Supplementary Information

Supplemental Materials and Methods

Zebrafish lines used:

gdf6a^{u768} (this study)

gdf6a^{u900} (this study)

Tg[atoh7:GFP]^{rw021} (Masai et al., 2003)

Tg[vsx2:GFP]^{nns1} (Vitorino et al., 2009)

Tg[atoh7:GAP-RFP]^{cu2} (Poggi et al., 2005)

Tg[RARE:YFP]^{id1} (Perz-Edwards et al., 2001)

radar (rdr^{s327}*)* (Gosse and Baier, 2009)

Cloning, Sequencing, and Genotyping Oligonucleotides

For *gdf6a*^{u768} cloning, TOPO-TA plasmids containing inserts were sequenced with SP6 and T7 primers.

Oligo Name	*Oligo Sequence*	*gdf6a region*
gdf6aF cDNA	5'-ACTTCACTTCAGACACGCAACACGCC-3'	*Entire cDNA*
gdf6aRcDNA	5'- CTACCTGCAGCCACACTGTTCTACC-3'	
gdf6a1F	5'-ACTTCACTTCAGACACGCAACACG-3'	*5'UTR and 1*st *exon*
gdf6a 1R	5'-CTTGCTTCACAAGTCTTTAAAGTTTGAG-3'	
gdf6a 2F	5'-CTCTTGTTCCATCTTGTGTGTATATTATC-3'	*5' portion of 2*nd *exon*
gdf6a 2iR	5'-GAAACCAAGTTGCTTTAGGTCGATC-3'	
gdf6a 2iF	5'-GATCGACCTAAAGCAACTTGGTTTC-3'	*3' portion of 2*nd *exon*
gdf6a 2R	5'-GACGTTGTTCCCAGAGTCTATGTAC-3'	

For *gdf6a*^{900} cloning, TOPO-TA plasmids containing inserts were sequenced with M13 forward and M13 reverse primers.

Oligo Name	*Oligo Sequence*	*gdf6a region*
gdf6a 1F	5'-TGAGACACGGCTCCACTTTA-3'	1st exon
gdf6a 1R	5'-CCTTTTCCCTTGTCCACGA-3'	
gdf6a 2F	5'-GTGAATCTGACTGCAATTATTTCTCT-3'	2nd exon
gdf6a 2R	5'-CGCATCGTTCAAGTGTTAGC-3'	

Genotyping of *gdf6a*^{u768} was carried out initially by PCR and digestion as designed by dCaps Finder (Neff et al., 2002). A 226 bp region of *gdf6a* containing a FauI restriction site was amplified with oligonucleotides 5'-GTCGCACCTAGAGCCGACCAACCCC-3'
and 5'-CTTGGTGCCATACTCTGCTGTGTG-3'. Digestion with FauI generated two bands in wild-type siblings, three bands in heterozygous carriers, and a single band in fish homozygous for the *gdf6a*^{u768} allele. For *gdf6a*^{u900}, the mutant single nucleotide polymorphism was used to develop a Kbioscience Allele-specific Polymorphism (KASP, LGC genomics) assay; the same was subsequently done for *gdf6a*^{u768}. The assay identifier numbers are 1077647146 and 1077647136 for *gdf6a*^{u768} and *gdf6a*^{u900}, respectively.

For generating antisense RNA in situ probes:

col1511b: A DNA fragment containing the last 1000 bp of the *col15a1b* gene was amplified from cDNA with oligonucleotides: 5'-ATGAGGGAAATCCTGGGTTGTCAG-3' and
5'-GGATCCATTAACCCTCACTAAAGGGAATCACAGGGTTTCCATAGCCAG-3'.

crabp2a: The entire coding sequence of *crabp2a* plus 100 bp of 3'UTR was amplified from cDNA with oligonucleotides: 5'-CCCGATTTTGCTGGTACCTGGA-3' and
5'-CATTAACCCTCACTAAAGGGAACTCTTGTACACACC-3'.

rdh10: The first 1000 bp of *rdh10a* cDNA were amplified with oligonucleotides:
5'- CGGCACGGCATTAAACATGGTG-3' and
5'-CATTAACCCTCACTAAAGGGAATGGCACCCTCTAGATC-3'. Underlined sequences correspond to the T3 promoter sequence used for in vitro transcription of antisense RNA probes.

