Apoptotic and Proliferative Defects Characterize Ocular Development in a Microphthalmic BMP Model

Curtis R. French,[1] Tara R. Stach,[2] Lindsey D. March,[3] Ordan J. Lehmann,[1,2] and Andrew J. Waskiewicz[3]

[1]Department of Ophthalmology, University of Alberta, Edmonton, Alberta, Canada
[2]Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada
[3]Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Correspondence: Andrew J. Waskiewicz, CW405, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G2E9; aw@ualberta.ca.

CRF and TRS are joint first authors.

Submitted: January 16, 2013
Accepted: May 26, 2013

Citation: French CR, Stach TR, March LD, Lehmann OJ, Waskiewicz AJ. Apoptotic and proliferative defects characterize ocular development in a microphthalmic BMP model. *Invest Ophthalmol Vis Sci*. 2013;54:4636–4647. DOI:10.1167/iovs.13-11674

Purpose. Vision is critically dependent on ocular size, which is regulated by environmental and genetic factors. Mutation of human *Growth and Differentiation Factor 6* (*GDF6*) or zebrafish *gdf6a* results in a spectrum of small eye phenotypes (microphthalmia, anophthalmia, and coloboma). However, current models do not explain their etiology fully. As such, analyses of apoptosis and cell cycle regulation were undertaken in a zebrafish *gdf6a* mutant.

Methods. Microarray analysis was performed at 2 days after fertilization to uncover novel *gdf6a*-dependent cell cycle regulators. Altered expression of Gdf6a targets was confirmed by in situ hybridization, and resulting changes in cell proliferation were assessed by phosphohistone H3 immunohistochemistry. Analysis of apoptosis was evaluated through activated Caspase 3 immunohistochemistry and chemical inhibitors of cell death.

Results. Reduced numbers of retinal progenitor cells are observed at 24 hours post fertilization (hpf), resulting in microphthalmic eyes in *gdf6a*$^{-/-}$ embryos. At 28 hpf, a wave of apoptosis occurs; however, apoptosis inhibition does not rescue eye size, indicating a limited contribution. Mutants display altered proliferation and expression levels of cell cycle regulators, including members of the *forkhead box i* (*foxi*) transcription factor family expressed in the ciliary marginal zone. Notably, inhibition of *foxi2* in *gdf6a*$^{-/-}$ embryos further reduces eye size.

Conclusions. These data support a model whereby the *gdf6a*$^{-/-}$-induced microphthalmia is based on early regulation of retinal progenitor cell number, and later by regulation of proliferation in the ciliary marginal zone. *Foxi* genes represent downstream effectors of Gdf6a function in the CMZ required for eye size determination.

Keywords: bone morphogenetic protein, forkhead box, Gdf6, apoptosis, proliferation

Microphthalmia, the presence of a small eye, and anophthalmia, the complete lack of ocular tissue, represent part of a phenotypic spectrum of congenital anomalies present in up to 11% of blind children.[1–3] Their complex etiology includes environmental and genetic factors,[1,4] with the latter encompassing coding mutations, copy number variations, and position effects induced by chromosomal translocations. Integrative studies of eye development in zebrafish, chick, mouse, and fly models have identified numerous genes essential for eye formation in which mutations contribute to microphthalmia and anophthalmia. The coordinated action of inductive signals, tissue interactions, and morphogenic movements are a prerequisite for normal ocular development and vision.

Initially, homeodomain transcription factors, such as *Rax, Six3, Pax6,* and *Otx2* specify the presumptive ocular field that evaginates from the diencephalon to form the optic vesicle.[5–7] Heterozygous *OTX2* and *RAX* mutations in human, mouse (*Rx*), and zebrafish (*rx3*) result in anophthalmia or bilateral microphthalmia, with *rx3* mutants exhibiting defects in cellular movement and proliferation.[7–9] Once the optic vesicles contact the surface ectoderm, *Six3, Pax6,* and *Sox2* have critical roles first in lens placode formation and induction, and subsequently in lens development.[7,10,11] The importance of lens induction is illustrated by reduced levels of mouse *Six3, Pax6*, or *Sox2* resulting in microphthalmia or anophthalmia.[1,5,10,12] In mammals, *Pax6*, *Sox2*, and *Otx2* contribute to the specification of the optic vesicle, whose inner layer becomes the neural retina, and requires *Pax6* and *Sox2* expression for accurate progenitor cell specification.[13,14] Mouse and chick analyses similarly demonstrate the requirement of *Mitf* and *Otx2* for RPE specification, which is essential for normal ocular growth.[7,15,16]

Central to the determination of eye size is the control of retinal progenitor cell proliferation and survival. Genes regulating both processes cause microphthalmia in patients and model organisms when mutated.[17–20] Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose at the interface between neural retina and ciliary epithelium, a region called the ciliary marginal zone (CMZ).[21] Thus in zebrafish, mutation of genes that maintain this cell population also may result in microphthalmia,[22] yet little is known

concerning the molecular regulation of cell proliferation in the CMZ.

The bone morphogenetic proteins, and growth and differentiation factors (BMPs/GDFs) have well-recognized ocular developmental roles. BMPs regulate apoptosis and proliferation in the eye, with mutation or copy number variation of 3 BMPs (*GDF3, GDF6*, and *BMP4*) associated with microphthalmia, anophthalmia, or coloboma (MAC) in patients. The contribution of this gene family is extended by the large number of paralogs implicated in eye development through model organism analysis (e.g., *bmp2, bmp7*, and *gdf11*).[23–29] Similarly, perturbed function of multiple BMP antagonists, including *gremlin*, *chordin-like 1*, and *Bambi*, induce ocular anomalies in model organisms and for some (*CHRDL1*), comparable and severe patient anterior segment phenotypes have been defined.[30–32] We demonstrated previously *gdf6a*$^{-/-}$ mutant zebrafish lack Smad 1/5/8 phosphorylation in the developing retina, and we and others demonstrated that *gdf6a* lies at the top of the hierarchy of genes controlling the patterning of the dorsal-ventral axis of the developing retina.[33,34] In addition to this fundamental role in retinal patterning, *gdf6a* mutants display bilateral microphthalmia.[33–36]

Since the cellular mechanisms that underlie this microphthalmic phenotype have yet to be elucidated, we hypothesized that aberrant regulation of cell cycle and apoptosis might contribute, and examined during *gdf6a*$^{-/-}$ ocular development in zebrafish. We demonstrated that *gdf6a*$^{-/-}$ eyes are microphthalmic at early developmental stages due to reduced retinal progenitor cell number. We showed that rescue of elevated ocular apoptosis during development does not restore normal eye size in *gdf6a*$^{-/-}$ larvae. Microarray analysis revealed aberrant expression of genes with roles in cellular proliferation in the CMZ, which included members of the *forkhead box* (*fox*) family of transcription factors. Notably, several of these genes have fundamental roles in development and cell cycle control, and contribute to ocular disorders.[37,38] We find a significant reduction in CMZ proliferation in *gdf6a*$^{-/-}$ larvae, with a requirement of *foxi1* and *foxi2* in mediating dorsal-ventral polarity of the CMZ, and in Gdf6a-mediated eye size determination.

Methods

Zebrafish Husbandry, Morpholino Injections, and In Situ Hybridization

Zebrafish were cared for according to standard protocols, and embryos grown in embryo media at either 25.5°C, 28.5°C, or 33°C to be staged appropriately.[39] Zebrafish embryos grown past 24 hours post fertilization (hpf) were treated with 0.003% 1-phenyl 2-thiourea (PTU; Sigma-Aldrich, St. Louis, MO) to prevent pigment formation. The AB strain of wild-type (WT) fish and the *gdf6a*^{s327} mutant line[34] were used. The latter, hereafter described as *gdf6a*$^{-/-}$, encodes a S55X truncation producing a 54 amino acid peptide lacking the mature domain. For inhibition of *foxi2,* 4 ng of a translation blocking morpholino (MO) (TCGATGGTGTTCATATCTCCAGTGC) were injected into 1- to 2-cell stage embryos. In situ hybridization (ISH) was performed as described previously,[40,41] with embryos fixed overnight at 4°C in 4% paraformaldehyde (PFA) and permeabilized by incubation in 10 μg/mL Proteinase K for 20 minutes. Animal care protocols were approved by the University of Alberta Biosciences Animal Care Committee and comply with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Microarray Analysis

Microarrays were performed using Agilent's 4X44K whole genome chips (version 2; Agilent Technologies, Santa Clara, CA). Four biologic replicates were performed, with each array using RNA isolated from separate matings. RNA was isolated from 30 phenotypically WT and 30 *gdf6a*$^{-/-}$ eyes at 2 days post fertilization (dpf) using the RNAqueous kit (Ambion, Austin, TX), and then amplified and labeled (low input linear amplification kit; Agilent Technologies). Labeled RNA was hybridized for 17 hours at 65°C and then processed per the manufacturer's protocol (Agilent Technologies), scanned on a GenePix 4000 scanner (Molecular Devices, Sunnyvale, CA) before data extraction using Agilent Technologies' Feature Extraction Software.

