Antagonism between Gdf6a and retinoic acid pathways controls timing of retinal neurogenesis and growth of the eye in zebrafish

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ABSTRACT

Maintaining neurogenesis in growing tissues requires a tight balance between progenitor cell proliferation and differentiation. In the zebrafish retina, neuronal differentiation proceeds in two stages with embryonic retinal progenitor cells (RPCs) of the central retina accounting for the first rounds of differentiation, and stem cells from the ciliary marginal zone (CMZ) being responsible for late neurogenesis and growth of the eye. In this study, we analyse two mutants with small eyes that display defects during both early and late phases of retinal neurogenesis. These mutants carry lesions in gdf6a, a gene encoding a BMP family member previously implicated in dorsoventral patterning of the eye. We show that gdf6a mutant eyes exhibit expanded retinoic acid (RA) signalling and demonstrate that exogenous activation of this pathway in wild-type eyes inhibits retinal growth, generating small eyes with a reduced CMZ and fewer proliferating progenitors, similar to gdf6a mutants. We provide evidence that RA regulates the timing of RPC differentiation by promoting cell cycle exit. Furthermore, reducing RA signalling in gdf6a mutants re-establishes appropriate timing of embryonic retinal neurogenesis and restores putative stem and progenitor cell populations in the CMZ. Together, our results support a model in which dorsally expressed gdf6a limits RA pathway activity to control the transition from proliferation to differentiation in the growing eye.

KEY WORDS: Retinoic acid, BMP, Gdf6a, Ciliary marginal zone, Neurogenesis, Retinal stem cells, Zebrafish

INTRODUCTION

The balance between cell proliferation and differentiation is spatially and temporally regulated during development, ensuring the generation of tissues with the correct proportion of differentiated cells (Schmidt et al., 2013; Urban and Guillemot, 2014). In the vertebrate retina, this process begins when retinal progenitor cells (RPCs) successively exit the cell cycle to generate retinal ganglion cells (RGCs), cone photoreceptors, interneurons, rod photoreceptors and Müller glia (Cepko, 2014). This progression of fate specification and the timing of neurogenic decisions require cells to integrate intrinsic information and extrinsic signals (Cepko, 2014; Cerveny et al., 2012). Cell cycle regulators and a variety of transcription factors are implicated in the cell-autonomous progression from RPC to post-mitotic neuron, and secreted signals can influence the transition from proliferation to differentiation (Agathocleous and Harris, 2009; Bassett and Wallace, 2012).

In vertebrate eyes, neurogenesis proceeds in waves. In zebrafish, neuronal differentiation spreads across the developing retina from a ventral to dorsal pathway of RPCs cells adjacent to the optic stalk (Rappaport, 2006). This spread of neurogenesis is preceded by comparable waves of expression of neurogenesis-related genes including the proneural gene atoh7 and the paired-class cone-rod homeobox gene crx (Masai et al., 2000; Shen and Raymond, 2004). Differentiation of distinct neuronal types continues to propagate circumferentially in successive waves, ultimately filling the central retina by 48 hours post fertilisation (hpf). After this point, neurogenesis occurs radially, with new neurons added to the eye in successive rings from a peripheral area of the retina termed the ciliary marginal zone (CMZ). The CMZ contains a stem cell niche that allows continued growth and neurogenesis in the eye (Centanin et al., 2011; Stenkamp, 2007).

Retinal neurogenesis occurs in a context where extrinsic signals impart nasotemporal and dorsoventral (DV) positional identity to newly generated neurons. In fish, the opposing actions of Fgf and Shh initiate nasotemporal patterning (Hernandez-Bejarano et al., 2015) whereas opposing Bmp and Hedgehog (Hh) signals (Yang, 2004) establish DV identities. For instance, Gdf6a (BMP13 homologue) and other BMPs are expressed in dorsal domains and influence dorsal identity within the retina (French et al., 2009; Gosse and Baier, 2009; Kruse-Bend et al., 2012; Williams et al., 2008). Mutations in gdf6 genes have also been linked to other ocular anomalies, including microphthalmia and coloboma (Asai-Coakwell et al., 2013; den Hollander et al., 2010; French et al., 2009). It is likely that the connection between axial patterning signals and positional identity is maintained within the CMZ as newly generated RGCs must integrate into existing circuitry and project to topographically appropriate target regions within the brain.

The RA signalling pathway also influences retinal development. RA-synthesising enzymes are expressed predominantly in the ventral retina and RA signalling is required for morphogenesis of the ventral eye, including choroid fissure closure (Lupo et al., 2011; Marsh-Armstrong et al., 1994). Expression of the RA synthesis enzyme aldh1a3 is expanded dorsally in eyes of gdf6a morphant
embryos (French et al., 2009) raising the possibility that interactions between RA and Gdf6a signals occur during retinal development.