Permutation testing in R:

General R code for conducting permutation testing using mosaic and dplyr packages in R-markdown is pasted below. Note that "Size" for all eyes analyzed was measured in ImageJ, exported to excel and then imported into R for analysis. Note that "type" refers to genotype and/or drug treatment and was designated as either egh (*gdf6a* mutants), sib, AM580, DMSO (n=10;Figure 4). See Supplemental Figure 4 for related histogram and results text for associated p-value. Calculated t-value = 0.1566654.

```
eyesize_data <- read.csv("eyesize_data.csv")
means <- mean(Size ~ type, data = eyesize_data)
egh_mean <- means[1]
sib_mean <- means[2]
AM580_mean <- means[3]
DMSO_mean <- means[4]
T_stat <- (egh_mean/sib_mean) - (AM580_mean/DMSO_mean); T_stat
eyesize_data$Set <- ifelse(eyesize_data$genotype %in% c("egh","sib"), 1, 2)
```

```
reduced <- select(eyesize_data, type, Size, Set)
num_sims <- 10000
T_sim_stats <- numeric(length = num_sims)
for(i in 1:num_sims){
shuffled <- shuffle(reduced, groups = reduced$Set)
shuffled$genotype <- c(rep("egh", 10), rep("sib", 10), rep("AM580", 10),
rep("DMSO", 10))
sim_means <- mean(Size ~ genotype, data = shuffled)
egh_sim_mean <- sim_means[1]
sib_sim_mean <- sim_means[2]
AM580_sim_mean <- sim_means[3]
DMSO_sim_mean <- sim_means[4]
T_sim_stats[i] <- (egh_sim_mean/sib_sim_mean) - (AM580_sim_mean/DMSO_sim_mean)}
histogram(~T_sim_stats, groups = T_sim_stats >= T_stat | T_sim_stats <= -T_stat,
type = "count", n = 60)
p_value <- prop(~T_sim_stats >= T_stat) + prop(~T_sim_stats <= -T_stat); p_value
```

