RESEARCH ARTICLE

Molecular and Cellular Characterization of a Zebrafish Optic Pathway Tumor Line Implicates Glia-Derived Progenitors in Tumorigenesis

Staci L. Solin1, Ying Wang1, Joshua Mauldin1, Laura E. Schultz1, Deborah E. Lincow1, Pavel A. Brodskiy1, Crystal A. Jones1, Judith Syrkin-Nikolau1, Jasmine M. Linn1, Jeffrey J. Essner1, Jesse M. Hostetter2, Elizabeth M. Whitley2, J. Douglas Cameron3, Hui-Hsien Chou1, Andrew J. Severin4, Donald S. Sakaguchi1, Maura McGrail1*

1. Department of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa, United States of America,
2. Department of Veterinary Pathology, Iowa State University, Ames, Iowa, United States of America,
3. Department of Ophthalmology and Visual Neurosciences, University of Minnesota, Minneapolis, Minnesota, United States of America,
4. Genome Informatics Facility, Office of Biotechnology, Iowa State University, Ames, Iowa, United States of America

Abstract

In this study we describe the molecular and cellular characterization of a zebrafish mutant that develops tumors in the optic pathway. Heterozygous Tg(flk1:RFP)is18 transgenic adults develop tumors of the retina, optic nerve and optic tract. Molecular and genetic mapping demonstrate the tumor phenotype is linked to a high copy number transgene array integrated in the lincRNA gene lincRNAis18/Zv9_00007276 on chromosome 3. TALENs were used to isolate a 147kb deletion allele that removes exons 2–5 of the lincRNAis18 gene. Deletion allele homozygotes are viable and do not develop tumors, indicating loss of function of the lincRNAis18 locus is not the trigger for tumor onset. Optic pathway tumors in the Tg(flk1:RFP)is18 mutant occur with a penetrance of 80–100% by 1 year of age. The retinal tumors are highly vascularized and composed of rosettes of various sizes embedded in a fibrous matrix. Immunohistochemical analysis showed increased expression of the glial markers GFAP and BLBP throughout retinal tumors and in dysplastic optic nerve. We performed transcriptome analysis of pre-tumorous retina and retinal tumor tissue and found changes in gene expression signatures of radial glia and astrocytes (slc1a3), activated glia (atf3, blbp, apoeb), proliferating neural progenitors (foxd3, nestin, cdh2, her9/ges1), and glioma markers (S100b, vim). The transcriptome also revealed activation of cAMP, Stat3 and Wnt signal transduction pathways. qRT-PCR confirmed >10-fold overexpression of the Wnt pathway signal transduction genes in tumors.
components \textit{hbefa}, \textit{ascf1a}, and \textit{insm1a}. Together the data indicate Müller glia and/or astrocyte-derived progenitors could contribute to the zebrafish \textit{Tg(flk1:RFP)is18} optic pathway tumors.

\section*{Introduction}

Glia play critical roles in the function and maintenance of the nervous system. They are involved in neuronal homeostasis and repair, but can also undergo reprogramming in response to injury to generate progenitors that repopulate missing neurons and glia [1]. In the retinas of mice, frog and fish one population of cells that can be reprogrammed in response to injury is Müller glia [2]. In normal retina Müller glia have stem-like behaviors, dividing asymmetrically to produce progenitors of the rod photoreceptor lineage [3]. After photoreceptor or retinal neuron damage, Müller glia can dedifferentiate and produce progenitors that give rise to the major neural retinal cell types. The zebrafish retina has been used extensively as a model system to investigate the molecular mechanisms required for this process [4–6]. Major signal transduction pathways activated in reprogrammed Müller glia in zebrafish include EGF [7], Stat3 [8–10] and Wnt [11, 12]. Understanding how these signaling pathways promote glia reprogramming and neural regeneration is important for advancing treatments of central nervous system injury and disease.

In this study we present the characterization and molecular cloning of a zebrafish transgenic line \textit{Tg(flk1:RFP)is18} that develops highly penetrant tumors in the retina and optic tract with features of retinoblastoma and fibrous glioma. The tumor phenotype is linked to a high copy number array of an RFP expressing reporter transgene in line \textit{Tg(flk1:RFP)is18}. The transgene integrated in a long intergenic noncoding RNA gene \textit{lincRNAis18}, which was previously identified in the zebrafish embryonic transcriptome as \textit{Zv9_00007276} and \textit{Zv9_00007274} [13]. Isolation of a second targeted deletion allele of the \textit{lincRNAis18} gene did not result in tumor formation, suggesting loss of function of the locus is not the initiating event that triggers tumor onset. Histological, cytological, and transcriptome analyses in pre-tumorous retina and tumor tissue reveal gene signatures of radial glia, neural progenitors, and injury induced activation of glia and astrocytes. The \textit{Tg(flk1:RFP)is18} tumors are similar to the zebrafish model of optic pathway glioma driven by activated Sonic hedgehog signaling in neural progenitors [14]. Our analyses indicate the \textit{Tg(flk1:RFP)is18} tumors originate from neural progenitors derived in part from an activated glial cell population that includes reprogrammed Müller glia.
Results

Isolation and molecular mapping of the optic pathway tumor line Tg(flk1:RFP)is18

We isolated a transgenic zebrafish line in which heterozygous adults develop neoplasia of the retina, optic nerve and optic tract. Transgenic line Tg(Tol2<flk1:RFP-CAAX> is18 (abbreviated as Tg(flk1:RFP)is18) was generated using a Tol2 transposon reporter construct that expresses membrane-targeted RFP-CAAX throughout vascular endothelial cells (Fig. 1A, B). Heterozygous Tg(flk1:RFP)is18 adults developed large ocular tumors that first became evident at approximately 5 months of age (Fig. 1C) with a penetrance of >80%. Multigenerational genetic analysis demonstrated that the tumor phenotype was linked to inheritance of the Tg(flk1:RFP)is18 transgene, as determined by RFP expression (Fig. 1D). In each generation non-transgenic siblings were healthy and showed no evidence of tumor formation. These results indicated that the tumor phenotype was due either to the location of the Tg(flk1:RFP)is18 transgene in the genome or the presence of the transgene itself. Attempts to map the Tg(flk1:RFP)is18 transgene integration site by standard ligation mediated PCR and inverse PCR methods suggested that during isolation of the line, multiple copies of the entire pTol2<flk1:RFP> construct, including the vector backbone, had integrated as a concatemer array. We confirmed by genomic Southern blot that the Tg(flk1:RFP)is18 line contains an array with ~100 copies of the Tol2<flk1:RFP> construct (Fig. 1E). Genomic DNA from Tg(flk1:RFP)is18 individuals from 3 consecutive generations hybridized with an RFP-specific probe revealed multiple bands, including the expected 1.3 kb EcoRI-ScaI fragment from the Tol2<flk1:RFP> construct. In each generation non-transgenic siblings that lacked RFP expression in vascular tissue did not inherit the concatemer (Fig. 1E). Together these results provided strong evidence that the transgene in line Tg(flk1:RFP)is18 is a stable, high-copy number concatemer that displays Mendelian transmission from one generation to the next.