Immunohistochemistry

For analysis of apoptosis, embryos fixed in 4% PFA were permeabilized with ice-cold acetone (7 minutes), washed in water (5 minutes), before four 5-minute washes in PBS with 0.5% Tween-20 (PBST). For analysis of cell proliferation, fixed embryos were permeabilized using Proteinase K (10 μg/mL for 20 minutes at 2 dpf or 45 minutes at 4 dpf), refixed (20 minutes in 4% PFA), washed four times in PBST (5 minutes each), and then treated with 95°C 10 mM citric acid buffer (10 minutes). Slides containing 15 μM sections were treated with 95°C citric acid buffer, but were not permeabilized. Embryos and slides were blocked for 1 hour at room temperature in 5% normal goat serum and 2% BSA. Primary antibodies used were rabbit anti-activated Caspase 3 (1/1000; BD Biosciences, San Jose, CA), and rabbit antiphosphohistone H3 (1/1000; BD Biosciences). Embryos and slides were incubated in secondary antibody (goat anti-rabbit Alexa Fluor 488, 1/1000; goat anti-rabbit Alexa Fluor 568; Molecular Probes, Eugene, OR) for 2 hours at room temperature. All embryos were washed for 5 minutes in PBST, then 4 times for 10 minutes in PBST after primary and secondary antibody incubations. Hoechst 33258 nuclear stain (1/1000; Molecular Probes) was added to the second 10-minute wash after secondary antibody incubation. Embryos and slides were mounted in Prolong Gold (Molecular Probes) for visualization.

Genotyping

The offspring of a *gdf6a*$^{+/-}$ heterozygous incross were genotyped by high resolution melt (HRM) analysis performed on genomic DNA, extracted in 10 μL of 50 mM NaOH (95°C, 10 minutes, neutralized with 1 μL Tris-HCl, pH 8.0). PCR was performed using primers optimized for HRM (GCGTTTGATG GACAAAGGTC; CCGGGTCCTTAAAATCATCC), MeltDoctor HRM Master Mix (Applied Biosystems, Foster City, CA) and an ABI 7500 HT Fast RT PCR machine (Applied Biosystems) or Qiagen Rotor Gene Q qPCR machine (1 cycle, 95°C for 10 minutes; 40 cycles, 95°C for 15 seconds and 60°C for 20 seconds; 1 cycle, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, and 60°C for 15 seconds; Qiagen, Inc., Valencia, CA). Results were analyzed via HRM 2.0 Software (Applied Biosystems) or Qiagen software v2.02 (Qiagen, Inc.) and variants initially were confirmed by Sanger sequencing. Quantification of average eye size was performed using arbitrary units in ImageJ (National Institutes of Health [NIH], Bethesda, MD), with *gdf6a*$^{+/-}$ control-treated eyes quantified as 100% eye size.

Pharmacologic Treatment

Embryos were treated from 5 to 31 hpf with 0.1 μM P7C3 (Asinex, Moscow, Russia)[42] or dimethyl sulfoxide (DMSO) as a

vehicle control. Then, 28 hpf embryos were fixed and whole-mount immunofluorescence was performed using anti-active Caspase 3 antibody (BD Pharmingen, San Diego, CA) with the nuclear counterstain Hoechst 33258. Dissected eyes were visualized using confocal microscopy (Zeiss LSM 700 on Axio Observer.Z1; Carl Zeiss Microscopy, LLC, Thornwood, NY). All photographed eyes were genotyped by PCR and sequencing. For quantification of eye size, embryos treated with P7C3 from 5 to 31 hpf were transferred into regular embryo media and grown to 3 dpf. Embryos were photographed and genotyped individually (as described above). Eye area was quantified using (ImageJ; NIH). Student's *t*-tests were performed with Bonferonni correction for multiple comparisons.

Results

Loss of *gdf6a* Expression Results in Microphthalmia

The zebrafish *gdf6a*$^{-/-}$ line studied contains a c.164C > A mutation that creates a premature stop codon, resulting in premature truncation of the protein, with loss of the mature domain of the peptide.[34] Homozygous mutant embryos display bilateral microphthalmia, and in comparison with WT or heterozygotes, the eye is noticeably smaller at 2 dpf (Figs. 1A, 1B), with microphthalmia more pronounced by 4 dpf (Figs. 1C, 1D). Previous studies demonstrated *gdf6a*'s expression adjacent to the developing eye field at the 3 somite stage, and from the 10 somite stage onwards in the dorsal retina and dorsal epidermis,[33,34] indicating that specification of retinal progenitor cells may influence eye size. From 2 to 4 dpf, *gdf6a* is expressed within the dorsal CMZ (Figs. 1E–H), highlighting a potential role for defective cell proliferation in the determination of *gdf6a*$^{-/-}$ eye size.

Reduced Number of Retinal Progenitor Cells in *gdf6a*$^{-/-}$ Embryos at 24 hpf

As eye size is noticeably smaller in *gdf6a*$^{-/-}$ embryos by 24 hpf, we conducted an analysis of the number of retinal progenitor cells and proliferation at this time point. Although the expression of a subset of genes that control proliferation is reduced in homozygous mutants (*myca*, Figs. 2A, 2B and *mych,* Figs. 2C, 2D), the number and percent of proliferating cells at this time point are unchanged (Figs. 2E–H). Thus, it is unlikely that early small eye phenotypes are due to a proliferation defect. However, the number of progenitor cells clearly is reduced in *gdf6a*$^{-/-}$ eyes (111.7 ± 8.0) when compared to WT siblings (125 ± 7.7, $P = 0.00017$, *t*-test, Fig. 2G), indicating that processes occurring before 24 hpf influence eye size in *gdf6a*$^{-/-}$ embryos.

Inhibition of the Increased Rates of Apoptosis in the *gdf6a*$^{-/-}$ Eye Does Not Rescue Eye Size

Previous characterization of *gdf6a*$^{-/-}$ embryos[36] and our findings (Fig. 3) show high rates of apoptosis throughout the developing eye. To determine if this contributes to microphthalmia, as suggested previously,[36] *gdf6a*$^{-/-}$ embryos were treated with either P7C3, a pharmacologic agent that inhibits apoptosis,[42] or DMSO control. Analysis of ocular activated Caspase-3 staining at 28 hpf indicated that heterozygous embryos have low levels of apoptosis when incubated with either P7C3 or DMSO (Figs. 3A, 3C). High levels of apoptosis are observed in control DMSO-treated *gdf6a*$^{-/-}$ embryos (mean number of foci [μ] = 76, $n = 34$), which is decreased significantly when treated with P7C3 (μ = 16, $n = 33$, $P < 0.00001$, *t*-test; Figs. 3B, 3D). This rescue of apoptosis in *gdf6a*$^{-/-}$ did not result in any significant rescue of eye size at 3 dpf, as P7C3-treated *gdf6a*$^{-/-}$ eyes still were significantly smaller than their control-treated heterozygous siblings (50.3% of eye size, *t*-test $P < 0.00000001$) or P7C3-treated heterozygous siblings (62% of eye size, $P < 0.001$, *t*-test, Fig. 3E). These data demonstrated that *gdf6a*$^{-/-}$-induced apoptosis is not a major contributor to the microphthalmia phenotype, as partial rescue does not measurably affect ocular size. Incubation of embryos in P7C3 may affect other aspects of retinal development in addition to apoptosis, as evidenced by the subtle change in eye size of treated heterozygous eyes (Fig. 3E).

Genes With Roles in Cell Cycle Progression Have Reduced Expression in *gdf6a*$^{-/-}$ Eyes

To investigate whether later developmental events contributed to microphthalmia in *gdf6a* mutants, we assessed transcriptome changes at 2 dpf via microarray analysis. Using cut-offs of ≥1.75-fold change in expression, 226 transcripts were down-regulated and 90 transcripts were upregulated in *gdf6a*$^{-/-}$ eyes (Supplementary Tables S1 and S2, respectively). Notably, the microarray results revealed significant alteration in expression of genes with roles in cell differentiation and patterning, including *atonal homolog 7*, *t-box 2b*, and *t-box 4, forkhead box N4,* and *H6 family homeobox 1*, with the findings validated by in situ hybridization (Fig. 4, Supplementary Figs. S1A–J). To identify *gdf6a*-dependent transcripts that have a role in proliferation, we focused on genes with ocular expression in the CMZ. Many genes responsible for regulation of cell cycle were down-regulated in *gdf6a*$^{-/-}$ embryos (Fig. 5), suggesting that they contributed to the microphthalmia phenotype. Indeed, in situ hybridization demonstrated the expression of six genes reduced or eliminated specifically in the CMZ of *gdf6a*$^{-/-}$ eyes, but minimally changed or unaffected in other areas of the embryo. This is compatible with a *gdf6a* specific role in control of ocular cell cycle. Altered genes include *carbamoyl-phosphate synthetase 2, aspartate transcarbamylase,* and *dihydroorotase* (*cad,* Figs. 5A–D), *ubiquitin-like with PHD and ring finger domain containing 1* (*uhfr1,* Figs. 5E–H) and *retinal homeobox1* (*rx1,* Figs. 5I–L). Expression of *minichromosome maintenance complex component 3* (*mcm3,* Figs. 5M–P) and *proliferating cell nuclear antigen* (*pcna,* Figs. 5Q–T) is highly down-regulated in the outer retina and CMZ in *gdf6a*$^{-/-}$ embryos, but minimally changed elsewhere. Similar results are seen for three zebrafish paralogs of the cell cycle regulator, *C-Myc*. The expression of *myca,* found at high levels in CMZ, was strongly reduced in *gdf6a*$^{-/-}$ eyes (Figs. 5U–X). This also was the case for *mycn* (Figs. 5Y–B') and *mych* (Figs. 5C'–F'), which displayed strongly reduced expression in the CMZ of *gdf6a*$^{-/-}$ eyes, but were minimally affected in other areas of the embryo. Reduction in ocular expression of genes with roles in cell cycle progression suggested a role for *gdf6a* in cellular proliferation during eye development, and led to examination of possible anomalies in *gdf6a*$^{-/-}$ larvae.