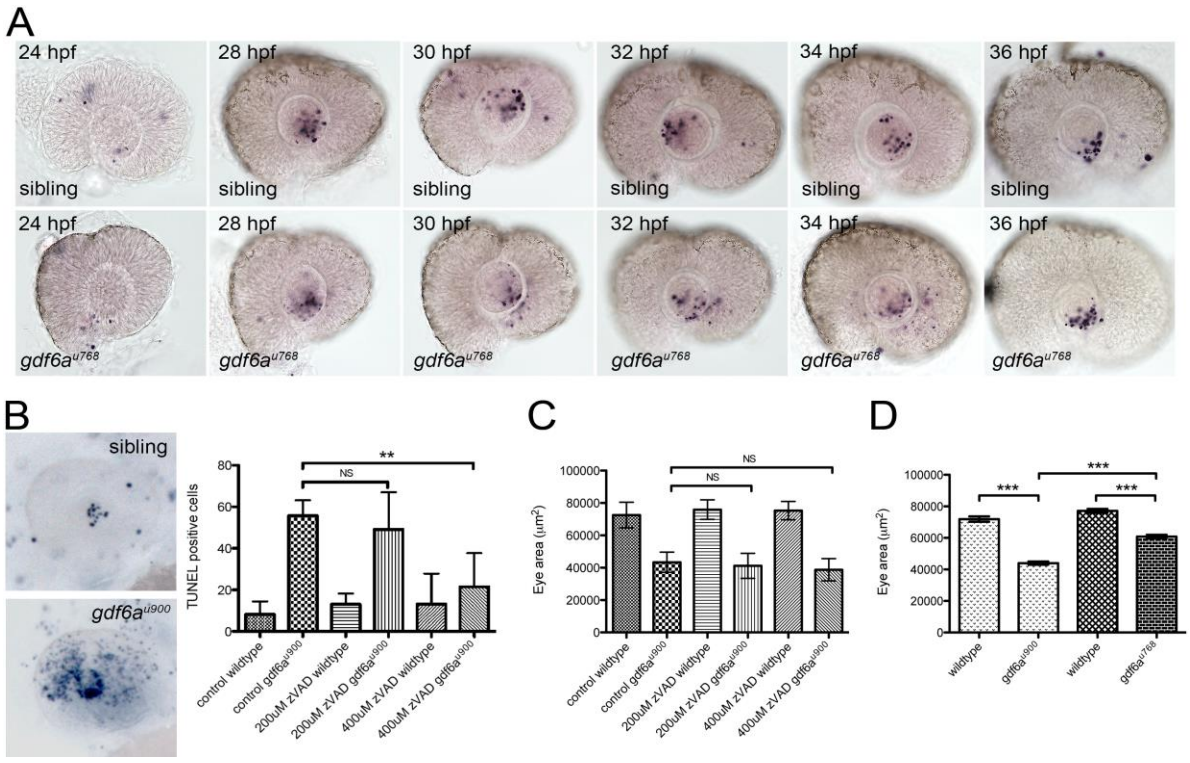


Fig. S1. *gdf6a* mutants exhibit microphthalmia independently of cell death. (A) Lateral views of wild-type (top row) and *gdf6a*^{u768} mutant (lower row) eyes showing apoptotic cells between 24 hpf - 36 hpf. No obvious difference in numbers is apparent between wild-type and mutants ($p \geq 0.07$ for all time points; $n \geq 10$ embryos per stage per genotype).

(B) Left: lateral views showing apoptotic cells (purple) wildtype (top) and *gdf6a*^{u900} mutant eyes. Right: Graph showing that the elevated number of TUNEL+ cells (blue) in *gdf6a*^{u900} mutant eyes at 32 hpf (bottom image) is reduced to near wild-type levels by incubating embryos with 400 µM Z-VAD-FMK caspase inhibitor from 14 hpf - 31 hpf (**Student's t-test, $p<0.05$; $n \geq 5$ embryos per genotype; NS, no significant difference).

(C) Graph showing that *gdf6a*^{u900} mutants exhibit significantly smaller eyes than their siblings at 3 dpf independently of Z-VAD-FMK treatment ($n \geq 7$ embryos; NS, no significant difference).

(D) Graph showing that both alleles of *gdf6a* are associated with smaller eyes, with *gdf6a*^{u900} mutant eyes smaller than those in *gdf6a*^{u768} mutants (***Student's t-test, $p<0.0001$; $n \geq 5$ embryos).

