R., Lingam, V., & Sarangapani, S. (2015). Genetic association of SNPs near ATOH7, CARD10, CDKN2B, CDC7 and SIX1/SIX6 with the endophenotypes of primary open angle glaucoma in Indian population. *PLoS ONE, 10*(3), e0119703.

Pierrou, S., Hellqvist, M., Samuelsson, L., Enerback, S., & Carlsson, P. (1994). Cloning and characterization of seven human forkhead proteins: Binding site specificity and DNA bending. *EMBO Journal, 13*(20), 5002–5012.

Prasov, L., Masud, T., Khaliq, S., Mehdi, S. Q., Abid, A., Oliver, E. R., et al. (2012). ATOH7 mutations cause autosomal recessive persistent hyperplasia of the primary vitreous. *Human Molecular Genetics, 21*(16), 3681–3694.

Quigley, H. A., & Broman, A. T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. *British Journal of Ophthalmology, 90*(3), 262–267.

Rapaport, D. H., Wong, L. L., Wood, E. D., Yasumura, D., & LaVail, M. M. (2004). Timing and topography of cell genesis in the rat retina. *The Journal of Comparative Neurology, 474*(2), 304–324.

Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S. A., et al. (2007). p53 activation by knockdown technologies. *PLoS Genetics, 3*(5), e78.

Saleem, R. A., Banerjee-Basu, S., Berry, F. B., Baxevanis, A. D., & Walter, M. A. (2001). Analyses of the effects that disease-causing missense mutations have on the structure and function of the winged-helix protein FOXC1. *American Journal of Human Genetics, 68*(3), 627–641.

Seo, S., Singh, H. P., Lacal, P. M., Sasman, A., Fatima, A., Liu, T., et al. (2012). Forkhead box transcription factor FoxC1 preserves corneal transparency by regulating vascular growth. *Proceedings of the National Academy of Sciences of the United States of America, 109*(6), 2015–2020.

Skarie, J. M., & Link, B. A. (2009). FoxC1 is essential for vascular basement membrane integrity and hyaloid vessel morphogenesis. *Investigative Ophthalmology & Visual Science, 50*(11), 5026–5034.

Smith, R. S., Zabaleta, A., Kume, T., Savinova, O. V., Kidson, S. H., Martin, J. E., et al. (2000). Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. *Human Molecular Genetics, 9*(7), 1021–1032.

Ster, A. M., Popp, R. A., Petrisor, F. M., Stan, C., & Pop, V. I. (2014). The role of oxidative stress and vascular insufficiency in primary open angle glaucoma. *Clujul Medical, 87*(3), 143–146.

Strungaru, M. H., Dinu, I., & Walter, M. A. (2007). Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations. *Investigative Ophthalmology & Visual Science, 48*(1), 228–237.

Tamimi, Y., Skarie, J. M., Footz, T., Berry, F. B., Link, B. A., & Walter, M. A. (2006). FGF19 is a target for FOXC1 regulation in ciliary body-derived cells. *Human Molecular Genetics, 15*(21), 3229–3240.

Thisse, C., & Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols, 3*(1), 59–69.

Topczewska, J. M., Topczewski, J., Shostak, A., Kume, T., Solnica-Krezel, L., & Hogan, B. L. (2001). The winged helix transcription factor Foxc1a is essential for somitogenesis in zebrafish. *Genes & Development, 15*(18), 2483–2493.

Venturini, C., Nag, A., Hysi, P. G., Wang, J. J., Wong, T. Y., Healey, P. R., et al. (2014). Wellcome Trust Case Control Consortium BGG: Clarifying the role of ATOH7 in glaucoma endophenotypes. *British Journal of Ophthalmology, 98*(4), 562–566.

Vinothkumar, S., Rastegar, S., Takamiya, M., Ertzer, R., & Strahle, U. (2008). Sequential and cooperative action of FGFS and SHH in the zebrafish retina. *Developmental Biology, 314*(1), 200–214.

Willardsen, M., Hutcheson, D. A., Moore, K. B., & Vetter, M. L. (2014). The ETS transcription factor Etv1 mediates FGF signaling to initiate proneural gene expression during Xenopus laevis retinal development. *Mechanisms of Development, 131*, 57–67.

Willardsen, M. I., Suli, A., Pan, Y., Marsh-Armstrong, N., Chien, C. B., El-Hodiri, H., et al. (2009). Temporal regulation of Ath5 gene expression during eye development. *Developmental Biology, 326*(2), 471–481.

Williams, A. L., & Bohnsack, B. L. (2015). Neural crest derivatives in ocular development: Discerning the eye of the storm. *Birth Defects Res C Embryo Today, 105*(2), 87–95.

Wu, F., Kaczynski, T. J., Sethuramanujam, S., Li, R., Jain, V., Slaughter, M., et al. (2015). Two transcription factors, Pou4f2 and Isl1, are sufficient to specify the retinal ganglion cell fate. *Proceedings of the National Academy of Sciences of the United States of America, 112*(13), E1559–1568.

Yang, Z., Ding, K., Pan, L., Deng, M., & Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. *Developmental Biology, 264*(1), 240–254.