Ocular Proliferation During Development Is Reduced in *gdf6a*$^{-/-}$ Larvae

The decrease in ocular expression of multiple genes with roles in the cell cycle in *gdf6a*$^{-/-}$ implicated altered levels of cellular proliferation in the microphthalmia phenotype. Accordingly, the number of dividing cells was assayed using antiphosphohistone H3 (PH3) antibodies at two time points: 2 dpf, when proliferation occurs primarily in the inner neural layer, and 4 dpf, when postmitotic retinal layers are present in *gdf6a* mutants, and there is a greater contribution from the CMZ. To

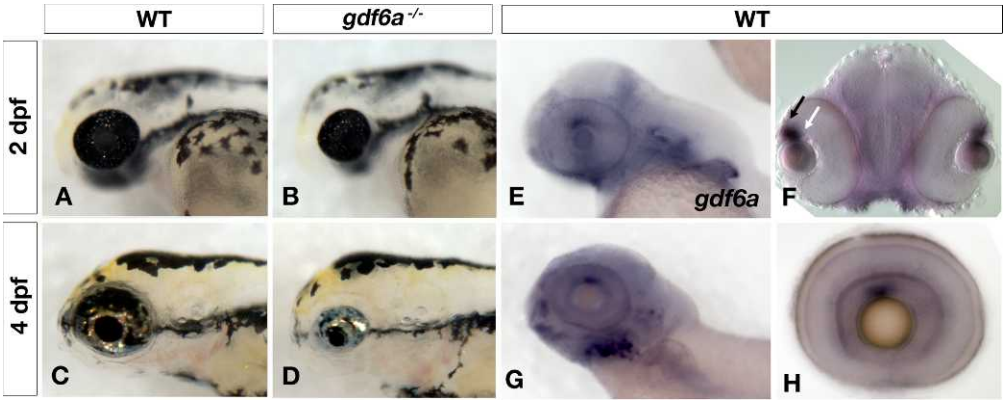

Figure 1. Analysis of *gdf6a* expression and ocular size. At 2 dpf *gdf6a*$^{-/-}$ embryos exhibit microphthalmia (**A**, **B**) that is more evident by 4 dpf (**C**, **D**). At 2 dpf, *gdf6a*'s ocular expression is confined to a small area of the dorsal retina (**E**), and in cross-section this domain includes the dorsal CMZ (*between black and white arrows*) and ganglion cell layer (**F**). Expression remains in the dorsal retina (**G**), with low levels of expression detected throughout the ganglion cell layer and CMZ at 4 dpf (**H**). Live pictures taken at ×90, in situ pictures taken at ×200.

address potential confounding effects from reduced ocular size, the percentage of proliferating cells was quantified in addition to the total number of cells per section. While there is a difference in the number of proliferating cells per section at 2 dpf (*gdf6a*$^{-/-}$: number of cells $\mu = 28$, $n = 7$; WT: $\mu = 47.5$, $n = 6$, $P = 0.0016$, *t*-test), there is no significant difference in the proportion of PH3-positive cells per section (*gdf6a*$^{-/-}$: percent of cells $\mu = 10.0\%$, $n = 7$; WT: $\mu = 11.7\%$, $n = 6$; $P = 0.262$, *t*-test; Figs. 6A, 6B, 6E, 6F, 6I). By 4 dpf there is a statistically significant difference in the total number of PH3-positive cells in the CMZ (*gdf6a*$^{-/-}$: $\mu = 1.1$, $n = 9$); WT: $\mu = 8$, $n = 6$; $P = 0.00004$, *t*-test). These findings are validated by the proportion of positive cells (*gdf6a*$^{-/-}$: mean = 0.7%, $n = 6$; WT: mean = 3.4%, $n = 6$, $P = 0.0017$, *t*-test, Figs. 6C, 6D, 6G, 6H, 6I). The strongly reduced proportion of proliferating cells at 4 dpf suggested that loss of *gdf6a* during ocular development results in decreased proliferation in the CMZ, and, taken together with previous results, suggested that *gdf6a* has a role in regulation of proliferation and apoptosis in the eye during development.

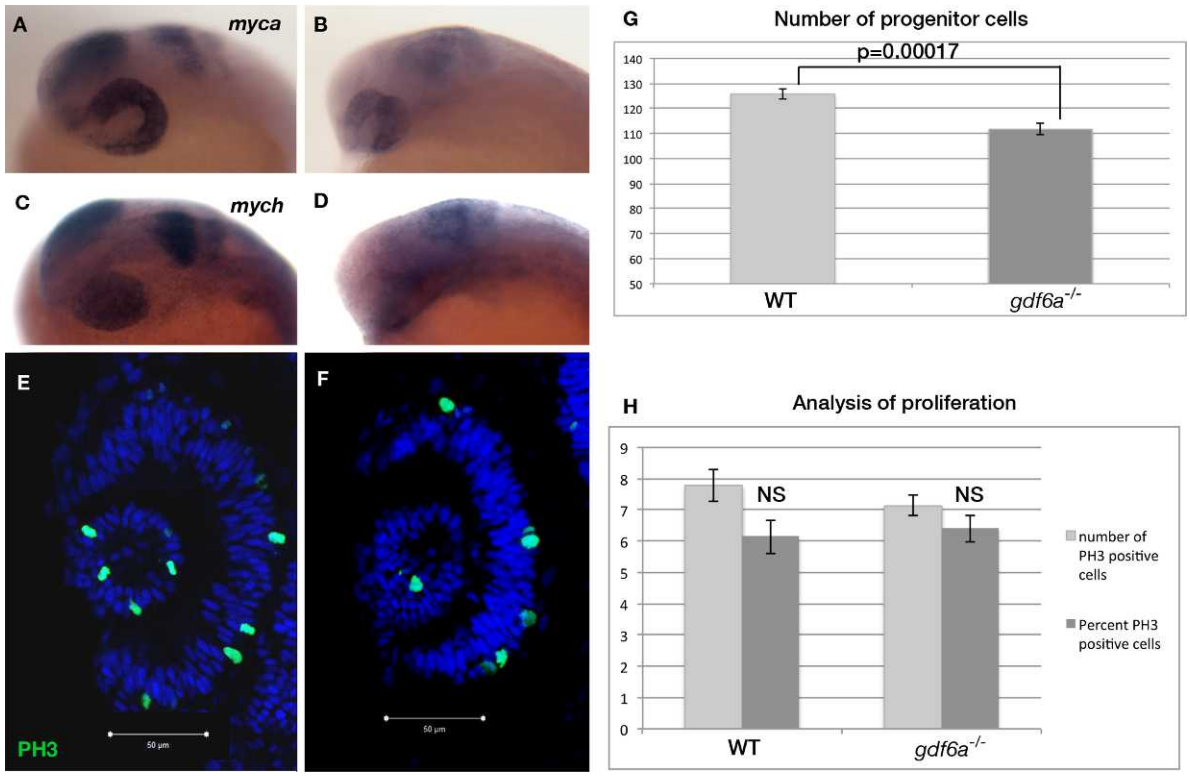

Figure 2. Reduced number of retinal progenitors contribute to microphthalmia in *gdf6a*$^{-/-}$ embryos. While a difference in the expression of cell cycle regulators, such as *myca* (**A**, **B**) and *mych* (**C**, **D**), is observed in *gdf6a* mutants at 24 hpf, no defects in the number or percentage of proliferating cells is observed (**E**, **F**). These data are quantified in (**H**). However, by this time there is a clear reduction in the number of retinal progenitor cells in *gdf6a* mutant eyes ($P = 0.00017$, *t*-test, [**G**]), indicating that reduced cell number contributes to the observed microphthalmia at early stages of development. Graphic data are presented as mean ± SE. In situ pictures taken at ×200.