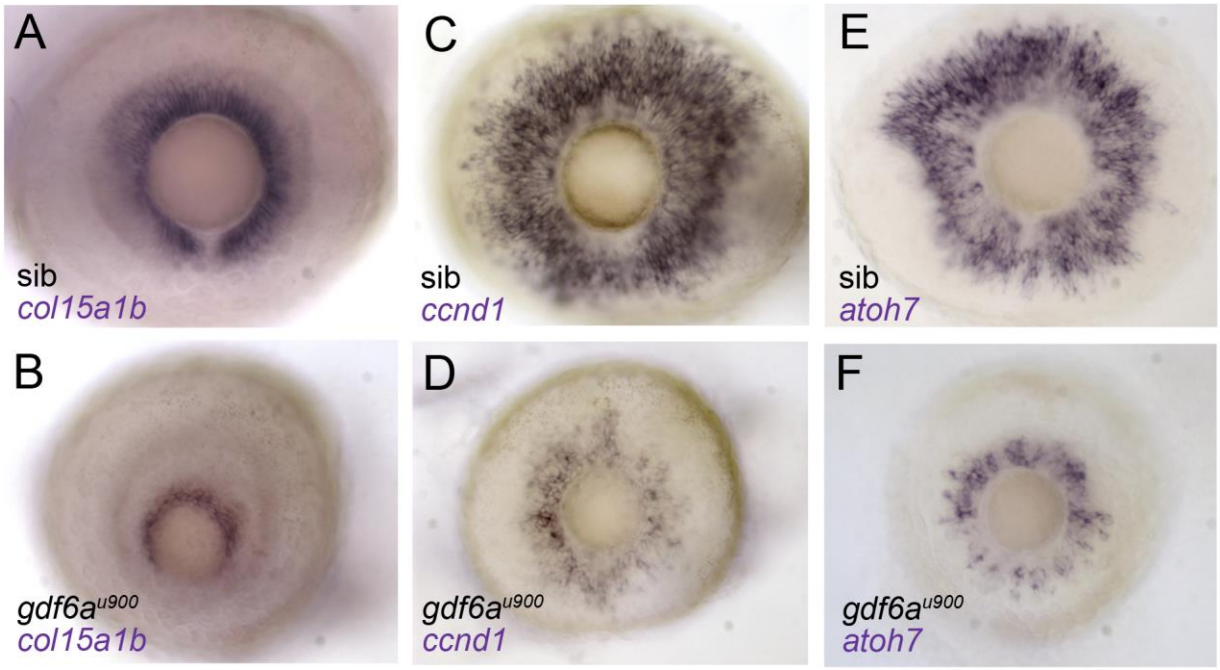


Fig. S2. *gdf6a*^{u900} mutant eyes show reduced expression of CMZ genes

Lateral views of 3dpf whole-mount *gdf6a*^{u900} mutant and sibling eyes stained for expression of *col15a1b* (A, B), *ccnd1* (C, D) and *atoh7* (E, F).

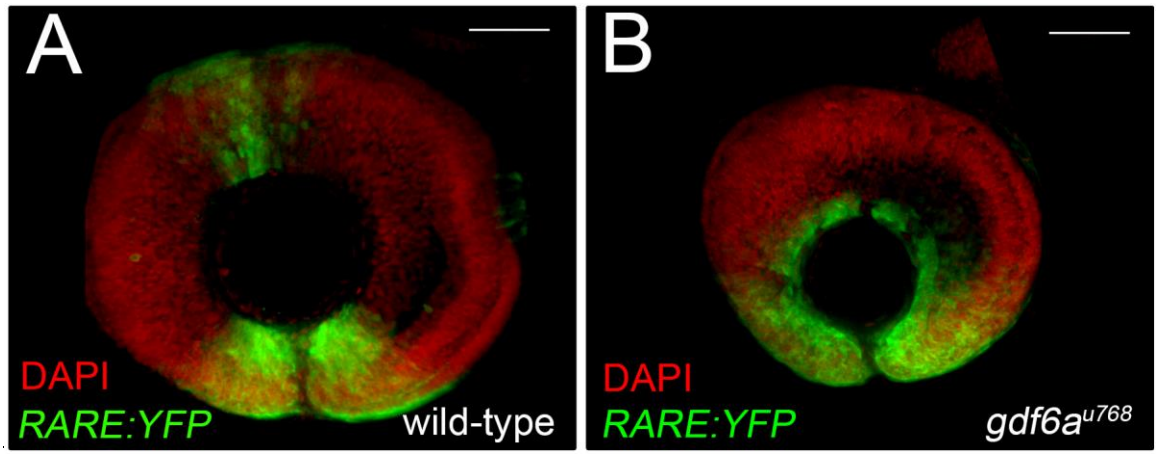


Fig. S3. *gdf6a* mutant eyes activate a RA responsive transgene in the CMZ.
Lateral views of eyes of 60hpf wildtype and *gdf6a*^{u768} mutant retinas carrying the *RARE:YFP* transgene. (A) The wildtype eye displays strong ventral transgene expression and only a discrete dorsal domain. (B) The mutant eye exhibits strong GFP expression in the peripheral-most compartment of the CMZ, with fewer cells expressing the reporter in the dorsal retina.