To clone the genomic DNA flanking the Tg(flk1:RFP)is18 concatemer integration site and map its location in the zebrafish genome, we designed a custom SureSelect Target Enrichment kit (Agilent Technologies) with single stranded biotinylated RNA probes that tile across the entire pTol2<flk1:RFP> vector (S1 A Figure; Table 1). Random sheared Tg(flk1:RFP)is18 genomic DNA was hybridized to the biotinylated bait library in order to capture all of the concatemer transgene sequences. Since the genomic DNA was randomly sheared, we predicted a percentage of captured sequences would span the junction of the ends of the concatemer and the genomic DNA. Five independent genomic DNA samples isolated from muscle or tumor tissue, representing 3 Tg(flk1:RFP)is18 individuals from 3 different generations, were captured and used to produce barcoded Illumina libraries for multiplex paired end sequencing (S2 Table). Sequences from the captured libraries were filtered to remove reads in which both ends mapped to the zebrafish genome or the pTol2<flk1:RFP> construct. The remaining paired end reads contained Tol2<flk1:RFP> transgene sequences at...
one end and zebrafish genomic sequences at the other. Four candidate integration sites that contained 9 or more reads from multiple samples (S3 Table) were chosen for further analysis. Three of the four sites mapped to regions with highly
repetitive DNA, indicating non-specific capture of during hybridization. The fourth site had 80 reads that aligned to either side of position 24.212 Mb on chromosome 3 of the v9 zebrafish genome (S1 B Figure). This location maps in between the HoxBa cluster and the Hp1 heterochromatin binding protein family member cbx1a (Fig. 2A). The integration site was confirmed by sequencing of PCR amplification products that span the Tg(flk1:RFP)is18 transgene-genomic DNA junction (S1 C, D Figure). An 8 bp duplication was present at the integration site, which indicated a simple Tol2 transposon integration (S1 C Figure). However, additional molecular analyses confirmed that the high copy array detected by genomic southern (Fig. 1E) is also tightly linked to the integration site on chromosome 3.

Genomic Southern blot analysis of restriction fragment length polymorphism caused by the transgene integration confirmed the location of the Tg(flk1:RFP)is18 chromosome haplotype in the region surrounding the integration site is Z7419-L, G39247-S, Z5197-L, cbx1a STS-L.

Table 1. Linkage of Tg(flk1:RFP)is18 transgene integration site to position 24.219 Mb Chromosome 3.

| Primer pair | Marker* | Chr3 Position | Genotype | Haplotype Analysis* | Linkage Analysis§ |
|-------------|---------|---------------|----------|--------------------|-------------------|
| 1           | Z7419   | 23158018      | is18+/F5#3 female | 10 is18+/ siblings | 100 is18/+ offspring |
|             |         |               | WIK male     | 4/10 L/S           | 100/100           |
| 2           | G39247  | 24163628      | L/S         | 10/10 L/S          | 100/100           |
| 3           | transgene - | 24219944 | +           | 10/10              | 100/100           |
| 4           | transgene | -             | -           | 10/10              | 100/100           |
| 5           | Z5197   | 24324461      | L/S         | 10/10 L/S          | 100/100           |
| 6           | cbx1a STS | 24360325 | L/M         | 6/10 L/M           | NA                |
| 7           | Z7486   | 59983229      | L/S         | 3/10 L/L           | NA                |
|             |         |               | L/S         | 4/10 L/L           | NA                |
|             |         |               | L/S         | 3/10 S/S           | NA                |
|             |         |               | L/S         | 4/10 S/S           | NA                |

*Microsatellite STS markers Z7419, G39247, Z5197, Z7486. cbx1a STS is a Short Simple Repeat located in the 5th intron of the cbx1a gene. Genotype determined by size of PCR amplification products after gel electrophoresis. L, long; M, middle; S, short allele PCR products. is18 transgenic genotype (+ or −) confirmed by PCR with primers that amplify across the genomic DNA-transgene junction (3) or amplify an internal fragment of the transgene concatemer (4).

Genotype of is18+/ female and WIK male was determined. Segregation of STS markers in 10 is18+/ and 10+/+ siblings was used to determine the haplotype of the chromosome containing the is18 transgene integration. The Tg(RFP)is18 chromosome haplotype in the region surrounding the integration site is Z7419-L, G39247-S, Z5197-L, cbx1a STS-L.

$100 is18+/ and 100+/+ siblings from a cross between a Tg(flk1:RFP)is18+/ female and WIK wild type male were genotyped by PCR. 1/100 Tg(flk1:RFP)is18/+ progeny was homozygous Z7419 M/M and heterozygous G39247 L/S. This indicates a single recombination event between markers Z7419 and G39247 and genetic map distance of 1 centimorgan.

doi:10.1371/journal.pone.0114888.t001
Figure 2. The Tg(flk1:RFP)is18 transgene integration site on chromosome 3 disrupts expression of lincRNAis18.v2. (A) Integration site of the transgene array at 24.2 Mb on chromosome 3. The integration sits downstream of the HoxBa cluster and upstream relative to the heterochromatin binding family member cbx1a. lincRNAis18 exon I overlaps with a 5′ regulatory region of the cbx1a gene. Positions of upstream and downstream microsatellite markers, G39247 and Z5197, and an STS/STR in cbx1a, are shown. (B) RT-PCR
analyses with primers in exon 1 and exon 8 of \textit{lincRNAis18.v2} showing expression of \textit{lincRNAis18.v2} is disrupted in 6 dpf homozygous \textit{Tg(flk1:RFP)is18} larvae. 5 individual larvae of each genotype are shown. The genotype of each larva was confirmed (Figure S6). Control, expression of ribosomal protein S6 kinase b, polypeptide 1, \textit{rps6kb1}. (C) TALENs targeting exons 2 and 5 of \textit{lincRNAis18}. (D) Predicted structure of the \textit{lincRNAis18}\textsubscript{e2e5del} deletion allele. Sequence of amplicon spanning exon 2 – exon 5 junction from F1 \textit{lincRNAis18}\textsubscript{e2e5del}/+ adult genomic DNA.