In this study, we sought to identify signals that regulate eye growth and found that, in addition to their roles in DV patterning, opposing Gdf6a and RA signals influence proliferation and differentiation of RPCs. Through a forward genetic screen in zebrafish, we identified two point mutations in gdf6a that lead to reduction in size of the CMZ and subsequent small eye phenotypes. These phenotypes are accompanied by an expansion of RA pathway activity within the CMZ and precocious differentiation of RPCs. We find that activating the RA pathway in wild-type eyes phenocopies gdf6a mutants and that abrogation of RA signalling ameliorates the gdf6a mutant phenotype. Together, our results show that signals that impart DV identity in the eye also regulate production of neurons and retinal growth, revealing an unappreciated link between the pathways that regulate patterning, proliferation, and stem/progenitor cell maintenance in the developing eye.

**RESULTS**

**gdf6a mutants exhibit small eyes independent of apoptosis**

Clutches of 72 hpf embryos from families of zebrafish carrying N-ethyl-N-nitrosurea (ENU)-induced mutations (Valdivia et al., 2011) were examined for ocular abnormalities, including microphthalmia. We recovered two non-complementing, recessive mutations, u768 and u900, that when homozygous led to small eyes with ventrally displaced lenses and, in some cases, coloboma (Fig. 1A). Although two homozygous mutant phenotypes are fully penetrant, u900 was examined for ocular abnormalities, including microphthalmia. We recovered two non-complementing, recessive mutations, u768 and u900, that when homozygous led to small eyes with ventrally displaced lenses and, in some cases, coloboma (Fig. 1A). Although both homozygous mutant phenotypes are fully penetrant, u900 mutants consistently display smaller eyes than u768 mutants (Fig. S1D).

Both mutants carry lesions in gdf6a, a gene that is expressed dorsally both in the developing retina (French et al., 2009; Gosse and Baier, 2009) and the CMZ (Fig. 1B). Using bulk segregant analysis, complementation testing, and sequencing, we identified u768 as a missense mutation that results in the substitution of a threonine for an absolutely conserved alanine at position 350 in the Gdf6a protein (Fig. 1C). It is likely that this mutation generates a hypomorphic Gdf6a protein. Sequencing identified u900 as a nonsense mutation that converts the codon for Gln179 to a Stop (gdf6a<sup>u900</sup>; Fig. 1C). This mutation is predicted to generate a truncated protein lacking the conserved carboxy-terminal signalling domain.

Previous studies have shown that loss of Gdf6a function is associated with a transient wave of retinal apoptosis, suggesting that Gdf6a is required for RPC survival (den Hollander et al., 2010; Gosse and Baier, 2009). However, in gdf6a<sup>u768</sup> mutants, the number of apoptotic retinal cells between 24 and 36 hpf was similar to wild type [gdf6a<sup>u768</sup>=5.25±2.75 (mean±s.d.); wild-type siblings=3.4±1.76; P=0.12; n=13 embryos of each genotype; Fig. 1D; Fig. S1A], and retinal neurons appeared healthy, suggesting that cell death is not the main driver of the small eye phenotype in this mutant. Although gdf6a<sup>u900</sup> mutants displayed increased levels of retinal apoptosis at around 30 hpf (Fig. S1B), blocking cell death with the pan-caspase inhibitor Z-VAD-FMK reduced apoptotic levels but did not rescue the subsequent small eye size, corroborating previously published data (French et al., 2013) (Fig. S1C).

Functional studies have also implicated Gdf6a in patterning the DV axis of the retina (French et al., 2009; Gosse and Baier, 2009; Rissi et al., 1995). However, the u768 allele exhibits overtly normal expression of DV markers at 24 hpf (Fig. 1E) suggesting that the small eye phenotype arises in this mutant allele in the absence of major defects in the establishment of axial patterning of the retina. Together, these data suggest that a mechanism other than programmed cell death and/or abnormal retinal patterning is responsible for the small eye phenotype in gdf6a mutants.

**The CMZ is reduced in gdf6a mutants**

As the CMZ is the primary source of new neurons and retinal growth in fish larvae (Centanin et al., 2011; Raymond et al., 2006), we examined expression of markers for subdomains of the CMZ in