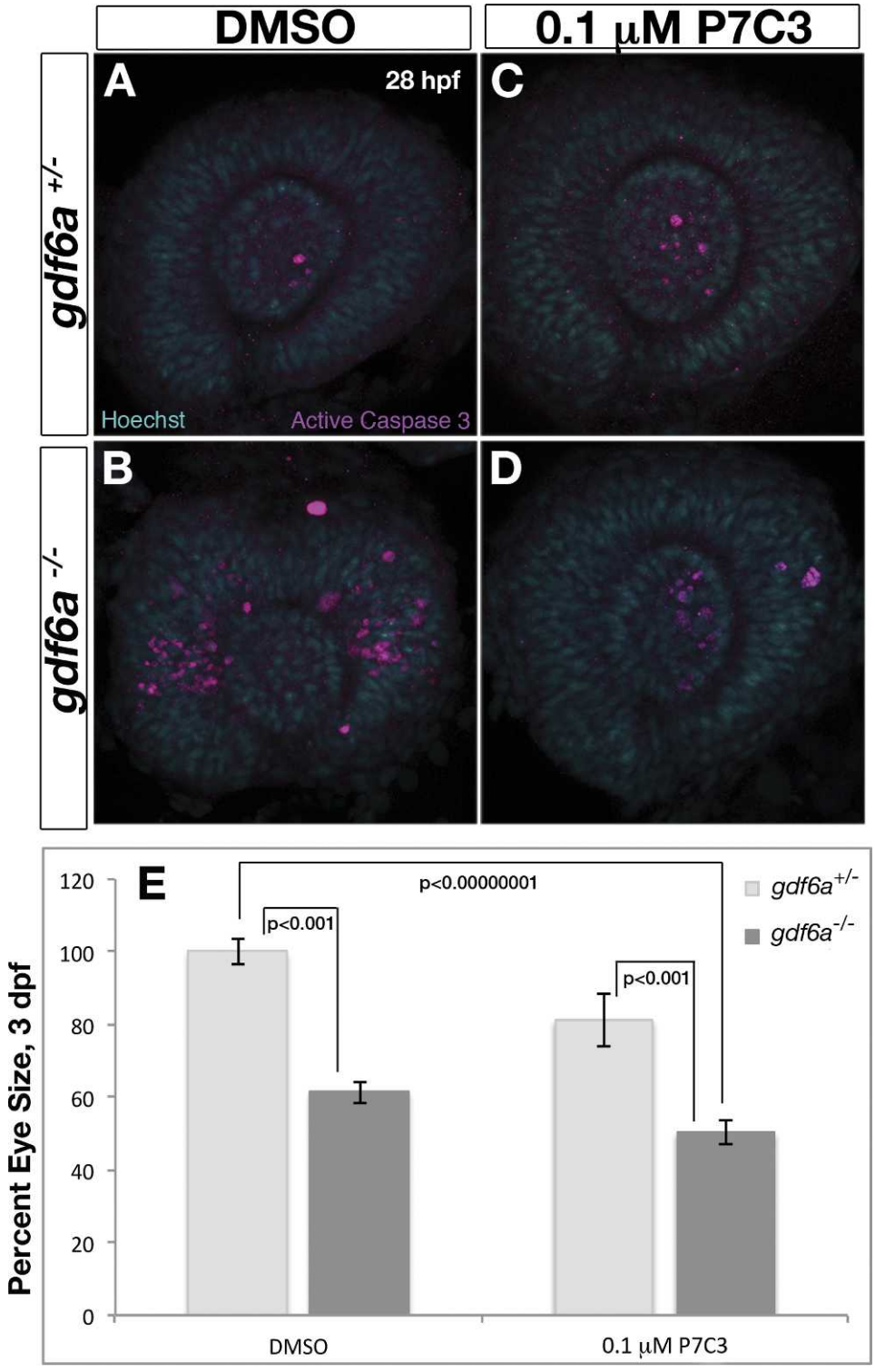

Figure 3. Inhibition of apoptosis in *gdf6a*$^{-/-}$ eyes does not rescue microphthalmia. In contrast to the low levels of ocular apoptosis observed in *gdf6a*$^{+/-}$ embryos treated with DMSO (**A**) or the apoptosis inhibitor P7C3 (**C**), *gdf6a*$^{-/-}$ embryos exhibit high levels of retinal apoptosis (**B**). Treatment of *gdf6a*$^{-/-}$ embryos with P7C3 results in significant reduction in the number of cells undergoing apoptosis in the eye at 28 hpf ($P < 0.00001$, *t*-test, [**D**]). At 3 dpf, no rescue of eye size is observed, as P7C3 treated *gdf6a*$^{-/-}$ eyes remain significantly smaller than DMSO-treated ($P < 0.00000001$, *t*-test) or P7C3-treated ($P < 0.001$, *t*-test) heterozygous siblings. Graphic data are presented as mean ± SE. Confocal pictures taken at ×400.

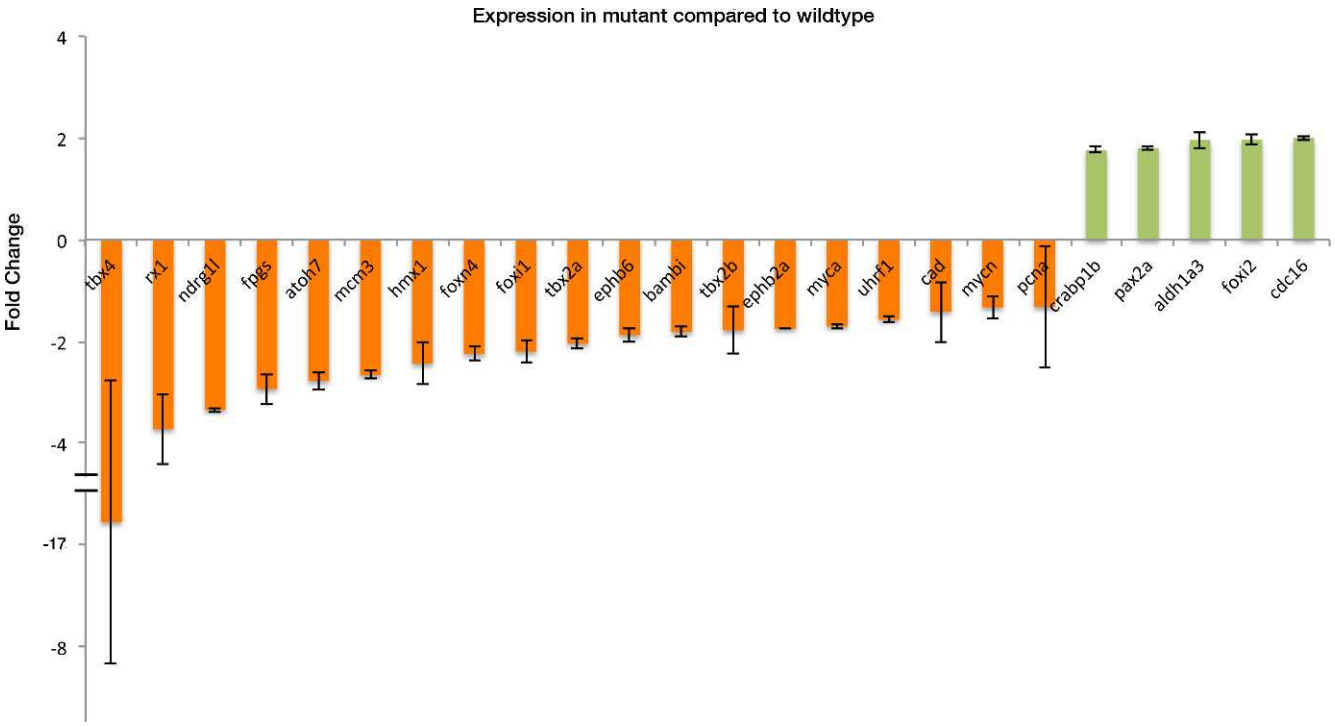

FIGURE 4. Summary of genes in which expression is dysregulated maximally in *gdf6a*$^{-/-}$ mutants. Graphic representation of microarray data illustrating the fold changes in expression in 24 genes that are up- (*green*) or down-regulated (*orange*) in *gdf6a*$^{-/-}$ mutants. Note the down-regulation of genes with roles in the cell cycle, such as *fpgs*, *mcm3, myca*, *mycn*, and *pcna*, along with alterations in three forkhead box transcription factors, *foxn4*, *foxi1*, and *foxi2.* Data are presented as mean ± SE.

Expression of the Forkhead Box Transcription Factors *foxi1* and *foxi2* Are Altered in *gdf6a*$^{-/-}$ Eyes

In addition to identifying altered expression of genes with roles in the cell cycle, microarray analysis also revealed genes with uncharacterized roles in ocular development and deregulated expression in *gdf6a*$^{-/-}$ embryos. The forkhead genes *foxi1* and *foxi2* showed decreased (2.20-fold) and increased (1.97-fold) expression, respectively (Fig. 4). As these genes are members of the forkhead box family of transcription factors that have well documented roles in ocular development,[43–46] we conducted additional studies to elucidate potential roles in determining ocular size. In situ analysis confirmed the microarray findings, and demonstrates that the two transcription factors are expressed in converse patterns in the dorsal (*foxi1*) and ventral (*foxi2*) CMZ (Figs. 7A, 7B, 7E, 7F). In WT embryos, *foxi1* is expressed in the dorsal retina in a pattern similar to *gdf6a* (Figs. 1E, 1F, 7A, 7B), with loss of *gdf6a* resulting in complete loss of *foxi1* expression in the dorsal CMZ (Figs. 7C, 7D). The expression domain of *foxi2* is the mirror image of *foxi1* (Figs. 7E, 7F), and with loss of *gdf6a,* this ventral retinal expression of *foxi2* expands to encompass the dorsal CMZ where *foxi1* normally would reside (Figs. 7G, 7H). These findings indicated that *foxi1* and *foxi2* lie downstream of *gdf6a*. Their specific dorsal or ventral CMZ expression patterns are compatible with a role regulating proliferation in the CMZ, consistent with previously characterized roles of *fox* transcription factors in regulation of the cell cycle.[38] Accordingly, we used gene-specific morpholino injections to block *foxi2* function in *gdf6a*$^{-/-}$ embryos and nonmutant siblings, and assessed consequent ocular size.