doi:10.1371/journal.pone.0114888.g002

Zebrafish \textit{Tg(flk1:RFP)is18} transgene integration in the \textit{lincRNAis18} gene

We searched NCBI, Ensembl, and the recent zebrafish embryonic transcriptome study [13] for long intergenic noncoding RNAs (lincRNAs) that map to the region between the \textit{HoxBa} cluster and \textit{cbx1a} on chromosome 3 and identified two. One of the genes, \textit{si:ch211-84g22.1} ENSDARG00000097724, was located \textasciitilde 14 kb 3\textsuperscript{9} to the integration site (Fig. 2A). The second lincRNA was identified by a \textasciitilde 930 bp transcript FDR202-P00026-DEPE-F\_N10 FDR202 EH545544/Zv9\_00007276 containing 8 exons that map to a genomic region spanning \textasciitilde 350 kb (Fig. 2A). The 5\textsuperscript{9} region and first exon overlaps the \textit{cbx1a} gene, and downstream exons 6, 7, and 8 map in the intergenic regions between genes in the \textit{HoxBa} cluster (Fig. 2A). A shorter transcript of \textasciitilde 480 bp (\textit{si:ch211-246i5.4} ENSDARG00000097621/ FDR202-P00032-DEPE-R EH568666.1/Zv9\_00007274) terminates in an alternative third exon. Neither transcript is predicted to encode a polypeptide longer than 73 amino acids (reading frame 3). We named the locus encoding these transcripts \textit{lincRNAis18}. The shorter transcript is designated \textit{lincRNAis18.v1} (Fig. 2A), the longer transcript is designated \textit{lincRNAis18.v2} (Fig. 2A). The \textit{Tg(flk1:RFP)is18} concatemer integration site is in the second intron of \textit{lincRNAis18.v2} (Fig. 2A). Since the concatemer contains many copies of the SV40 bidirectional polyadenylation sequence, the transgene is predicted to cause premature transcriptional termination of \textit{lincRNAis18.v2}. Together, the data showed that the optic pathway tumor phenotype was linked to the integration of the \textit{Tg(flk1:RFP)is18} concatemer in the second intron of \textit{lincRNAis18.v2}.

We performed a number of experiments to determine whether \textit{lincRNAis18} is involved in formation of ocular tumors in \textit{Tg(flk1:RFP)is18} heterozygotes.
examined the expression pattern of lincRNAis18 by in situ hybridization and RT-PCR to determine whether it is expressed in the zebrafish retina. In situ hybridization on adult retina detected a low level of lincRNAis18 expression in the inner nuclear layer and the ganglion cell layer (S5 A Figure), indicating it is not highly expressed in the retina. RT-PCR on an adult tissue panel revealed high levels of lincRNAis18.v2 transcripts in the male and female germline and lower levels in muscle and retina (S5 B Figure). High levels of maternally supplied lincRNAis18.v2 were observed in the developing embryo before the onset of zygotic transcription (S5 C Figure) then rapidly declined later stages. Multiple alternatively spliced forms of lincRNAis18 were cloned from wild type adult retina, ovary and embryonic and larval stages (S5 Figure). Exons 1, 2 and 8 were consistently present in all isoforms, indicating a possible functional role of these exons. Although a low level of lincRNAis18.v2 expression was detected in the retina, the specificity of expression in this tissue, and its absence from other adult tissues except muscle, suggested it may have a role in retina function.

To further examine the coding potential of lincRNAis18, we searched the recently published ribosome profiling datasets of expressed transcripts from 8 early developmental stages of zebrafish [15]. The datasets included 2–4 cell, 256 cell and 1000 cell embryos in which a high level of maternally supplied lincRNAis18 is present. None of the ~900 million 35 bp sequence reads fully aligned with the lincRNAis18 sequence, providing further evidence that lincRNAis18 represents a novel lincRNA that most likely is not translated into a functional protein. Database searches showed lincRNAis18 has no significant homology with lincRNAs from other species. Despite the lack of homologous sequences in other vertebrate species, the high levels of lincRNAis18 expression in the embryo suggests it could play an important role in early zebrafish development.

To determine whether the transgene integration in Tg(flk1:RFP)is18 disrupts expression of lincRNAis18.v2, we examined lincRNAis18.v2 expression by RT-PCR in homozygous Tg(flk1:RFP)is18/Tg(flk1:RFP)is18 individuals. Progeny from an intercross of Tg(flk1:RFP)is18/+ heterozygous adults were sorted into RFP-expressing and RFP-negative classes. Genomic DNA and total RNA were isolated from each individual for genotyping and RT-PCR. Expression of lincRNAis18.v2 was undetectable in 5/5 homozygous is18/is18 larvae (Fig. 2B). The genotype of each individual was confirmed by PCR (S6 Figure). These data demonstrate that integration of the Tg(flk1:RFP)is18 transgene disrupts expression of the long form of lincRNAis18, lincRNAis18.v2.

To determine whether homozygosity results in additional phenotypes, all surviving progeny from the is18/+ heterozygous intercross were raised and sacrificed at 4 weeks of age for PCR genotyping of genomic DNA. Some larvae began to develop edema by 7 dpf and as the larvae were aged many developed edema and did not survive. At 4 weeks of age, each of the 96 surviving RFP-expressing juveniles was sacrificed and genotyped. All were heterozygous for a wild type and a Tg(flk1:RFP)is18 chromosome (data not shown), indicating that homozygous Tg(flk1:RFP)is18/Tg(flk1:RFP)is18 individuals die from edema.
between 7 dpf and 4 weeks of age. These data indicate that homozygous Tg(flk1:RFP)is18 larvae lack expression of lincRNAis18.v2 (Fig. 2B), due to premature transcription in the transgene, as predicted by the location of the transgene integration in intron 2. However, this does not rule out the possibility that the onset of edema and homozygous lethal phenotype might be caused by the presence of the Tg(flk1:RFP)is18 transgene concatemer or its effect on nearby genes.