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**Fig. 1. Small-eye phenotype in gdf6a mutants is independent of programmed cell death in developing retina.** (A) Lateral views of wild-type and gdf6a mutants showing small eyes. (B) Lateral view of dorsal gdf6a expression (purple) in wild-type retina. (C) Domain structure of Gdf6a protein indicating the N-terminal signal peptide (amino acids 1-25, light blue), the furin protease recognition site (amino acids 280-285, orange), and the premature stop codon of gdf6a<sup>u900</sup> and missense mutation (Ala350Thr) in gdf6a<sup>u768</sup> (asterisks). (D) Lateral views of eyes of siblings (sib) and gdf6a mutants. Similar levels of TUNEL<sup>+</sup> apoptotic cells (purple) are evident in the gdf6a<sup>u768</sup> and sibling eyes at 30 hpf; most apoptotic cells are in the lenses (arrows) and only few in the retinae (arrowheads). TUNEL<sup>+</sup> cells are undetectable in eyes of either genotype by 2.5 dpf (lower panels). (E) Lateral views of whole-mount eyes of 24 hpf gdf6a<sup>u768</sup> and sibling embryos showing expression (purple) of markers for dorsal (tbx5a) and ventral (vax2) retinal character.
gdf6a mutants and found striking differences compared with wild-type eyes as early as 2.5 days post fertilisation (dpf). The most peripheral col15a1b-expressing region, which contains presumptive stem cells (Cerveny et al., 2010; Gonzalez-Nunez et al., 2010), is reduced in gdf6au768 mutants with fewer col15a1b-positive cells in the dorsal CMZ and virtually no col15a1b expression in the ventral CMZ (Fig. 2A,B). Similarly, all other subdomains of this germinal zone—proliferating progenitors (expressing ccnd1), specified precursors (expressing the transcription factor atoh7), and differentiating neurons (expressing the cyclin-dependent kinase inhibitor cdkn1c) (Cerveny et al., 2010)—are reduced (Fig. 2C-H).

To characterise the mutant CMZs further, we examined expression of Tg[vsx2:GFP]. vsx2 encodes a homeodomain transcription factor expressed throughout the CMZ, in Müller glia and in a subset of bipolar cells (Vitorino et al., 2009). Consistent with fewer cells within the CMZ, we observed a smaller vsx2:GFP-expressing region with only a few cells separating the retinal margin and atoh7:GAP-RFP-expressing RGCs in 60 hpf gdf6au900 mutant retinae (Fig. 2I,J).

Consistent with decreased growth from a smaller CMZ, gdf6a mutant eyes contained fewer proliferative cells in both S phase [marked by bromodeoxyuridine (BrdU) staining] and M phase [marked by phospho-histone H3 (PH3) staining] of the cell cycle (Fig. 2K-N) by 2.5 and 3 dpf (Fig. 2O; n=10 embryos for each genotype). These findings indicate that reduced Gdf6a function is correlated with decreased cell proliferation in the CMZ.

To gauge expression levels of CMZ markers quantitatively, we used real-time PCR (qPCR) in dissected mutant and wild-type sibling larval retinae. Both mutant alleles show nearly identical reduction in transcript levels of genes expressed in proliferating progenitors (ccnd1) and committed precursors (e.g. atoh7) (Fig. 2P). Although in situ hybridisation clearly shows fewer col15a1b-positive cells, qPCR detected only a modest reduction in transcript levels. Not all genes analysed, however, showed decreased expression. A control gene, nr2f5, encoding a transcriptional target of RA signalling (Love and Prince, 2012) was elevated nearly 2.5-fold in gdf6a mutant eyes (Fig. 2P), suggesting increased RA pathway activity.
**gd6a mutants display RA pathway activity throughout the peripheral CMZ**

RA synthesis and target genes are ectopically activated throughout the circumference of the peripheral-most compartment of the CMZ of gd6a mutants. Expression of two genes involved in RA synthesis, retinol dehydrogenase (rdh10a) and a retinaldehyde dehydrogenase (aldh1a3), is increased and expanded dorsally in the gd6au768 mutants (Fig. 3, top two rows). Consequently, and as expected from qPCR experiments, n2f5 is transcribed in more cells of the presumptive CMZ (Fig. 3, middle row). Consistently, gd6au768 mutants carrying an RA-responsive YFP transgene (Perz-Edwards et al., 2001) exhibit expanded YFP expression in the peripheral compartment of the CMZ (Fig. S3). Genes likely to limit RA activity, such as the cytosolic RA binding protein crabp2a (Cai et al., 2012) and the RA-degrading enzyme cyp26a1 (Isken et al., 2008), are expressed in fewer cells in gd6a mutant eyes (Fig. 3, bottom two rows). Together, these data indicate that RA signalling is enhanced in the CMZ of gd6a mutants.