Injection of *foxi2* Morpholino Into *gdf6a* Mutants Further Reduces Eye Size

Expression of *foxi* mRNAs delineates dorsal and ventral domains of the ciliary marginal zone. We assessed whether *foxi2* and *gdf6a* function in the same genetic pathway by morpholino inhibition of *foxi2* in *gdf6a*$^{-/-}$ and *gdf6a*$^{+/-}$ embryos. Normally, the ocular size of *gdf6a*$^{+/-}$ embryos closely resembles that of *gdf6a*$^{+/+}$ (Figs. 8A and 1A, respectively), and genetic differences are discernible only upon genotyping. While injection of 4 ng of *foxi2* morpholino into WT embryos resulted in no discernible phenotype (data not shown), injection into *gdf6a*$^{+/-}$ embryos resulted in a small eye phenotype by 2 dpf ($\mu = 76.6\%$ of eye size, $n = 15$, $P = 0.004$, *t*-test, Figs. 8C, 8E), similar to that of *gdf6a*$^{-/-}$ ocular size (Fig. 8B). Moreover, injection of *gdf6a*$^{-/-}$ embryos with *foxi2* morpholino resulted in a further reduction in eye size relative to uninjected *gdf6a*$^{-/-}$ embryos (Figs. 8D, 8E, $\mu = 77\%$ of *gdf6a*$^{-/-}$ eye size, $n = 10$, *gdf6a*$^{-/-}$; $n = 15$ *gdf6a*$^{-/-}$ + Foxi2^{MO}, $P = 0.00014$, *t*-test). On the basis of these findings, we observed a synergistic phenotype when we injected *foxi2* morpholino into *gdf6a*$^{+/-}$ embryos, consistent with these genes functioning on the same or converging genetic pathways.

DISCUSSION

During zebrafish ocular development, the neural retina is formed as progenitor cells exit the cell cycle and differentiate into populations of retinal neurons.[47] Throughout the life of the fish, a population of self-renewing cells remains at the ciliary margin and adds new retinal neurons as the eye grows.[21,48] We demonstrated that *gdf6a*, already known to

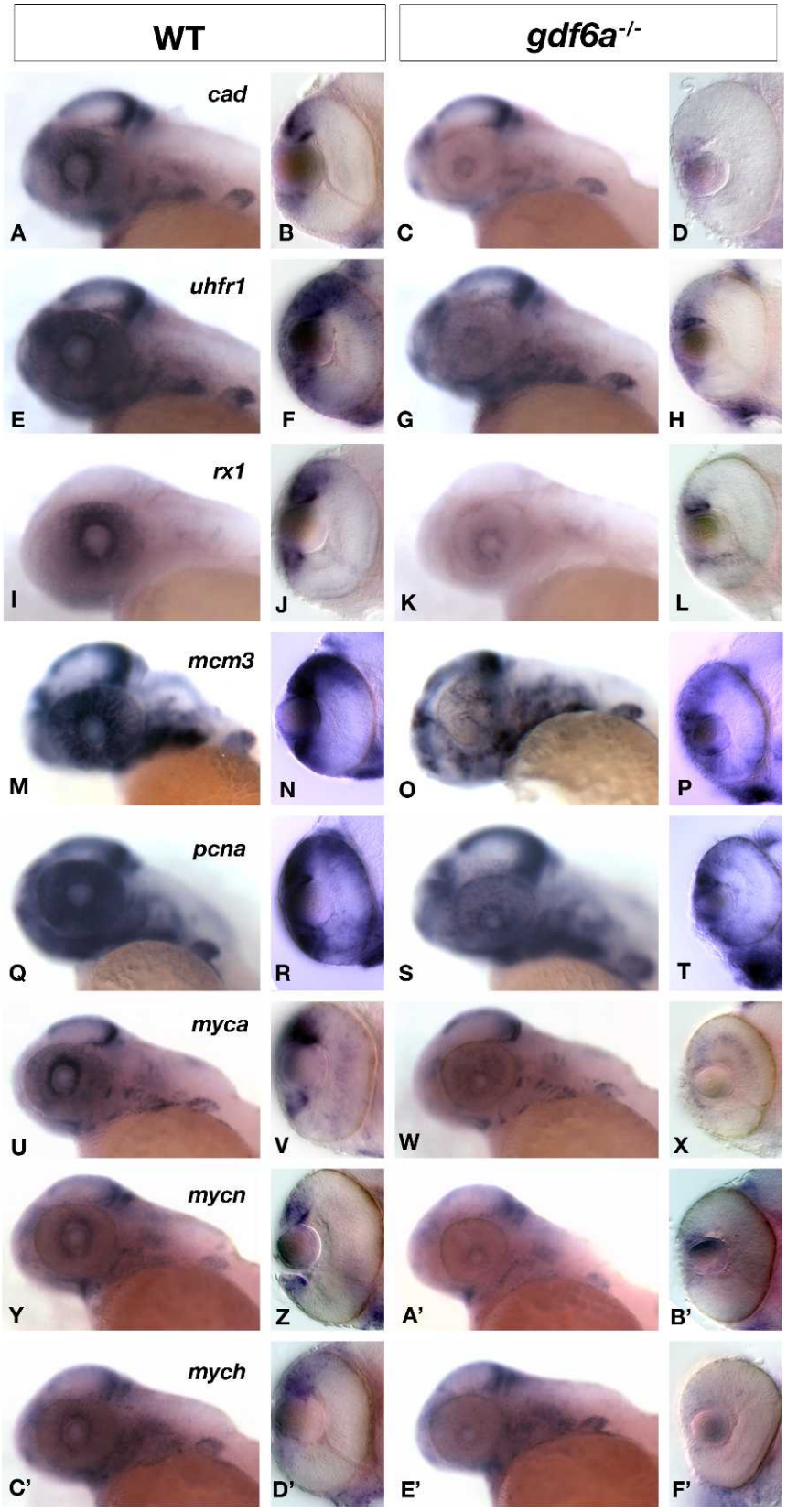

FIGURE 5. In situ hybridization validation of Gdf6a responsive genes with known roles in cell cycle. *Gdf6a*$^{-/-}$ embryos have ocular specific reduction in expression of genes with roles in the cell cycle. The expression of *cad* is lost almost completely in *gdf6a*$^{-/-}$ (**A–D**), while expression of *uhfr1* (**E–H**) and *rx1* (**I–L**) are down-regulated. The cell cycle regulators *mcm3* (**M–P**) and *pcna* (**Q–T**) are expressed at high levels throughout the eye, including the CMZ at 2 dpf, and are down-regulated in *gdf6a*$^{-/-}$ embryos. The expression of three zebrafish *C-Myc* paralogs is found in the CMZ at 2 dpf, and is highly down-regulated (*myca* [**U–X**], *mycn* [**Y–B'**]) or eliminated *(mych* [**C'–F'**]) in *gdf6a*$^{-/-}$ eyes. Whole embryo pictures are taken at ×90, and cross-section pictures are taken at ×200.