To test the hypothesis that disruption of expression of lincRNAis18.v2 is homozygous lethal and involved in tumor formation, we isolated a second allele using TALEN genome editing to create a deletion in the lincRNAis18 gene (Fig. 2C). TALENs targeting exons two and five (S7 Figure) were co-injected to create a deletion that removes 147 kb of genomic DNA and fuses exon two to exon five. Exons further upstream or downstream were not targeted in order to avoid affecting the genomic region surrounding the cbx1a and HoxBa cluster genes. We recovered 1 founder out of 27 that transmitted the deletion allele to F1 progeny. Sequencing of the exon 2 - exon 5 junction fragment and genotyping with STR markers confirmed the identity of F1 individuals carrying the deletion allele lincRNAis18^e2e5del (Fig. 2D). To test whether the deletion allele was homozygous lethal, heterozygous lincRNAis18^e2e5del F1 adults were intercrossed and the progeny were raised to adulthood. Genotyping identified homozygous F2 adults (S8 Figure), demonstrating that deletion of the genomic sequences between exons two and five of lincRNAis18 did not result in lethality. By 8 months neither heterozygous nor homozygous lincRNAis18^e2e5del adults developed ocular tumors. Together, these data suggest that tumor onset in Tg(flk1:RFP)is18 individuals is most likely not due to a loss of function of the lincRNAis18 gene, or disruption of expression of lincRNAis18.v2. However, since the deletion allele that was generated is predicted to produce a transcript containing exons 1, 2/5, 6, 7 and 8, it does not represent a null allele, and may retain some function that prevents homozygous lethality and tumor onset. Alternatively, because the Tg(flk1:RFP)is18 transgene mutation segregates as a dominant allele, it might create a dominant effect on the expression of other lincRNAis18 transcript isoforms. It is also possible that the presence of the high copy number transgene in Tg(flk1:RFP)is18 adults induces ocular tumor formation through an oncogenic mechanism that induces overexpression of nearby genes. Transcriptome analyses presented below do not support the latter mechanism.

Histopathology of the zebrafish Tg(flk1:RFP)is18 optic pathway tumors reveals features of retinoblastoma and fibrous glioma

Histopathology of the tumor positive fish revealed large intraocular masses that filled the vitreal space and displaced the lens (Fig. 3B–D). By one year of age affected individuals frequently developed tumors in both eyes (Fig. 3D). Advanced retinal tumors were not characterized by necrosis or high mitotic activity. The tumors were composed of neuroepithelial-like cells with areas of dysplastic tissue forming rosettes of various sizes (Fig. 3J, M arrowheads). Blood
vessels were present throughout the tumors, indicating neovascularization in the advanced tumors. There were extensive glial fibrillar structures within the tumors (Fig. 3J, M arrow) and the optic nerve and tract (Fig. 3K, L). In advanced tumors the optic nerves and tract were completely replaced by neoplastic tissue, and the architecture of the brain lobes was highly disrupted (Fig. 3C, D, K, L). Cells having a “salt and pepper” chromatin dispersion pattern were present, again consistent with neuro-ectodermal tumor cell cytomorphology (Fig. 3N, arrowheads). Areas of necrosis were present throughout the optic lobe (Fig. 3P arrows). Examination of tumors in younger adults revealed relatively normal organization of the retina at the ciliary marginal zone. In a 5-month old adult with a large ocular mass (Fig. 3B), the neural retinal layers were observed to be intact at the
ciliary marginal zone (Fig. 3I). These initial examinations indicated the ocular masses likely originated from cells located in the differentiated tissue of the neural retina, not from the retinal stem cell population that resides at the ciliary marginal zone. Overall, the retinal tumors appeared similar to fibrous glioma with features present in human retinoblastoma.

Evidence for involvement of glia in Tg(flk1:RFP)is18 tumor proliferation

To gain further insight into the identity of the Tg(flk1:RFP)is18 retinal tumors we examined the expression of retinal neural markers in cryosections of tumor tissue. The calcium binding protein Recoverin is normally expressed in all photoreceptors in the retina (Fig. 4A), while the antibody RT97, which broadly recognizes neuronal intermediate filament proteins, also labels photoreceptor outer segments (Fig. 4A). In tumors Recoverin was detected in the cell bodies and RT97 labeled structures projecting into the center of the rosettes (Fig. 4E–H). RT97 also strongly labeled the fibrous stroma of the tumor (Fig. 4E–F), indicating the presence of neuronal processes in this matrix. The synaptic vesicle marker SV2 was also detected throughout the fibrous stroma (G). These analyses were consistent with the histopathology suggesting the tumors likely originate from the neural retina.

To examine the proliferative cell populations in the Tg(flk1:RFP)is18 retinal tumors, we labeled adult fish with BrdU for 2 hours, followed by a 4 hour recovery period. In wild type retina BrdU incorporation was detected at the ciliary marginal zone where progenitor cells reside (Fig. 4D, arrow). Very rarely BrdU was detected in single cells in more central retina, consistent with the slow cycling of the Müller glia-rod photoreceptor lineage. We examined BrdU incorporation in advanced tumors and found robust labeling throughout the tumor mass with some overlap with rosettes (Fig. 4H). BrdU did not consistently co-label cells expressing a specific retinal neural cell type marker, making it difficult to conclusively determine the identity of proliferating cells in very advanced tumors. Moreover, histopathology did not indicate high mitotic activity in the advanced tumors (Fig. 3). This suggests the possibility that the large number of cells that incorporated BrdU was not due to DNA synthesis during S phase but was the result of the activity of global DNA repair processes.