**Enhanced RA pathway activity inhibits eye growth**

We next asked whether excessive RA affects eye size. We first tested whether all wild-type retinal progenitors respond to RA. Embryos carrying the Tg[RARE:YFP] RA reporter transgene (Perz-Edwards et al., 2001) were incubated with 13-cis RA, which can be locally converted to all-trans RA by UV-driven photo-isomerisation to activate the RA pathway (Xu et al., 2012). In 24-36 hpf wild-type embryos, only ventral retinal cells activate the RA pathway reporter transgene in the absence of exogenous all-trans RA (Fig. 4A). UV-mediated activation of RA signalling, however, demonstrated that most, if not all, RPCs are capable of responding to RA. Mediated activation of RA signalling, however, demonstrated that most, if not all, RPCs are capable of responding to RA. RA-mediated activation of RA signalling, however, demonstrated that most, if not all, RPCs are capable of responding to RA. RA synthesis and target genes are ectopically activated throughout the CMZ of gd6a mutants. Expression of two genes involved in RA synthesis, retinol dehydrogenase (rdh10a) and a retinaldehyde dehydrogenase (aldh1a3), is increased and expanded dorsally in the gd6au768 mutants (Fig. 3, top two rows). Consequently, and as expected from qPCR experiments, n2f5 is transcribed in more cells of the presumptive CMZ (Fig. 3, middle row). Consistently, gd6au768 mutants carrying an RA-responsive YFP transgene (Perz-Edwards et al., 2001) exhibit expanded YFP expression in the peripheral compartment of the CMZ (Fig. S3). Genes likely to limit RA activity, such as the cytosolic RA binding protein crabp2a (Cai et al., 2012) and the RA-degrading enzyme cyp26a1 (Isken et al., 2008), are expressed in fewer cells in gd6a mutant eyes (Fig. 3, bottom two rows). Together, these data indicate that RA signalling is enhanced in the CMZ of gd6a mutants.

**Inhibition of the RA pathway restores the CMZ in gd6a mutants**

To evaluate the extent to which misregulation of RA pathway activity contributes to the imbalance in proliferation and differentiation, and subsequent growth of gd6a mutant eyes, we asked whether reducing RA pathway activity could restore the CMZ in gd6a mutant retinae. When clutches of embryos from a gd6au768 carrier in-cross were incubated with the panRAR inverse agonist BMS493 (Germain et al., 2009), mutant eyes contained more cells expressing col15a1b, ccnd1 and atoh7 in the peripheral retina (Fig. 5A-F). We also quantified transcript levels for CMZ markers in BMS493-treated eyes relative to DMSO-treated eyes and confirmed in situ hybridisation data that ccnd1 and atoh7 levels were increased (Fig. 5G). Because changes in col15a1b detected by qPCR were modest, we measured the percentage area of col15a1b-positive cells in control and BMS-treated eyes and found that treated eyes contained a nearly 40% larger col15a1b-positive area than did controls (Fig. S5C). Sibling eyes with the same treatment exhibited a noticeable expansion of col15a1b in the ventral retina (Fig. S5B). Together, these data suggest that RA limits expression of CMZ

![Fig. 3. Expression of genes encoding RA pathway components is expanded dorsally in the CMZ of gd6a mutants.](image-url)
markers in gdf6a mutants and contributes to CMZ maintenance during normal development.

**Retinal precursors precociously generate neurons in gdf6a mutants**

The reduced CMZs in gdf6a mutants could result from changes in cell cycle kinetics within the CMZ and/or altered timing of cell cycle exit and transition of post-mitotic cells into the mature retina. To distinguish between these possibilities, all S-phase cells were first labelled with BrdU for 4 h prior to fixation. Next, BrdU incorporation in cells that had progressed to M phase (PH3+) was evaluated. Wild-type progenitors can progress from S-phase to M-phase in as little as 2.5 h (Cerveny et al., 2010), and so this labelling regime results in 100% of PH3+ cells being labelled by BrdU. In both wild-type sibling and gdf6a768 mutant eyes, we found that all PH3+ cells were also BrdU+ (Fig. 6A,B; n=16 eyes), suggesting that cell cycle kinetics were not dramatically changed in mutant CMZ.

RPCs exit the cell cycle and differentiate in a stereotypical pattern (Cepko et al., 1996; Harris, 1997), with proliferative RPCs ultimately restricted to the CMZ by ∼2.5 dpf (Stenkamp, 2007). To gauge the balance between proliferation and differentiation after the onset of retinal neurogenesis in gdf6a mutants, we labelled all S-phase cells with BrdU for 10 h beginning at ∼50 hpf. With this labelling regime, only a small proportion of cells in wild-type eyes (primarily RGCs) are BrdU negative and are likely to have exited the cell cycle prior to BrdU treatment (Fig. 6C; red cells; Ohnuma et al., 1999). By contrast, all layers of gdf6a mutant retinae contained a much larger proportion of BrdU-negative cells (Fig. 6D, white arrows). We observed this striking pattern of precocious cell cycle exit and differentiation in all embryos (n=60 total genotyped embryos; 14 gdf6a768 mutants). These data indicate that the pace of neurogenesis is accelerated in gdf6a mutant retinae.