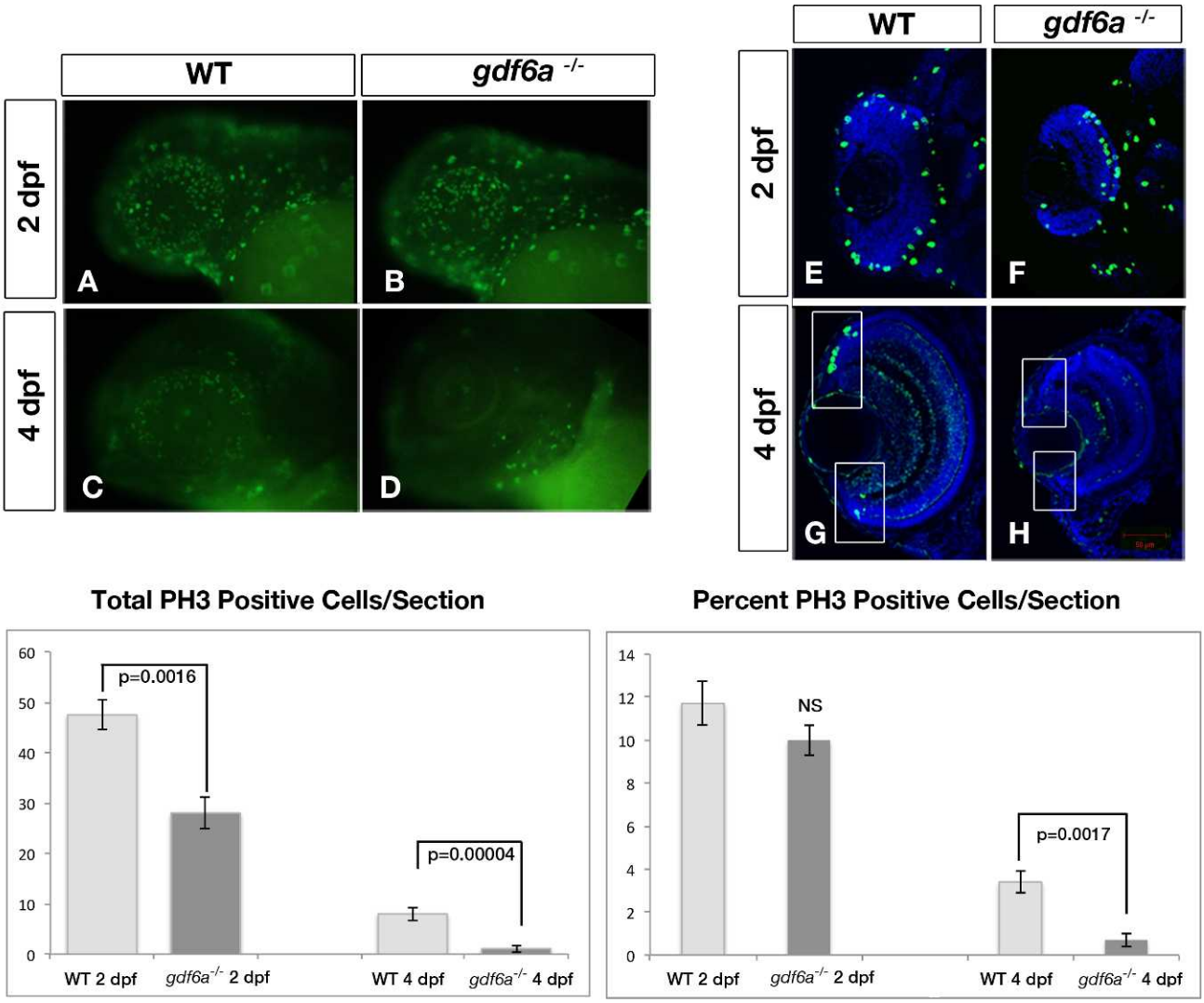

FIGURE 6. Gdf6a regulates cell proliferation in the developing eye. The natural reduction in the level of proliferation between 2 and 4 dpf in WT (**A**, **C**) is accelerated in *gdf6a*$^{-/-}$ embryos (**B**, **D**). Cross-sections of WT embryos at 2 dpf show proliferating cells around the periphery of the eye (**E**), and confined to the CMZ at 4 dpf (*white boxes*, [**G**]). There is a significant reduction of the number of proliferating cells at 2 and 4 dpf (**F**, **H**, **I**) in the whole eye and in the CMZ (*white boxes*, [**H**]). To correct for the reduced number of cells in *gdf6a*$^{-/-}$ embryos, data also were quantified as the percentage of proliferating cells. Only at 4 dpf is the percentage of proliferating cells significantly different between WT and *gdf6a*$^{-/-}$ embryos (**I**). Total number of PH3-positive cells per section of ocular tissue shows a significant reduction at 2 dpf (*gdf6a*$^{-/-}$: $\mu = 28$, $n = 7$; WT: $\mu = 47.5$, $n = 6$, $P = 0.0016$, *t*-test, [**I**]) and 4 dpf (*gdf6a*$^{-/-}$: $\mu = 1.11$, $n = 9$; WT: $\mu = 8$, $n = 6$, $P = 0.00004$, *t*-test, [**I**]). Quantification of the percent of PH3-positive cells shows no significant difference at 2 dpf (*gdf6a*$^{-/-}$: $\mu = 10.0\%$, $n = 7$; WT: $\mu = 11.7\%$, $n = 6$, $P = 0.262$, *t*-test), but a significant difference at 4 dpf between *gdf6a*$^{-/-}$ and WT (*gdf6a*$^{-/-}$: $\mu = 0.68\%$, $n = 6$; WT: $\mu = 3.37\%$, $n = 6$, $P = 0.0017$, *t*-test). Graphic data are presented as mean ± SE. Whole embryo pictures are taken at ×90.

initiate dorsal retina patterning and having a key role in lens development,[33,34] also regulates the cell cycle during retinal development. Parallels existed between the requirement of *gdf6a* for initiation of dorsal retina identity, and *gdf6a*'s comparable role in specifying the dorsal identify of the CMZ. Notably, the CMZ has a polarized axis specified by *foxi1* and *foxi2*, with a requirement of *foxi2* in maintenance of ocular size in *gdf6a*$^{+/-}$ fish.

Mutations in *GDF6* resulted in microphthalmia, anophthalmia, and coloboma,[27,28] phenotypes that are recapitulated in mouse, frog, and zebrafish model systems.[33-36] While the early role of *gdf6a* for dorsal retinal identity is well documented, few studies have addressed the mechanism underlying the small eye phenotype. Microphthalmia is observed as early as 24 hpf, and becomes more pronounced at later developmental stages. By 24 hpf, *gdf6a*$^{-/-}$ eyes contain less progenitor cells than their WT or heterozygous counterparts, indicating a potential role for *gdf6a* in retinal precursor cell specification, or optic cup evagination as one facet of the microphthalmic phenotype. Consistent with this hypothesis, it has been shown that other BMP ligands, such as Bmp2b, have a role in selecting the eye field from surrounding forebrain tissue.[49] Given *gdf6a's* expression adjacent to the early eye fields,[33] it also may have a role in such processes.

The microphthalmia observed in *gdf6a*-deficient zebrafish and *Xenopus* embryos had been attributed to increased levels of retinal apoptosis.[35,36] Despite these reports, we found that inhibition of this apoptosis with P7C3, and anti-apoptotic compound thought to protect mitochondrial membrane integrity,[42] does not rescue microphthalmia. Although incubation with this agent may have other effects on retinal cell development that were not tested, it clearly is able to inhibit

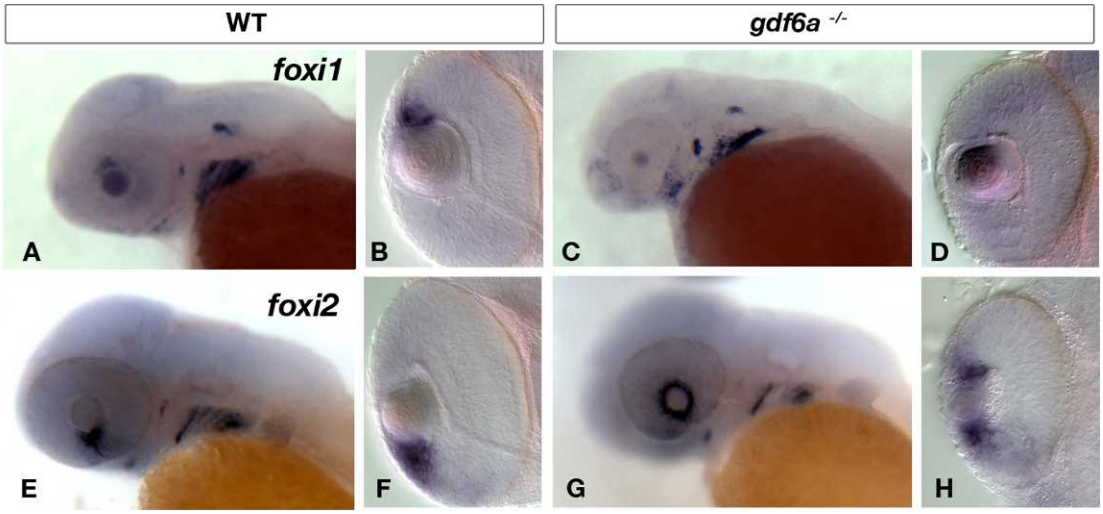

FIGURE 7. Altered *foxi* expression in the ciliary marginal zone of *gdf6a*$^{-/-}$ eyes. At 48 hpf *foxi1* is expressed in the dorsal retina, CMZ, lens, otic vesicle, and pharyngeal arches of WT fish (**A**, **B**), while in *gdf6a*$^{-/-}$ mutants *foxi1* expression is absent in the dorsal retina, reduced in the pharyngeal arches, and unchanged in the lens and otic vesicle (**C**, **D**). Expression of *foxi2* at 48 hpf in WT embryos is in the ventral retinal and surrounding the choroid fissure (**E**, **F**), while in *gdf6a*$^{-/-}$ mutants it expands to the CMZ surrounding the lens with reduced expression also noted in the pharyngeal arches (**G**, **H**). In situ pictures taken at ×90.

retinal apoptosis with no associated increase in eye size. This lack of eye size rescue via incubation with P7C3 also has been observed up to 7 dpf.[50] Although this result does not exclude an apoptotic contribution to the *gdf6a*$^{-/-}$ small eye phenotype, additional factors clearly are involved.

Increasing evidence supports a distinct role for *gdf6a* in regulating targets that control ocular proliferation during development. Indeed, there are significant alterations in the transcriptome after loss of *gdf6a* that are involved in cell cycle progression, such as *mcm3, pcna, myca, mycn*, and *mych. MYC* genes have well-documented roles in the regulation of proliferation in normal and tumorigenic cells, and the *MYC* proto-oncogene is a known downstream target of the TGF-β signaling pathway.[51] *Myc* family members previously have implicated roles in ocular development, and are responsible for microphthalmic phenotypes seen in *meis1* mutant zebrafish, as *myca* expression is lost from the developing eye, and coinjection of *c-myc* mRNA with *meis1* MO rescues cell cycle defects and eye size.[52] It recently has been demonstrated that knock-down of *hmx1* results in microphthalmia in part due to the failure of retinal progenitors to exit the cell cycle.[53] Since *hmx1* is one of the most down-regulated genes on the microarray, it is likely that this also may contribute to *gdf6a*$^{-/-}$-induced microphthalmia. Similarly, *cad* expression is down-regulated in *gdf6a* mutants, consistent with *cad* mutant phenotypes that involve small eyes and reduced retinal proliferation.[54] Taken together, our results indicated that Gdf6a regulates ocular proliferation through multiple genetic path-