Numerous studies have demonstrated that the Müller glia can be activated in response to retinal injury and reprogrammed to produce progenitors that repopulate all retinal neural cell types [16]. To determine if the Tg(flk1:RFP)is18 tumors showed evidence of reprogrammed Müller glia-derived progenitors, we examined the expression of Müller glia and activated Müller glia markers by immunolocalization. The glial specific marker GFAP was detected in the Müller glia end feet located in the ganglion cell layer in wild type retina (Fig. 4I–L). Very little GFAP expression was detected in the glia of the wild type optic nerve (Fig. 4K, arrow). In the Tg(flk1:RFP)is18 retinal tumors intense labeling of GFAP was detected extending throughout the tumor mass (Fig. 4M, N), indicating a
glial cell type. Increased expression was also evident in the dysplastic optic nerve at the lamina cribrosa of an eye with an advanced tumor (Fig. 4O), indicating expansion of the astroglia of the optic nerve. To visualize activated Müller glia, we examined immunolocalization of Brain Lipid Binding Protein/fatty acid binding protein (BLBP/fabp). In wild type retinas BLBP is expressed in progenitor cells at the ciliary marginal zone (Fig. 4L) but is absent from Müller glia. In the Tg(flk1:RFP)is18 retinal tumors an increase in BLBP was detected throughout the tumor and did not appear to co-localize with GFAP positive Müller glia cells (Fig. 4L, P). This suggested BLBP was labeling a population of cells distinct from mature Müller glia. Together these results indicate Tg(flk1:RFP)is18 tumors might

Figure 4. Characterization of lincRNAis18 tumors indicates a glial cell origin. Immunolabelling and in situ hybridization of cryosections from wild type retina (A–D, I, J, M–P) and advanced lincRNAis18 tumors (E–H, K, L, O–T). Cells in rosettes in the tumors label with neurofilament marker RT-97 (red) and photoreceptor marker recoverin (green) (E, F). The synaptic vesicle marker SV2 (green), which is enriched in the retinal plexiform layers (C), was distributed throughout the fibrous tumor mass (G). (D) BrdU (green) incorporated into proliferating progenitor cells at the ciliary marginal zone of the wild type retina (arrow). (H) Intense labeling of BrdU incorporation was detected in cells forming rosettes and throughout the tumor mass. In wild type the glial marker GFAP is most evident in the Müller glia end feet that sit in the retinal ganglion cell layer (I–L). GFAP expression is absent in the oligodendrocytes and astrocytes of the optic nerve (I, K arrow). In contrast, GFAP was readily detected in long streaks throughout the tumor tissue (M, N, P), and was expressed by cells in the mutant optic nerve (O, arrow). BLBP is present at the ciliary marginal zone of wild type retina (L) and appeared present throughout the tumor mass (P). A, E, I, M, Differential interference contrast (DIC) overlay on immunofluorescence labeling images. CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; ON, optic nerve; ONH, optic nerve head; ONL, outer nuclear layer. All scale bars represent 50 μm, except in panels K and O scale bars represent 100 μm.
be the result of abnormal levels of glial proliferation, with features similar to activated Müller glia and the neural progenitors derived from them.

**Differential gene expression analysis of Tg(flk1:RFP)is18 retinal tumor progression**

To identify the molecular signatures that correlate with onset and tumorigenesis of Tg(flk1:RFP)is18 tumors we performed differential gene expression analysis by RNA-Seq. Libraries were prepared from 6 month old age-matched wild type retina (Wild Type), pretumor retina from heterozygous Tg(flk1:RFP)is18 adults (Pretumor), and advanced tumor tissue from heterozygous Tg(flk1:RFP)is18 adults (Tumor) (S6 Table). The data was mapped to the zebrafish v9 genome, gene model assembly version 71 (S7 Table). The gene models included 1133 recently identified lincRNAs expressed during early zebrafish development [13]. Genes with FPKM (Fragments Per Kilobase per Million sequenced reads) value of $\geq 1$ in Wild Type retina were examined for significant changes in expression level in Pretumor and Tumor samples (S7 Table). GO term analysis (Table 2) indicated translational activity was elevated in Pretumor and Tumor tissue. In Pretumor and Tumor tissue cellular respiration was decreased; photoreception and ion transport processes were also decreased in tumor. Consistent with the GO term analysis, the gene set that showed the greatest down-regulation between Wild Type and Tumor was enriched for genes that function in photoreceptor maintenance and photoreception (S8 Table), indicating that in the Tg(flk1:RFP)is18 tumors, normal photoreception and synaptic transmission are disrupted. As expected for proliferating tumor, mitosis and cell division processes were increased in Tg(flk1:RFP)is18 tumors. 252 of the 1133 lincRNAs identified in the zebrafish embryonic transcriptome were expressed in wild type retina (S7 Table). 11 of the 252 lincRNAs were significantly increased or decreased in expression level in Pretumor and/or Tumor tissue (S9 Table).

We examined the expression levels of genes that map in the region of the Tg(flk1:RFP)is18 transgene integration in order to determine if the array influenced expression of nearby genes. Only 5 reads in the wild type retina transcriptome mapped to lincRNAis18, indicating the majority of lincRNAis18 mRNA was lost during isolation of polyadenylated RNA for RNA-Seq library construction. We confirmed this by RT-PCR using the input RNA for library construction and samples of the resulting libraries. The presence of lincRNAis18 mRNA was detected by RT-PCR in the total RNA input sample, but was only detectable in the wild type RNA-Seq library after nested RT-PCR (S9 Figure). The lincRNA si:ch211-84g22.1, located in intron 2 of lincRNAis18 and positioned 14 kb 3’ to the Tg(flk1:RFP)is18 transgene integration site, was only represented by 4 reads in wild type retina. Expression levels of si:ch211-84g22.1 in Tg(flk1:RFP)is18 Pre-Tumor and Tumor tissue were increased (98 and 132 reads total), but did not significantly change between the two samples. These results raise the possibility that the dominant nature of the Tg(flk1:RFP)is18 transgene to induce tumors could be the result of activation of expression of the nearby
lincRNA *sich211-84g22.1*. Other genes located in the region of the *Tg(flk1:RFP)is18* transgene integration were examined for altered expression. No significant change in expression level was detected for genes that map within 1 Mb upstream or downstream of the transgene integration, including the *HoxBa* cluster and *cbx1a* genes. Overall, the analyses indicate the *Tg(flk1:RFP)is18* transgene integration is responsible for inducing tumor formation, but the mechanism does not involve altering the expression level of genes in cis other than the lincRNA *sich211-84g22.1*.