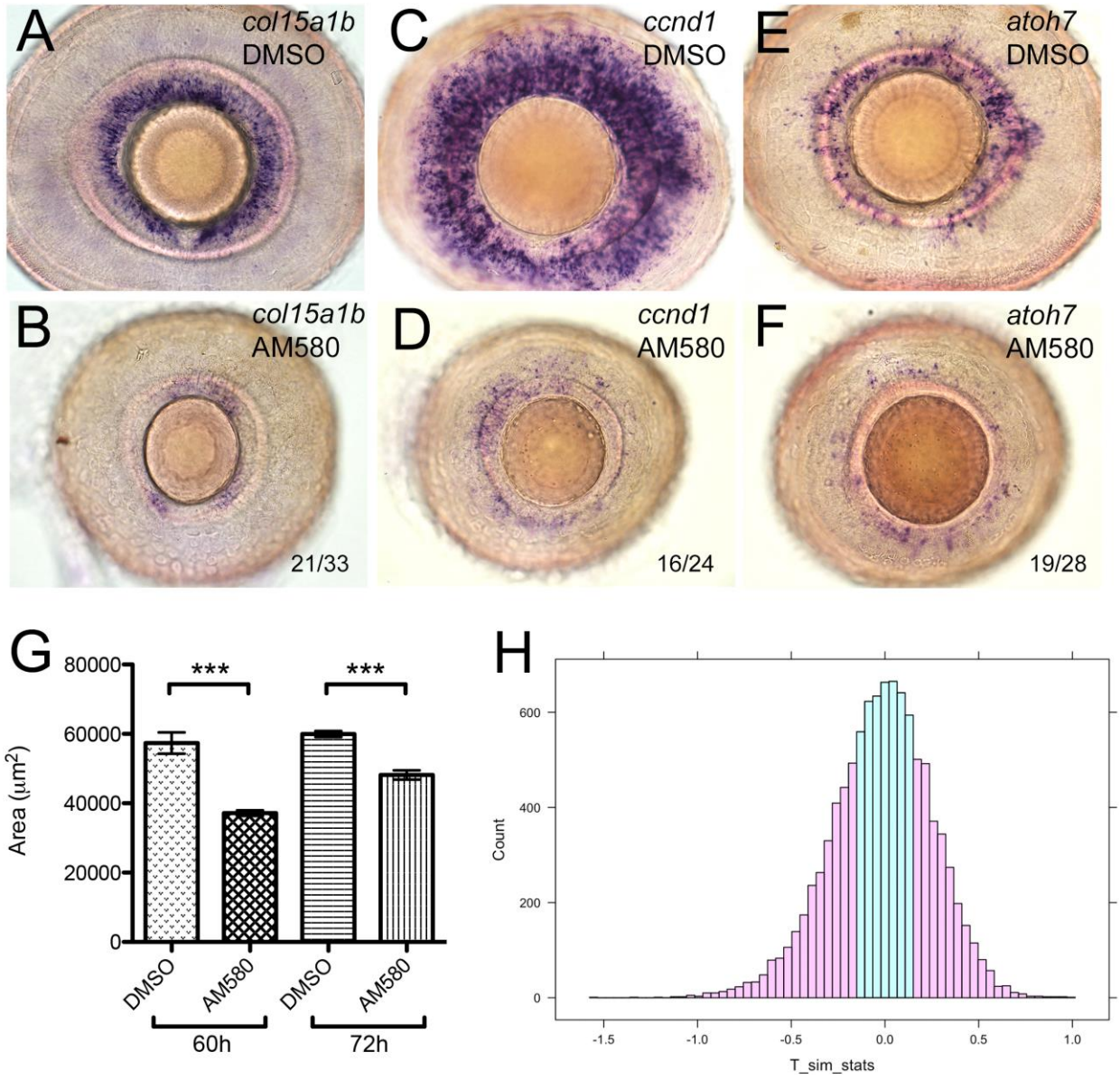


Fig. S4. AM580 treated fish phenocopy *gdf6a* mutants.