To characterise the precocious production of neurons in gdf6a mutants, we analysed the onset and progression of expression of atoh7 and crx, which both exhibit dynamic, fan-shaped ventral-nasal to dorsal-temporal patterns of expression (Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000; Shen and Raymond, 2004). By ∼28 hpf, atoh7 is expressed in a small patch of ventral RPCs in wild-type eyes (Fig. 6E) whereas a larger proportion of ventral and central RPCs express atoh7 in gdf6a mutant eyes (Fig. 6F). Likewise, expression of crx, which is initially transcribed in the ventral-nasal and ventral-central retina at ∼36 hpf in wild-type eyes (Fig. 6G), is more broadly expressed in gdf6a mutant eyes (Fig. 6H), similar to wild-type crx expression at 48 hpf (compare Fig. 6H and 6I). Quantitative PCR in gdf6a768 mutant retinae corroborated the upregulation of atoh7 and crx (1.5- and 2-fold greater, respectively), as well as of the RA-target gene nr2f5 (2.5-fold greater) (Fig. 6K).
Because RA pathway activity is enhanced in gdf6a mutant retinae, we examined whether RA could modulate cell cycle exit and neurogenesis of RPCs by pharmacologically activating or inhibiting RA signalling and then examining atoh7 and Tg[atoh7:GFP] expression. Similar to gdf6a mutant retinae, wild-type embryos incubated in AM580 exhibited significantly more atoh7+ cells (Fig. 7A-F). At 28 hpf, ~2% of the wild-type retinal volume was GFP+, whereas it was more than doubled in both gdf6a mutant and AM580-treated wild-type retinae (Fig. 7C,D,K). By 40 hpf, 3% of AM580-treated wild-type and mutants versus DMSO-treated wild type, gdf6au768 mutants, we imaged living gdf6au768 mutants treated with vehicle (DMSO;A,C,E) or the RAR inverse agonist BMS493 (15 µM; B,D,F) showing expression of col15a1b, ccnd1, and atoh7. Because RA pathway activity is enhanced in gdf6a mutants, we imaged living gdf6au768 mutants treated with vehicle (DMSO;A,C,E) or the RAR inverse agonist BMS493 (15 µM; B,D,F) showing expression of col15a1b, ccnd1, and atoh7. 

**Precocious RA-mediated differentiation depletes retinal progenitors**

Because RA pathway activity is enhanced in gdf6a mutant retinae, we examined whether RA could modulate cell cycle exit and neurogenesis of RPCs by pharmacologically activating or inhibiting RA signalling and then examining atoh7 and Tg[atoh7:GFP] expression. Similar to gdf6a mutant retinae, wild-type embryos incubated in AM580 exhibited significantly more atoh7+ cells (Fig. 7A-F). At 28 hpf, ~2% of the wild-type retinal volume was GFP+, whereas it was more than doubled in both gdf6a mutant and AM580-treated wild-type retinae (Fig. 7C,D,K). By 40 hpf, 3% of wild-type retinal volume was GFP+, but this percentage was nearly tripled in mutant or AM580-treated wild-type eyes (Fig. 7E,F,K). Pair-wise t-tests (n=5 for each condition and genotype) indicate that AM580-treated wild-type and gdf6au768 DMSO-treated eyes are not statistically different from one another at 28 hpf (P=0.188) or at 40 hpf (P=0.77) and that both are different from DMSO-treated wild-type atoh7+ retinal volumes at both developmental stages (28 hpf: P=0.017 for DMSO-treated u768 mutants versus DMSO-treated wild type, P=0.012 for AM580-treated wild type versus DMSO-treated wild type; 40 hpf: P=0.000003 for DMSO-treated u768 mutants versus DMSO-treated wild type, P=0.0001 for AM580-treated wild type versus DMSO-treated wild type).

Reducing RA signalling in gdf6a mutants with the RAR antagonist BMS614 decreased the number of GFP+ cells (Fig. 7G-J) such that DMSO-treated siblings and BMS614-treated gdf6au768 embryos contained nearly equivalent GFP+ retinal volumes (Fig. 7G-J,L). Pair-wise t-tests (n≥7 for each condition and genotype) indicate that BMS614-treated gdf6au768 and DMSO-treated sibling eyes contain GFP+ retinal volumes that are not statistically different from each other at 28 hpf (P=0.566) and 40 hpf (P=0.229). At 28 hpf, ~1.5% of the gdf6au768 retinal volume was GFP+, similar to DMSO-treated siblings and less than that of gdf6au768 untreated eyes (Fig. 7G-H,L; P=0.07 for BMS614-treated u768 mutants versus DMSO-treated u768 mutants). By 40 hpf, both BMS614-treated gdf6au768 mutant eyes and DMSO-treated sibling eyes contained similar GFP+ retinal volumes whereas DMSO-treated gdf6au768 mutant eyes contained substantially larger GFP+ retinal volumes (Fig. 7I,J,L; P=0.001 for BMS614-treated u768 mutants versus DMSO-treated u768 mutants).