**Human Mutation-Driver and Glioma Marker Genes**

We examined the *Tg(flk1:RFP)is18* tumor differentially expressed gene set for changes in tumor suppressors and oncogenes that identify disrupted signal transduction pathways in cancer. Although the ocular tumors showed features of retinoblastoma tumors, the expression level of the *rb1* tumor suppressor was unchanged in pretumor and elevated ~3 fold in tumor tissue ([S7 Table](#tab7)), indicating the tumors do not arise due to deletion of the *rb1* locus. 62 of the 138 designated Mutation-Driver human cancer genes ([17](#ref17)) showed at least a 2-fold change in expression ([Fig. 5A](#fig5a)). In the Wnt signaling pathway, two direct molecular targets of β-catenin transcriptional activation, the *myc* oncogene ([18](#ref18)) and cell cycle regulator *cyclinD1* ([19](#ref19)), showed significant increases in expression in *Tg(flk1:RFP)is18* tumor tissue. Two tumor suppressors in the PI3K-mTOR pathway altered in the majority of human gliomas ([20–23](#ref20-23)), PI3K regulatory subunit *pi3kr1* and the tsc1 repressor of mTOR, were decreased in expression level in the *Tg(flk1:RFP)is18* tumors ([Fig. 5A](#fig5a) and [S7 Table](#tab7)). The tumors showed a significant increase in expression of glioma diagnostic markers ([24, 25](#ref24-25)) [22](#ref22) *vimentin (vim)*, *S100β (s100β)* and the matrix metalloprotease *mmp9* ([Table 3](#tab3)).

### Table 2. Categories of biological processes altered in *Tg(flk:RFP)is18/+* Pre-tumor and Tumor tissues.

| DGE Group* | GO Process                                      | p-value   |
|------------|-------------------------------------------------|-----------|
| Increased in Pretumor | translation                                       | 4.50e-13  |
|           | ribonucleoprotein complex biogenesis             | 9.59e-8   |
|           | regulation of translational initiation           | 5.62e-5   |
|           | ribosome biogenesis                              | 9.28e-5   |
| Decreased in Pretumor | cellular respiration                              | 9.38e-7   |
|           | energy derivation by oxidation of organic compounds | 4.53e-5   |
|           | ATP metabolic process                            | 9.01e-5   |
| Increased in Tumor | ribonucleoprotein complex biogenesis             | 1.99e-13  |
|           | translation                                      | 3.86e-12  |
|           | mitotic cell cycle                               | 9.48e-9   |
|           | cell division                                    | 5.02e-7   |
| Decreased in Tumor | sensory perception of light stimulus             | 2.00e-4   |
|           | cellular respiration                             | 3.11e-3   |
|           | ion transport                                     | 3.67e-3   |

*DGE Group: Differential Gene Expression Group. Genes were grouped according to direction of change in expression level in Pretumor and Tumor tissue.*

doi:10.1371/journal.pone.0114888.t002
The zebrafish homolog of the putative tumor suppressor ajap1 [26] was also significantly decreased in expression in Tg(flk1:RFP)is18 tumor tissue (Table 3). qRT-PCR confirmed ajap1 decreased in expression 4-fold (Fig. 5C). These data are consistent with the histological and immunohistochemical data that suggests Tg(flk1:RFP)is18 retinal tumors express a glial gene signature.

**Glial activation and Neuronal Progenitors**

Two populations of glia cells in the retina may contribute progenitor cells that give rise to the Tg(flk1:RFP)is18 tumors. The Müller glia reside within the neural retina and in the mature retina function as late retinal progenitors that produce the rod photoreceptor lineage. The nerve fiber layer contains astrocytes that migrate into the retina from the optic nerve during embryogenesis. We examined the transcriptome data for changes in gene expression of markers for glia, glial progenitors, reactive astrocytes and activated Müller glia. Glutamate transporter GLAST/SLC1A3 has been identified as a marker of radial glia, glial progenitor cells, and glial progenitor-derived astrocytes [27]. Zebrafish slc1a3a and -b were both significantly increased in expression in the Tg(flk1:RFP)is18 tumors (Table 3). Activating transcription factor 3 (ATF3) is a member of the cAMP-response element binding protein family of transcriptional activators that bind the CRE (cAMP response element) enhancer and are activated in response to a rise in cytosolic cAMP levels. atf3 expression increases in the retinal ganglion and nerve fiber layers of the retina and the optic nerve after injury to the optic nerve in adult zebrafish [28]. In the Tg(flk1:RFP)is18 transcriptome atf3 expression levels increased significantly in Pretumor and Tumor tissue (Table 3). In contrast, bystin (bysl), whose expression increases substantially after optic nerve injury and marks reactive astrocytes [29], remained unchanged (S7 Table). Changes in expression levels of atf3 and bysl were confirmed by qRT-PCR (Fig. 5C). These data indicate reactive gliosis was not solely responsible for the increased expression of glial progenitor markers in the Tg(flk1:RFP)is18 ocular tumors. Many of the genes required for proliferation of neuroglial progenitor cells were increased in expression level in the Tg(flk1:RFP)is18 tumor tissue. This included nerve growth associated factor gap43, neural stem cell intermediate filament protein nestin, and neural cadherin 2 cdh2 (Table 3). Transcription factors required in neural stem cells (foxd3, sox21b, sox4a, sox4b, sox11a and sox11b) were also increased in Tg(flk1:RFP)is18 Tumor tissue (Fig. 5B; Table 2). In response to photoreceptor degeneration after retinal injury, the Müller glia become activated to reenter the cell cycle and produce retinal progenitors. The gene expression pattern that defines proliferating Müller glia (apoeb, blbp, cdh2, hes1, rlbp1a and –
Table 3. Differential Expression of Neural Progenitor, Müller glia Regeneration, and Glioma Genes in Tg(flk1:RFP)is18/+/ tumor progression.