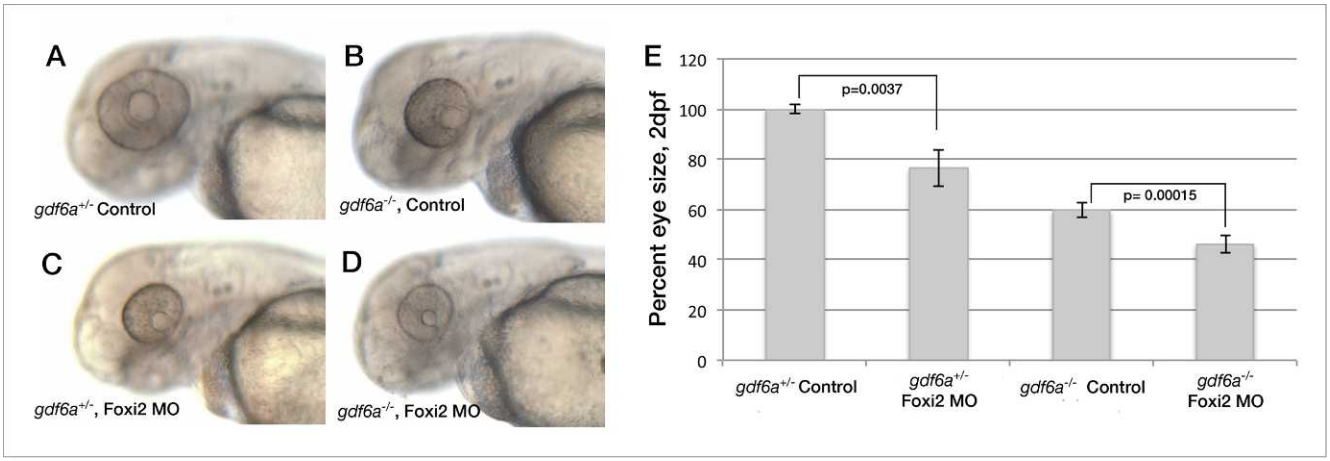

FIGURE 8. Loss of *foxi2* in *gdf6a*$^{+/-}$ and *gdf6a*$^{-/-}$ fish reduces eye size. Heterozygous (*gdf6a*$^{+/-}$) larvae have normal ocular size (**A**), in contrast to the microphthalmia evident in homozygous mutant (*gdf6a*$^{-/-}$) at 48 hpf (**B**). Foxi2 morpholino injection into *gdf6a*$^{+/-}$ embryos results in *gdf6a*$^{+/-}$ larvae developing microphthalmic eyes ([**C**], $n = 15$) almost indistinguishable from those of *gdf6a*$^{-/-}$ (**B**). Quantification of average eye size demonstrates that *gdf6a*$^{+/-}$ larvae injected with 4 ng of *foxi2*^{MO} have significantly smaller eyes than uninjected *gdf6a*$^{+/-}$ larvae ([**E**], $\mu = 76.6\%$ eye size, $n = 15$, $P = 0.004$, *t*-test). Similarly, a reduction in the eye size of *gdf6a*$^{-/-}$ embryos is observed when compared to uninjected (**D**). Quantification of this change reveals significantly smaller eyes of *gdf6a*$^{-/-}$ larvae injected with *foxi2*^{MO} when compared to uninjected *gdf6a*$^{-/-}$ larvae ([**E**], $\mu = 77\%$ of *gdf6a*$^{-/-}$ eye size, $n = 10$ *gdf6a*$^{-/-}$; $n = 15$ *gdf6a*$^{-/-}$ + Foxi2^{MO}, $P = 0.00014$, *t*-test). Graphic data are presented as mean ± SE. Live pictures taken are at ×90.

ways, similar to studies in mice where loss of Bmp receptors (*Bmpr1a* and *Bmpr1b*) affect eye size through multiple mechanisms.[55]

Although our data at 2 dpf were in accord with previous studies demonstrating no significant alteration in proliferation rates,[36] we observed a profound reduction in the number and proportion of proliferating cells at 4 dpf in the CMZ. From such findings, we concluded that the microphthalmia in *gdf6a*$^{-/-}$ larvae is due in part to significant decreased ocular proliferation. Although Gdf6a has been shown to regulate processes specifically in the dorsal retina,[33,34] the decrease in proliferation is uniform across the dorsal ventral axis. In addition, *gdf6a*$^{-/-}$ eyes clearly are smaller than their WT siblings at earlier stages, such as 24 hpf, and contain less progenitor cells, suggesting that specification of progenitors, or migration within the optic cup may influence early eye size in these animals. Apoptosis does not appear to have a major role in the induction of microphthalmia in *gdf6a* mutants, as rescuing apoptosis does not improve eye size significantly. However, the possibility that cells that fail to progress through the cell cycle undergo apoptosis (at stages not tested), remains to be explored. We also cannot rule out other processes, such as eye patterning and neural differentiation as contributing to *gdf6a*$^{-/-}$-induced microphthalmia, as Gdf6a clearly is involved in such processes.

The forkhead box transcription factors, *foxi1* and *foxi2*, are present at 28 hpf in WT embryos in multiple sites, including the dorsal and ventral CMZ, respectively, and the otic vesicle and pharyngeal arches. Zebrafish *foxi1* has a key role in otic placode formation and jaw development through maintenance of survival of neural crest cell populations.[56–60] Full elucidation of the developmental roles of *foxi2* has yet to occur; expression in the mouse neural retinal layer has been described, and *Xenopus* Foxi2 was shown recently to be required for activation of Foxi1e, critical for consequent ectodermal gene expression.[61,62] *gdf6a*$^{-/-}$ embryos have significant alterations in ocular expression of *foxi1* and *foxi2*, as *foxi1* is lost from the dorsal CMZ, and *foxi2* expands to encompass the ventral and dorsal CMZ. The highly specific expression pattern of these transcription factors, coupled with loss of *foxi1* when dorsal retinal identity is not initiated, suggests that the CMZ has similar dorsal-ventral patterning to the retina. We are aware of no previous reports of CMZ patterning and cellular identity, which implies that stem cells in these areas may have differential proliferative or inductive potential. With *foxi1*'s known role in maintenance of neural crest cell survival, and roles of other *fox*'s in cell cycle control,[57,60,61] we hypothesized that *foxi1* and *foxi2* have a role in regulation of cell cycle progression in the CMZ and consequent ocular size. We examined possible anomalies in ocular proliferation and apoptosis in the previously described *foo* mutant (*foxi1*^{bi3747Tg})[63] that contains a retrovirus-induced mutation, and observed no changes in ocular size and proliferation (data not shown). It is possible that *foxi1* has redundant roles with *gdf6a* and other dorsal genes. Alternatively, expansion of *foxi2* expression may compensate for loss of *foxi1*. If the latter is correct, then loss of *foxi2* should result in reduced eye size in *gdf6a* mutants. This, indeed, is the case as transient knockdown of *foxi2* coupled with loss of one functional copy of *gdf6a* resulted in the occurrence of microphthalmia, revealing a novel requirement for this gene in controlling ocular size. The further reduction of eye size in *gdf6a* homozygous mutants injected with Foxi2 morpholino, compared to that of homozygous mutants alone further supported our hypothesis of a compensatory role of *foxi2* in the determination of eye size in *gdf6a* mutants. Of great interest for the future is elucidation of developmental roles of *foxi2* and its transcriptional targets in the eye, as currently there is a lack in literature on this topic.

In summary, we have shown that central to microphthalmia in *gdf6a*$^{-/-}$ larvae is the reduction in ocular proliferation during development, due to significant changes in expression of genes responsible for cell cycle progression in the eye. Gdf6a-induced ocular apoptosis does not appear to have a primary role in the determination of eye size, as inhibition does not rescue microphthalmia. Lastly, we have demonstrated that the CMZ has dorsal-ventral identity specified by *foxi1* and *foxi2*, with *foxi2* having a role in the control of ocular size.

Acknowledgments

The authors thank Aleah McCorry for maintenance of fish stocks, and Herwig Baier (Max Planck Insitute for Neurobiology) for providing the zebrafish *dark half* (*gdf6a*) mutant line. Andrew J. Waskiewicz and Ordan J. Lehmann are recipients of Canada Research Chairs.

Supported by Canadian Institutes of Health Research (OJL, AJW), the Natural Sciences and Engineering Research Council (AJW), The Foundation Fighting Blindness (AJW), Alberta Innovates Health Solutions (TRS, CRF), and the University of Alberta (TRS). The authors alone are responsible for the content and writing of the paper.

Disclosure: **C.R. French**, None; **T.R. Stach**, None; **L.D. March**, None; **O.J. Lehmann**, None; **A.J. Waskiewicz**, None

References

1. Verma AS, Fitzpatrick DR. Anophthalmia and microphthalmia. *Orphanet J Rare Dis*. 2007;2:47.
2. Morrison D, FitzPatrick D, Hanson I, et al. National study of microphthalmia, anophthalmia, and coloboma (MAC) in Scotland: investigation of genetic aetiology. *J Med Genet*. 2002;39:16–22.
3. Parker SE, Mai CT, Canfield MA, et al. Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. *Birth Defects Res Clin Mol Teratol*. 2010;88:1008–1016.
4. Kallen B, Robert E, Harris J. The descriptive epidemiology of anophthalmia and microphthalmia. *Int J Epidemiol*. 1996;25:1009–1016.
5. Shaham O, Menuchin Y, Farhy C, Ashery-Padan R. Pax6: a multi-level regulator of ocular development. *Prog Ret Eye Res*. 2012;31:351–376.
6. Bardakjian TM, Schneider A. The genetics of anophthalmia and microphthalmia. *Curr Opin Ophthalmol*. 2011;22:309–313.
7. Fuhrmann S. Eye morphogenesis and patterning of the optic vesicle. *Curr Top Dev Biol*. 2010;93:61–84.
8. Ragge NK, Brown AG, Poloschek CM, et al. Heterozygous mutations of OTX2 cause severe ocular malformations. *Amer J Hum Genet*. 2005;76:1008–1022.
9. Schilter KF, Schneider A, Bardakjian T, et al. OTX2 microphthalmia syndrome: four novel mutations and delineation of a phenotype. *Clin Genet*. 2011;79:158–168.
10. Smith AN, Miller LA, Radice G, Ashery-Padan R, Lang RA. Stage-dependent modes of Pax6-Sox2 epistasis regulate lens development and eye morphogenesis. *Development*. 2009;136:2977–2985.
11. Liu W, Lagutin OV, Mende M, Streit A, Oliver G. Six3 activation of Pax6 expression is essential for mammalian lens induction and specification. *EMBO J*. 2006;25:5383–5395.
12. van Raamsdonk CD, Tilghman SM. Dosage requirement and allelic expression of PAX6 during lens placode formation. *Development*. 2000;127:5439–5448.