Further suggesting that precocious neurogenesis underlies the gdf6a mutant phenotype, we found that mutant eyes contain a higher proportion of GFP+ neurons in the RGC layer by 80 hpf (Fig. 7M-O; Fig. S6) and more GFP+ cells in the inner nuclear layer, suggesting that more atoh7+ positive cells are generated earlier (Fig. 7N, white arrows). Together, these observations suggest that RA can promote cell cycle exit and the production of atoh7+ positive neurons in developing retinae.

**DISCUSSION**

This study reveals that opposing activities of Gdf6a from the dorsal retina and RA from the ventral retina influence RPCs as they transition from proliferation to differentiation, ultimately regulating eye size. Our data demonstrate that in addition to previously demonstrated roles in DV patterning, the combined action of these pathways regulates when RPCs exit the cell cycle. Our genetic and pharmacological manipulations illustrate that Gdf6a-mediated inhibition of RA pathway activity modulates the timing of retinal neurogenesis and suggest that RA-mediated precocious differentiation of the RPC pool might underlie the microphthalmic phenotype in vertebrates carrying GDF6 mutations.

**Gdf6a regulates retinal growth**

Our study adds to others linking eye defects to abrogation of Gdf6 function in fish, mice and humans (Asai-Coakwell et al., 2007, 2013; den Hollander et al., 2010; Gosse and Baier, 2009; Hanel and Hensen, 2006), but the mechanisms connecting loss of gdf6a to microphthalmia have remained unclear. It has been suggested that apoptosis contributes to the small eye phenotype in gdf6a mutants (den Hollander et al., 2010; Gosse and Baier, 2009). Our study and another (French et al., 2013), provide evidence that microphthalmia occurs independently of cell death in gdf6a mutants. Pronounced differences in eye size are observed in...
gdf6au768 mutants, which exhibit levels of retinal apoptosis comparable to wild type, and in gdf6au900 embryos when apoptosis is blocked. Despite these differences in apoptosis, both gdf6a alleles exhibit similar changes in gene expression, proliferation and differentiation. Moreover, analyses of gdf6au768 mutants suggest that abnormal initiation of retinal DV patterning is not a prerequisite for gdf6a-linked microphthalmia.

Gdf proteins regulate cell cycle exit and differentiation through various mechanisms

Members of the BMP family regulate cell fate, proliferation, differentiation, and apoptosis during embryonic development. As a result, BMPs and their antagonists have been implicated in control of tissue and organ size (Beites et al., 2009; Sartori et al., 2013). The presence of fewer proliferating cells and the accelerated expression of differentiation genes in the early retinas of gdf6a mutants suggest that the BMP-related protein Gdf6a is part of a genetic circuit that balances progenitor cell proliferation and differentiation to control eye size in zebrafish. In mammals, GDF11 and GDF8 (MSTN) balance proliferation and differentiation in different contexts. Both act as auto-inhibitory feedback signals to directly and reversibly regulate cell cycle exit of neuronal progenitors in the olfactory epithelium (Wu et al., 2003) and myoblasts (McPherron and Lee, 1997), respectively. GDF11 also modulates retinal size in mice but
through a different mechanism. During early neurogenesis, expression of GDF11 and its inhibitor, follistatin, influence the timing of expression of Atoh7 (also known as Math5) without affecting expression of cell cycle regulators (Kim et al., 2005). Despite diverse mechanisms by which these GDF proteins act, they promote similar consequences for timing of cell cycle exit and differentiation.

**Gdf6a-antagonised RA pathway activity regulates cell cycle exit and differentiation**

Our study reveals that a key role for Gdf6a is to limit RA activity in RPCs. We show that enhancing RA activity accelerates the production of neurons from RPCs and this presumably depletes the pool of proliferative precursors. Such a role for RA is consistent with studies showing that RA promotes neuronal differentiation in vitro (reviewed by Janesick et al., 2015). In the vertebrate retina, RA can skew the fate of post-mitotic photoreceptor precursors towards rod and red-sensitive cone fates (Hyatt et al., 1996; Kelley et al., 1999; Mitchell et al., 2015; Stevens et al., 2011) and in vitro studies of oligodendrocyte precursors suggest that the timing of differentiation is modulated by retinoid signalling (Barres et al., 1994). Similarly, our data indicate that RA influences the timing of cell cycle exit and neuronal differentiation in the developing central retina and CMZ. Consequently, unlike other Gdfs that regulate timing of neurogenesis by promoting cell cycle exit (Gokoffski et al., 2011; Kim et al., 2005; Wu et al., 2003), our data suggest that Gdf6a is permissive for proliferation and, in balance with RA signalling, governs the timing of differentiation in the retina to preserve the pool of progenitors needed for multiple rounds of neurogenesis.