| Gene   | Gene Name                              | Zebrafish Gene ID and Symbol          | Fold Change |
|--------|----------------------------------------|---------------------------------------|-------------|
|        |                                        |                                       | WT –> Pretumor | WT – Tumor  |
| Neuroglial Progenitors                      |                                        |                                       |             |
| atf3   | activating transcription factor 3       | ENSDARG00000007823_atf3               | 7.7         | 63.1        |
| foxd3  | forkhead box D3 transcriptional activator | ENSDARG0000021032_foxd3            | 17.7        | 177.7       |
| gap43  | growth associated protein 43, nerve growth factor | ENSDARG0000015775_gap43            | 3.1         | 52.8        |
| nestin | neural stem cell intermediate filament protein | ENSDARG0000088805_nes              | 8.7         | 162.1       |
| slc1a3a| glutamate transporter GLAST/SLC1A3     | ENSDARG0000026218_slc1a3a           | 2.1         | 3.4         |
| slc1a3b| glutamate transporter GLAST/SLC1A3     | ENSDARG0000043148_slc1a3b           | 1.3         | 43.6        |
| sox4a  | SRY-box-related 4a transcriptional activator | ENSDARG0000004588_sox4a           | 2           | 5.4         |
| sox4b  | SRY-box-related 4b transcriptional activator | ENSDARG0000043235_sox4b           | 1.6         | 6.1         |
| sox11a | SRY-box-related 11a transcriptional activator | ENSDARG0000077811_sox11a           | 1.2         | 4.4         |
| sox11b | SRY-box-related 11b transcriptional activator | ENSDARG0000095743_sox11b           | 2.3         | 18.5        |
| sox21b | SRY-box-related 21b transcriptional activator | ENSDARG0000008540_sox21b          | 13          | 469         |
| Müller glia*                                |                                        |                                       |             |
| apoeb  | apolipoprotein Eb                       | ENSDARG00000040295_apoeb             | 10.7        | 13.7        |
| fabp7a | brain lipid binding protein             | ENSDARG0000007697_fabp7a            | 2.2         | 17.7        |
| cdh2   | neuronal cadherin 2                     | ENSDARG0000018693_cdh2              | 1.2         | 3.4         |
| her9   | hairy/enhancer of split 1 transcriptional repressor | ENSDARG0000056438_her9      | 1.3         | 12.6        |
| gfap   | glial fibrillary acid protein           | ENSDARG00000025301_gfap             | 2.3         | 2.5         |
| ribp1a | retinaldehyde binding protein 1         | ENSDARG0000012504_ribp1a            | 1.9         | 1.3         |
| ribp1b | retinaldehyde binding protein 1         | ENSDARG0000045808_ribp1b            | 2.4         | 2.1         |
| Müller glia regeneration#                   |                                        |                                       |             |
| ascl1a | achaete-scute like 1a transcriptional activator | ENSDARG0000038386_ascl1a         | 4.6         | 13.8        |
| dkk1b  | dickkopf 1, wnt antagonist              | ENSDARG00000045219_dkk1b           | 0.9         | 0.2         |
| hbegfa | heparin binding epidermal-like growth factor | ENSDARG0000075121_hbegfa       | 24.4        | 48.8        |
| hbegfb | heparin binding epidermal-like growth factor | ENSDARG0000031246_hbegfb       | 2.4         | 9.4         |
| insm1a | insulinoma 1a transcriptional repressor | ENSDARG0000091756_insm1a           | 2.3         | 12.7        |
| stat3  | signal transduction and activator of transcription 3 | ENSDARG0000022712_stat3         | 1.9         | 2.9         |
| Glioma genes§                               |                                        |                                       |             |
| ajap1  | Adherens Junction Associated Protein 1  | ENSDARG0000038655_ajap1            | 1           | 0.2         |
also showed increased expression in the Tg(flk1:RFP)is18 tumors. Reprogramming of Müller glia is mediated by activation of multiple signal transduction pathways [4]. The JAK/Stat cytokine signal transduction pathway transcription factor stat3, which is required for proliferation after light dependent retinal injury and regeneration [9, 10], was elevated nearly 3-fold in the tumor (Table 3). Wnt-β-catenin signal transduction is activated in Müller glia derived progenitors via a network involving heparin binding epidermal growth factor hbegf, the transcriptional activator ascl1a, and the transcriptional repressor insm1a [7, 12]. Each of these genes was significantly increased in the Tg(flk1:RFP)is18 Tumor, while the wnt antagonist dkk1b was significantly decreased in expression (Table 3). We confirmed the differential gene expression of hbegfa, ascl1a, and insm1a by qRT-PCR (Fig. 5C). Together, these data are consistent with the hypothesis that Tg(flk1:RFP)is18 retinal tumors might arise from neural progenitors derived from Müller glia and/or astroglia.

**Discussion**

In this report we describe the isolation and characterization of a zebrafish optic pathway tumor line that is linked to integration of a Tol2<flk1:RFP-CAAX> transgene concatenemer in line Tg(flk1:RFP)is18. By 1 year, greater than 80% of heterozygous Tg(flk1:RFP)is18 adults develop tumors in the retina, optic nerve and optic tract with features of retinoblastoma and fibrous glioma. Histological, immunohistochemical, and transcriptomic analyses of Tg(flk1:RFP)is18 ocular tumors are consistent with the tumor originating in part from a glial cell population in the retina that includes the Müller glia. Astrocytes residing within the optic nerve fiber layer may also contribute to the retinal tumor cell progenitor population. The dominant pattern of inheritance indicates the presence of the transgene results in an oncogenic mechanism that induces tumor onset. The Tg(flk1:RFP)is18 transgene integration is homozygous lethal, and animals die between 1 and 4 weeks of age. The transgene disrupts expression of the lincRNA gene, lincRNAis18 (Zv9_00007276) but elevates expression of the opposite strand lincRNA sic:ch211-84g22.1 ~25 fold. A second deletion allele generated using
TALEN genome editing that removes exons 2–5 of lincRNAis18 and the entire lincRNA sichel21-84g22.1 locus did not result in a tumor or lethal phenotype. This suggests that in line Tg(flk1:RFP)is18 the presence of the Tol2<flk1:RFP-CAAX> array is responsible tumor formation and lethality, however, the mechanism underlying tumor onset is not known.

Our study provides several pieces of evidence that the Tg(flk1:RFP)is18 retinal tumors likely originate from glial-derived neural progenitors in the retina. The Tg(flk1:RFP)is18 phenotype is very similar to the previously reported zebrafish optic pathway glioma model in which activation of Sonic hedgehog signaling in neural progenitors induces tumors [14]. Radial glia in the CNS are the source of neural stem cells and glial progenitors during development and in adult neurogenesis [30]. The dramatic increase in expression of the radial glial marker GLAST/slc1a3b [27] and the neural stem cell markers foxd3, nestin, sox4, sox11, and sox21 in Tg(flk1:RFP)is18 tumor tissue supports a neuroglial progenitor population. The CREB transcriptional activator ATF3 and the reactive astrocyte marker Bystin are associated with reactive gliosis and upregulated in the optic nerve in response to injury [28,29]. While atf3 was dramatically increased in expression in the Tg(flk1:RFP)is18 retinal tumors, zebrafish bystin-like (bysl) showed no change in expression level. Consequently, the activation of glia in the Tg(flk1:RFP)is18 model is not merely a result of reactive gliosis due to insult or injury in the retina. ATF3 is a member of the cAMP-response element binding protein family of transcriptional activators that bind the CRE (cAMP response element) enhancer and are activated in response to a rise in cAMP levels. In fetal brain synergism between the cytokine LIF and BMP signal transduction pathways is mediated by CREB, which acts as a bridge between STAT3 and smad1 to promote astrocyte formation from neural progenitors [31]. Consistent with increase in CREB family member ATF3, both zebrafish smad1 and stat3 expression levels were elevated in Tg(flk1:RFP)is18 tumors, indicating a role for cAMP signal transduction in tumor growth.