13. Matsushima D, Heavner W, Pevny LH. Combinatorial regulation of optic cup progenitor cell fate by SOX2 and PAX6. *Development*. 2011;138:443–454.
14. Wen J, Hu Q, Li M, et al. Pax6 directly modulate Sox2 expression in the neural progenitor cells. *Neuroreport*. 2008; 19:413–417.
15. Martinez-Morales JR, Dolez V, Rodrigo I, et al. OTX2 activates the molecular network underlying retina pigment epithelium differentiation. *J Biol Chem*. 2003;278:21721–21731.
16. Westenskow P, Piccolo S, Fuhrmann S. Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. *Development*. 2009;136:2505–2510.
17. Dhomen NS, Balaggan KS, Pearson RA, et al. Absence of chx10 causes neural progenitors to persist in the adult retina. *Invest Ophthalmol Vis Sci*. 2006;47:386–396.
18. Thiel G. How Sox2 maintains neural stem cell identity. *Biochem J*. 2013;450:e1–2.
19. Wu LY, Li M, Hinton DR, et al. Microphthalmia resulting from MSX2-induced apoptosis in the optic vesicle. *Invest Ophthalmol Vis Sci*. 2003;44:2404–2412.
20. Steingrimsson E, Copeland NG, Jenkins NA. Melanocytes and the microphthalmia transcription factor network. *Ann Rev Genet*. 2004;38:365–411.
21. Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ. Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Dev Biol*. 2006;6:36.
22. Nuckels RJ, Ng A, Darland T, Gross JM. The vacuolar-ATPase complex regulates retinoblast proliferation and survival, photoreceptor morphogenesis, and pigmentation in the zebrafish eye. *Invest Ophthalmol Vis Sci*. 2009;50:893–905.
23. Bakrania P, Efthymiou M, Klein JC, et al. Mutations in BMP4 cause eye, brain, and digit developmental anomalies: overlap between the BMP4 and hedgehog signaling pathways. *Amer J Hum Genet*. 2008;82:304–319.
24. Reis LM, Tyler RC, Schilter KF, et al. BMP4 loss-of-function mutations in developmental eye disorders including SHORT syndrome. *Hum Genet*. 2011;130:495–504.
25. Ye M, Berry-Wynne KM, Asai-Coakwell M, et al. Mutation of the bone morphogenetic protein GDF3 causes ocular and skeletal anomalies. *Hum Mol Genet*. 2010;19:287–298.
26. Trousse F, Esteve P, Bovolenta P. Bmp4 mediates apoptotic cell death in the developing chick eye. *J Neurosci*. 2001;21:1292–1301.
27. Asai-Coakwell M, French CR, Berry KM, et al. GDF6, a novel locus for a spectrum of ocular developmental anomalies. *Amer J Hum Genet*. 2007;80:306–315.
28. Asai-Coakwell M, French CR, Ye M, et al. Incomplete penetrance and phenotypic variability characterize Gdf6-attributable oculo-skeletal phenotypes. *Hum Mol Genet*. 2009;18:1110–1121.
29. Gonzalez-Rodriguez J, Pelcastre EL, Tovilla-Canales JL, et al. Mutational screening of CHX10, GDF6, OTX2, RAX and SOX2 genes in 50 unrelated microphthalmia-anophthalmia-coloboma (MAC) spectrum cases. *Brit J Ophthalmol*. 2010;94:1100–1104.
30. Huillard E, Laugier D, Marx M. Defects in chicken neuroretina misexpressing the BMP antagonist Drm/Gremlin. *Dev Biol*. 2005;283:335–344.
31. Webb TR, Matarin M, Gardner JC, et al. X-linked megalocornea caused by mutations in CHRDL1 identifies an essential role for ventroptin in anterior segment development. *Amer J Hum Genet*. 2012;90:247–259.
32. Paulsen M, Legewie S, Eils R, Karaulanov E, Niehrs C. Negative feedback in the bone morphogenetic protein 4 (BMP4) synexpression group governs its dynamic signaling range and canalizes development. *Proc Natl Acad Sci U S A*. 2011;108: 10202–10207.
33. French CR, Erickson T, French DV, Pilgrim DB, Waskiewicz AJ. Gdf6a is required for the initiation of dorsal-ventral retinal patterning and lens development. *Dev Biol*. 2009;333:37–47.
34. Gosse NJ, Baier H. An essential role for Radar (Gdf6a) in inducing dorsal fate in the zebrafish retina. *Proc Natl Acad Sci U S A*. 2009;106:2236–2241.
35. Hanel ML, Hensey C. Eye and neural defects associated with loss of GDF6. *BMC Dev Biol*. 2006;6:43.
36. den Hollander AI, Biyanwila J, Kovach P, et al. Genetic defects of GDF6 in the zebrafish out of sight mutant and in human eye developmental anomalies. *BMC Genet*. 2010;11:102.
37. Hannenhalli S, Kaestner KH. The evolution of Fox genes and their role in development and disease. *Nat Rev Genet*. 2009; 10:233–240.
38. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer*. 2007;7:847–859.
39. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995;203:253–310.
40. Gongal PA, Waskiewicz AJ. Zebrafish model of holoprosencephaly demonstrates a key role for TGIF in regulating retinoic acid metabolism. *Hum Mol Genet*. 2008;17:525–538.
41. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc*. 2008;3:59–69.
42. Pieper AA, Xie S, Capota E, et al. Discovery of a proneurogenic, neuroprotective chemical. *Cell*. 2010;142:39–51.
43. Berry FB, Lines MA, Oas JM, et al. Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Hum Mol Genet*. 2006;15:905–919.
44. Seo S, Singh HP, Lacal PM, et al. Forkhead box transcription factor FoxC1 preserves corneal transparency by regulating vascular growth. *Proc Natl Acad Sci U S A*. 2012;109:2015–2020.
45. Semina EV, Brownell I, Mintz-Hittner HA, Murray JC, Jamrich M. Mutations in the human forkhead transcription factor FOXE3 associated with anterior segment ocular dysgenesis and cataracts. *Hum Mol Genet*. 2001;10:231–236.
46. Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron*. 1995;14:1141–1152.
47. Cepko CL, Austin CP, Yang X, Alexiades M, Ezzeddine D. Cell fate determination in the vertebrate retina. *Proc Natl Acad Sci U S A*. 1996;93:589–595.
48. Wehman AM, Staub W, Meyers JR, Raymond PA, Baier H. Genetic dissection of the zebrafish retinal stem-cell compartment. *Dev Biol*. 2005;281:53–65.
49. Bielen H, Houart C. BMP signaling protects telencephalic fate by repressing eye identity and its Cxcr4-dependent morphogenesis. *Dev Cell*. 2012;23:812–822.
50. Asai-Coakwell M, March L, Dai XH, et al. Contribution of growth differentiation factor 6-dependent cell survival to early-onset retinal dystrophies. *Hum Mol Genet*. 2013;22:1432–1442.
51. Dang CV. MYC on the path to cancer. *Cell*. 2012;149:22–35.
52. Bessa J, Tavares MJ, Santos J, et al. meis1 regulates cyclin D1 and c-myc expression, and controls the proliferation of the multipotent cells in the early developing zebrafish eye. *Development*. 2008;135:799–803.
53. Boisset G, Schorderet DF. Zebrafish hmx1 promotes retinogenesis. *Exp Eye Res*. 2012;105:34–42.
54. Willer GB, Lee VM, Gregg RG, Link BA. Analysis of the Zebrafish perplexed mutation reveals tissue-specific roles for

Investigative Ophthalmology & Visual Science

de novo pyrimidine synthesis during development. *Genetics*. 2005;170:1827–1837.

55. Murali D, Yoshikawa S, Corrigan RR, et al. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. *Development*. 2005;132:913–923.
56. Solomon KS, Kudoh T, Dawid IB, Fritz A. Zebrafish foxi1 mediates otic placode formation and jaw development. *Development*. 2003;130:929–940.
57. Nissen RM, Yan J, Amsterdam A, Hopkins N, Burgess SM. Zebrafish foxi one modulates cellular responses to Fgf signaling required for the integrity of ear and jaw patterning. *Development*. 2003;130:2543–2554.
58. Hulander M, Wurst W, Carlsson P, Enerback S. The winged helix transcription factor Fkh10 is required for normal development of the inner ear. *Nat Genet*. 1998;20:374–376.
59. Solomon KS, Logsdon JM Jr, Fritz A. Expression and phylogenetic analyses of three zebrafish FoxI class genes. *Dev Dyn*. 2003;228:301–307.
60. Ohyama T, Groves AK. Expression of mouse Foxi class genes in early craniofacial development. *Dev Dyn*. 2004;231:640–646.
61. Wijchers PJ, Hoekman MF, Burbach JP, Smidt MP. Cloning and analysis of the murine Foxi2 transcription factor. *Biochim Biophys Acta*. 2005;1731:133–138.
62. Cha SW, McAdams M, Kormish J, Wylie C, Kofron M. Foxi2 is an animally localized maternal mRNA in Xenopus, and an activator of the zygotic ectoderm activator Foxi1e. *PLoS One*. 2012;7:e41782.
63. Amsterdam A, Burgess S, Golling G, et al. A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev*. 1999;13:2713–2724.

Investigative Ophthalmology & Visual Science