(A-F) Lateral views of eyes from wildtype embryos that were treated with 25 nM AM580 or an equal volume of vehicle control (DMSO) at 72 hpf for 12 hours and then assayed for expression of *col15a1b* (A-B), *ccnd1* (C-D), and *atoh7* (E-F) by in situ hybridization at 4.5 dpf. These representative images are similar to the phenotype observed when embryos were treated in the same way at 60 hpf and then assayed at 4.5 dpf. The numbers in the AM580-treated specimens show the number of embryos with the most severe phenotypes. The remaining embryos exhibited a noticeable, but more modest reduction in CMZ marker expression.

(G) Area measurements of eyes from wildtype embryos ($n \geq 14$ embryos for each condition) treated with 25 nM AM580 at either 60 hpf or 72 hpf for 12 hours and then fixed at 4.5 dpf were averaged and plotted with standard error bars (95% confidence limits; ***Student's t-test, $p \leq 0.000003$).
(H) Statistical analyses of eye size in mutant and drug treated embryos. R-generated histogram for permutation testing of changes in eye size of *gdf6a* mutants (as compared to siblings) relative to AM580 treated wild-types (as compared to DMSO-treated siblings). Pink shading indicates the number of data points along the simulated distribution that are as extreme or more extreme than the T value (0.15664) divided by the total number of simulations, giving us a visual for the calculated p value (0.5468) and indicating that the ratios compared are not statistically significantly different.