Although our data suggest that antagonism between Gdf6a and RA regulates RPC behaviours, Gdf6a could also interact with other signalling pathways to influence proliferation and differentiation. For instance, Fgf signalling maintains a subpopulation of RPCs in a proliferative state (Wong et al., 2015), and Hedgehog signalling is implicated in RPC proliferation and differentiation decisions (Borday et al., 2012; Locker et al., 2006; Masai et al., 2000). Cross-talk and integration between these pathways and BMP have been demonstrated in many diverse progenitor cell populations, including those in the developing neural tube (e.g. Horner and Caspary, 2011; Sasai et al., 2014).

**Intersection between morphogenetic and neurogenic effects of Gdf6a**

In addition to the requirement for Gdf6a to establish and maintain dorsal retinal character, we suggest an additional role for Gdf6a during eye development. Our data suggest that Gdf6a modulates RA pathway activity, which in turn influences the probability of RPC
cell cycle exit by regulating proliferative versus neurogenic divisions. Bolstering this idea, we observed enhanced and accelerated expression of the HLH transcription-factor-encoding gene atoh7, which is required for RGC production (Kay et al., 2001). Furthermore, we show that normal timing of atoh7 expression is restored when RA pathway activity is downregulated in gdf6a mutants. Two observations from studies in medaka fish also support a connection between RA, atoh7 expression, and eye size. First, a global analysis of the regulatory inputs driving retinal neurogenesis identified a potential positive role for RA upstream of atoh7 expression (Souren et al., 2009), and, second, a study that expanded atoh7 expression throughout the developing retina generated smaller eyes (Sinn et al., 2014).

How might an imbalance between RA and Gdf6a contribute to the small eyes in gdf6a mutants? We observe a smaller CMZ in mutant eyes as well as in eyes in which the RA pathway is activated early in development. Consequently, the failure of gdf6a eyes to grow robustly could be due to problems in both establishment of the CMZ and generation of neurons from the CMZ. Although studies have begun to examine the origins of the CMZ (Heermann et al., 2015; Kwan et al., 2012), how this stem cell niche is established and have begun to examine the origins of the CMZ (Heermann et al., 2015; Kwan et al., 2012), how this stem cell niche is established and maintained is not clear. One possibility is that in gdf6a mutants, precocious differentiation of RPCs depletes the pool of progenitors that ultimately populate the CMZ, thus generating a smaller CMZ.

Coupled with the possibility of fewer stem and progenitor cells in the CMZ of gdf6a mutants, defective Gdf6a/retinoid signalling within the CMZ is likely to further limit eye growth by exhausting the stem cell/progenitor population. Many signalling pathways required during early eye development, including BMP and RA pathways, remain active within the CMZ (Harris and Perron, 1998; Sharma et al., 2005; Shawi and Serluca, 2008). Previous work from our laboratories supports a model for differentiation dynamics within the zebrafish CMZ with at least two sets of environmental signals coexisting. One set of signals promotes proliferation of stem and progenitor cells and the other limits proliferation of rapidly cycling precursors by encouraging differentiation (Cerveny et al., 2010). Although a molecular basis for this so-called ‘environmentally driven differentiation’ has yet to be established, our current work suggests that Gdf6a and RA are two secreted signals that establish an appropriate balance between proliferation and differentiation in the CMZ. Together, our results support a model in which dorsally secreted Gdf6a balances RA pathway activity, controlling the transition from proliferation to differentiation during eye growth.

MATERIALS AND METHODS

Zebrafish lines

Eggs were collected by natural spawning, raised at 28.5°C in either fish water or E3 embryo medium (Nüsslein-Volhard and Dahm, 2002) and staged according to Kimmel et al. (1995). Transgenic and mutant lines are listed in supplementary Materials and Methods. To prevent pigment formation, 0.003% phenylthiourea (PTU, Sigma) was added to the fish water between 20 and 24 hpf.

ENU mutagenesis and eye screening

Mutagenesis was performed in wild-type male AB/TL fish by four rounds of 3 mM ENU treatment as previously described (Valdivia et al., 2011; van Eeden et al., 1999). Eyes of F3 larvae were screened for morphological abnormalities.

Genetic mapping, cloning and genotyping

The gdf6a<sup><i>r768</i></sup> mutation was mapped by bulk segregant analysis with simple sequence length polymorphisms to LG16 (Telbott and Schier, 1999). Markers Z13555 and Z45043 flanked a ∼2 Mb interval containing gdf6a.

gdf6a<sup><i>r768</i></sup> carriers were crossed to a reported gdf6a mutant line (rda<sup>3327</sup>, Gosse and Baier, 2009) for complementation testing. The gdf6a<sup>9900</sup> mutation was in turn complementation tested with gdf6a<sup>r768</sup>. To identify the molecular nature of both mutations, we performed RT-PCR (SuperScript-II Reverse Transcriptase and random primers; Invitrogen) on RNA isolated from 3 dpf wild-type and gdf6a<sup>r768</sup> larvae (Trizol, Invitrogen), and PCR on genomic DNA obtained using the HotSHOT method (Meeker et al., 2007) for gdf6a<sup>9900</sup>. Amplons were cloned into TOPO-TA vectors (Invitrogen) for sequencing. Oligonucleotides for cloning, sequencing and genotyping are listed in supplementary Materials and Methods.