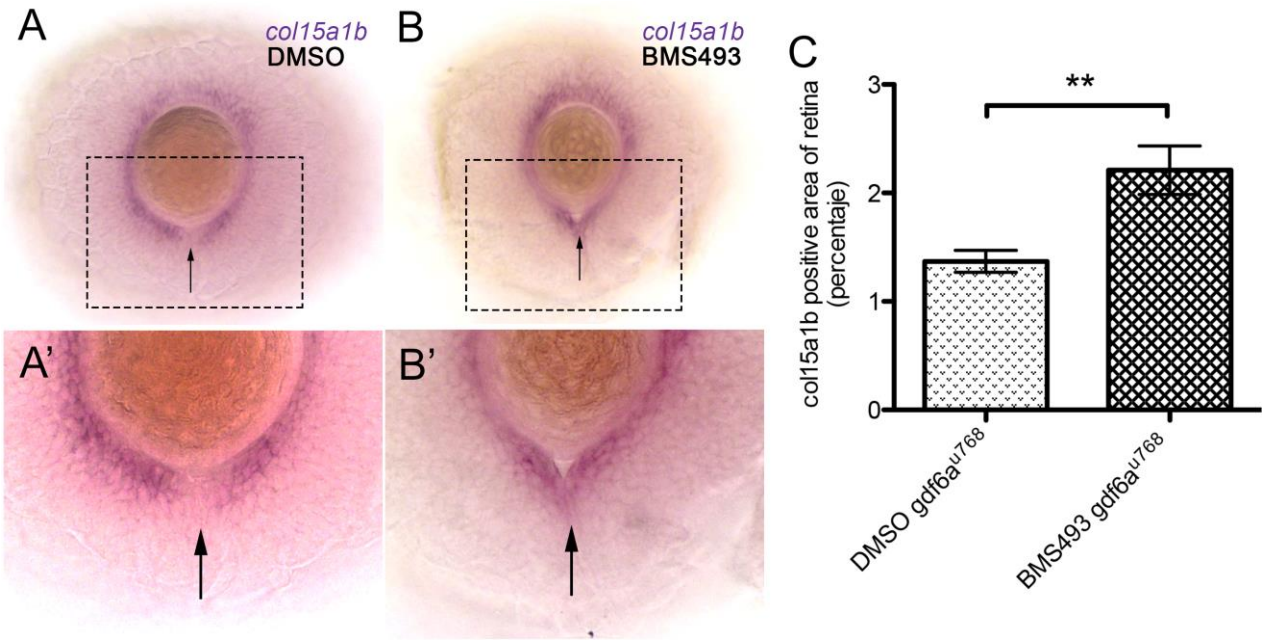


Fig. S5. Pharmacological inhibition of the RA pathway expands the CMZ ventrally along the choroid fissure.

(A-B) Lateral views of eyes from wild-type *gdf6a*^{u768} siblings treated with vehicle (DMSO, A) or the RAR inverse agonist BMS493 (15 µM, B) and assayed for expression of a marker of the putative stem compartment of the CMZ (*col15a1b*). Boxed regions in A and B were imaged with a 40x lens and are shown A' and B', respectively. Arrow indicates choroid fissure.

(C) Total eye area and *col15a1b*-positive area were measured for lateral views of either vehicle or BMS493-treated homozygous mutant eyes ($n \geq 11$ embryos), and percentage of eye area occupied by *col15a1b*-expressing cells was calculated for each eye. Mean percent *col15a1b*-positive areas were then graphed with standard error bars (95% confidence limits; **Student's t-test, p=0.0054).