Microinjections

Capped histone H2B-red fluorescent protein fusion (H2B-RFP) mRNA was prepared using the mMessage mMachine RNA Synthesis Kit (Ambion) according to the manufacturer’s instructions. One-cell-stage embryos resulting from gdf6a<sup>9900</sup> heterozygous in-crosses carrying the Tg<sup>[atoh7: GFP]</sup> transgene were injected with 150 pg of mRNA.

Histology

To prepare in situ hybridisation probes, DNA templates were generated by restriction digestion of plasmids carrying atoh7, cond1, cdkn1c, nr2f5, aldha1a2, aldha1a3 and cyp26a1 or by PCR from cDNA for coll15a1b, crabp2a and rdlh16a. For each template, the reverse strand oligonucleotide encodes a T3 polymerase priming site as well as the gene-specific sequence (Thistle and Thisset, 2008). All primers are provided in supplementary Materials and Methods. Digoxigenin-labelled RNA probes were transcribed using a DIG labelling kit (Roche) with the appropriate polymerases (Promega or NEB). Embryos were processed as previously described (Thistle and Thisset, 2008) and hybridisation signals were detected with anti-digoxigenin-AP antibody (1:3000; 11093274910, Roche) and NBT/BCIP substrate (1:3.5; Roche).

Apoptotic cell death was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). Embryos were fixed in 4% paraformaldehyde overnight and kept in methanol 100% until used. Following rehydration, embryos were treated following manufacturer instructions using the ApopTag kit (Millipore). An anti-digoxigenin-AP antibody (1:3000; Roche) was used for NBT/BCIP (Roche) detection.

For sectioning, embryos were fixed with 4% paraformaldehyde overnight at 4°C and then cryoprotected by sequential incubation in 15% and 30% sucrose dissolved in PBS supplemented with 0.5% Triton X-100 (PBST) for 12-16 h at 4°C. They were embedded in OCT, frozen on dry ice, and sectioned at 16-25 μm using a Leica cryostat. All immunostaining steps were performed at room temperature (~22°C).

BrdU incorporation was performed at room temperature as previously described (Cerveny et al., 2010). Briefly, 1 nl pulses of 10 mg/ml BrdU in E3 were injected into the hearts of embryos anaesthetised with MS-222 and immobilised in 1% low melting point agarose dissolved in E3. After injection at defined time points, embryos were liberated from agarose and incubated in E3 until fixation with 4% paraformaldehyde.

Primary antibodies used were: chicken or mouse anti-GFP (ab13970 and ab1218, Abcam; 1:1000); rabbit anti-RFP (PM005, Medical & Biological Laboratories Co.; 1:2500); mouse anti-BrdU (3262F, Millipore; 1:200); rabbit anti-PH3 (06-570, Millipore; 1:400).

Microscopy and image analysis

After in situ hybridisation or before immunostaining, embryo tails were genotyped and heads and/or dissected eyes were either imaged using a Nikon E1000 microscope equipped with DIC 20×0.5 NA and 40×1.15 NA objective lenses or subjected to immunohistochemistry. After immunohistochemistry or for time-lapse imaging of transgenic lines, sections or agarose-embedded embryos were imaged with Leica SPE (25±0.95 NA and 40±0.8 NA water immersion objectives) or Nikon A1+ (25±1.1 NA water immersion objective) confocal microscopes.

Digital images were processed with ImageJ and/or Imaris (Bitplane) software and compiled using Photoshop CS6 (Adobe). For some images,
white balance was digitally adjusted using the exposure option in Photoshop CS6. For quantifying TUNEL1+, PI3- and PH3+/BrdU+ cells, images were blind-counted using ImageJ. To quantify the volume of retinas containing GFP+ cells, the fluorescence of each z-plane of confocal stacks (~60 μm) was thresholded, images compiled and a contour surface drawn for both the entire eye and the fluorescent portion using the surface contour feature and either manual selection (for the entire eye) or auto selection (for GFP fluorescence) of Imaris. For eye and section size measurements, images taken from a lateral view at fixed magnification were opened in ImageJ. The freehand selection tool was used to select to outline the circumference of each eye and then the measure function was used to calculate the area. Data were exported to R or Prism (GraphPad) for statistical analysis and graphing. Either permutation testing with 10,000 input from other co-authors.

Author contributions

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Supplementary information
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References