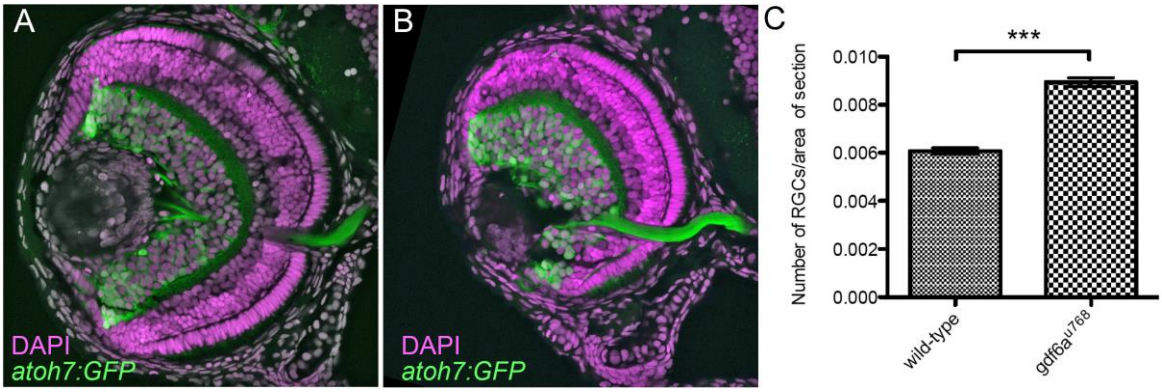
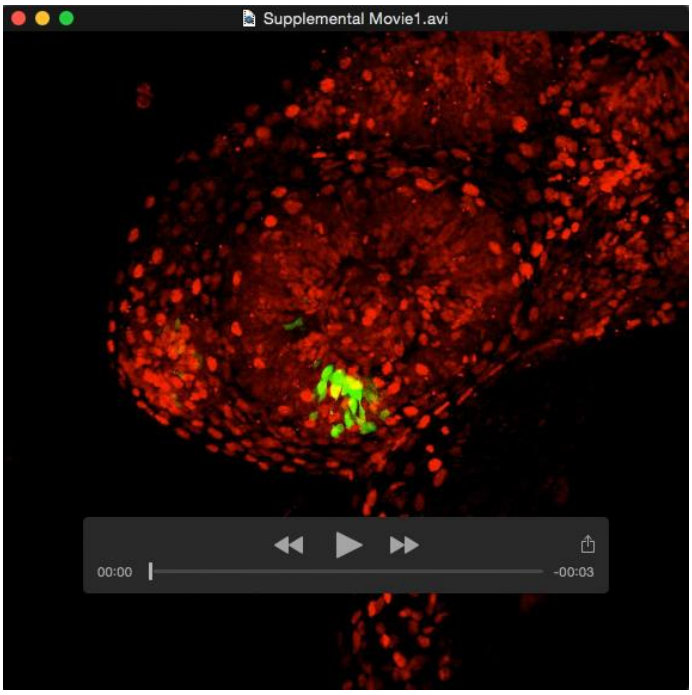
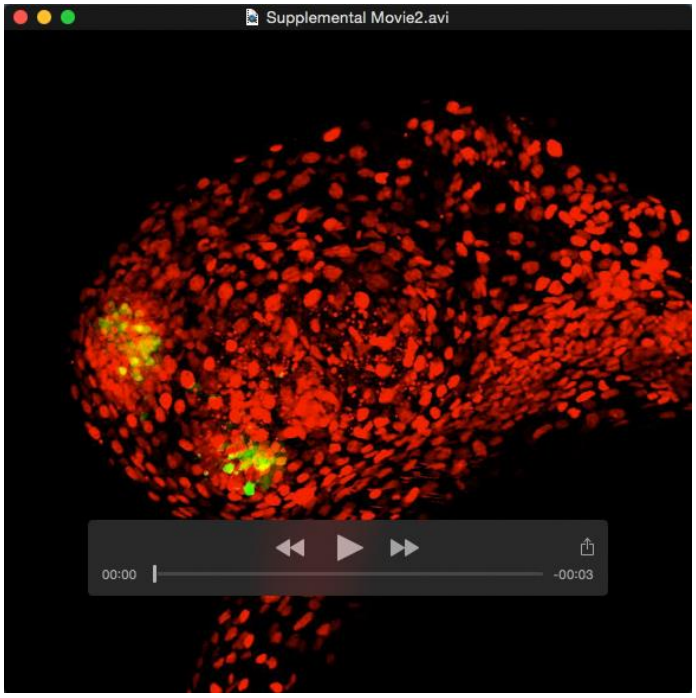


Fig. S6. *gdf6a*^{u768} mutant eyes contain increased relative numbers of *Tg[atoh7:GFP]*$^{+}$ cells. (A-B)Transverse cryosections of wild-type sibling (A) and *gdf6a*^{u768} (B) eyes at ~84hpf showing expression of the *atoh7:GFP* transgene (green) and counter stained nuclei with DAPI (magenta). (C) Graphical representation of the average number of *atoh7:GFP* positive cells per area of retina in wildtype and *gdf6a*^{u768} mutant eyes (n=7 embryos, standard error bars, 95% confidence limits, **Student's t-test p=0.00004).



Movie 1. Normal developmental pattern of *atoh7:GFP*+ cells birth within a transgenic retina.
In this movie of a lateral view of a wildtype eye, GFP+ cells spread circumferentially throughout the retinal neuroepithelium. See also figure 6.



Movie 2. Accelerated production of *atoh7:GFP*+ cells in *gdf6a*^{u900} mutant transgenic retina.
In this movie of a lateral view of an eye from a *gdf6a* mutant fish, more GFP cells appear within the retinal neuroepithelium than in the wild-type control. See also figure 6.

Supplemental References

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Masai, I., Lele, Z., Yamaguchi, M., Komori, A., Nakata, A., Nishiwaki, Y., Wada, H., Tanaka, H., Nojima, Y., Hammerschmidt, M., et al. (2003). N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. *Development* **130**, 2479-2494.

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