Activation of multiple signal transduction pathways required for inducing Müller glia proliferation and progenitor production was observed in the Tg(flk1:RFP)is18 ocular tumors. Müller glia markers, such as apolipoprotein Eb, apoeb, brain lipid binding protein, blbp, and hairy/enhancer of split 1 transcriptional repressor, hes1 [32], were significantly increased in the tumors. The JAK/Stat signal transduction and transcriptional activator stat3, which is required to stimulate Müller glia proliferation in the injured zebrafish retina [9,10], was elevated as well in tumor tissue. The insm1a, ascl1a/dkk1b, and hbegf pathways that active wnt signaling to induce Müller glia proliferation [7,11,12] were also significantly altered. Together these data support the hypothesis that the Müller glia in the Tg(flk1:RFP)is18 retinas dedifferentiate and produce transformed neuroglial progenitors. Retinal tumor progenitors might also arise from astrocytes located in the nerve fiber layer. Overall, the transcriptome data, histological analyses, and immunohistochemical labeling of tumor tissue provides significant support for the conclusion that transformed glia give rise to the Tg(flk1:RFP)is18 tumors. Identifying the mechanism that initiates glial progenitor
proliferation in the Tg(flk1:RFP)is18 retina will require additional studies. The zebrafish Tg(flk1:RFP)is18 line presents a highly penetrant and consistent retinal tumor model that will be useful for investigating the mechanisms driving glia activation and reprogramming in the vertebrate central nervous system.

**Materials and Methods**

**Zebrafish husbandry and genetics**

Zebrafish were reared in an Aquatic Habitat system (Aquatic Ecosystems, Inc., Apopka, FL). Fish were maintained on a 14-hr light/dark cycle at 27°C. Transgenic lines were established in a WIK wild type strain obtained from the Zebrafish International Research Center (http://zebrafish.org/zirc/home/guide.php). For in situ hybridization experiments, embryos were collected and maintained at 28.5°C in fish water (60.5 mg ocean salts/l) containing 0.003% 1-phenyl-2-thiourea (PTU) until harvesting. Embryos were staged according to published guidelines [33]. All experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee (Log # 11-06-6252-I) and are in compliance with American Veterinary Medical Association and the National Institutes of Health guidelines for the humane use of laboratory animals in research. Adult fish were anesthetized and euthanized in MS-222 Tricaine Methanesulfonate prior to sacrifice and tissue dissection for histopathology and immunolabeling.

**Animal Care and Humane Endpoint Establishment**

Transgenic fish predisposed to tumor formation were raised side by side with non-transgenic siblings. Heterozygous and homozygous transgenic fish and sibling fish were monitored daily during routine feeding for viability and morbidity, and monitored bi-weekly for gross presentation of ocular tumors. 50% of each generation of transgenic fish developed pericardial edema. All homozygous transgenic fish presented with pericardial edema beginning at 2–7 dpf and developing through 4 weeks of age. Fish presenting with edema were sacrificed before swimming and feeding behavior were adversely affected. Juvenile and adult fish were anesthetized and euthanized in MS-222 Tricaine Methanesulfonate according to experimental protocols approved by the Iowa State University Institutional Animal Care and Use Committee (Log # 11-06-6252-I) in compliance with the American Veterinary Medical Association and the National Institutes of Health guidelines for the humane use of laboratory animals in research.

NIH/Office of Animal Care and Use/Animal Research Advisory committee (ARAC) Guidelines for endpoint in neoplasia studies (oacu.od.nih.gov/ARAC/Guidelines for Endpoints in Animal Study Proposals) were used to establish a humane endpoint in the zebrafish heterozygous Tg(flk1:RFP)is18 glioma tumor model. Adult fish were monitored bi-weekly for general appearance and assessed
for size and length relative to non-transgenic siblings. Fish were sacrificed before tumor burden reached 3 mm in size/25 mg in weight, constituting less than 10% of the total body weight of an adult fish (300–500 mg), as outlined for mouse and rat studies [34]. No adverse affect on growth rate, feeding behavior or fertility was detected in Tg(flk1:RFP)is18 fish with a tumor burden less than 3 mm/10% of body weight. For transcriptome studies age matched 6 month old fish with tumor size ranging from undetectable to 2 mm in size were anesthetized and euthanized in MS-222 Tricaine Methanesulfonate. Retinal and tumor tissue was dissected for isolation of total RNA. In each generation of Tg(flk1:RFP)is18 fish, individuals presenting with ocular tumors were sacrificed when tumor burden reached 3 mm/25 mg of total body weight, or by 1 year of age, whichever endpoint was first reached.

Isolation of transgenic line Tg(flk1:RFP)is18

The endothelial specific membrane targeted RFP reporter construct flk1:RFP-CAAX was assembled using standard PCR cloning methods. The zebrafish promoter for the flk1 gene was amplified from WIK genomic DNA. The construct was cloned into the miniptol2 transgenesis vector [35]. Transgenics were isolated by co-injection of in vitro transcribed, capped, polyadenylated Tol2 transposase mRNA [35] and the pTol2<flk1:RFP-CAAX> construct into 1 cell zebrafish WIK embryos, as described previously [36]. Three independent lines expressing RFP in the endothelial cells of the vasculature were isolated. Line Tg(flk1:RFP)is18 contains a high copy number array Tol2<flk1:RFP-CAAX>. The Tg(flk1:RFP)is18 line is available upon request.

Tol2<flk1:RFP>is18 transgene integration site cloning

A custom RNA bait library (Agilent) was used for capture of the Tol2<flk1:RFP> transgene concatemer and flanking genomic DNA sequences following the Agilent SureSelect Targeted Capture protocol. Briefly, a panel of overlapping biotin-labeled RNA baits (S1 Table) complementary to the pTol2<flk1:RFP> construct was synthetized. Genomic DNA from 5 samples was subjected to shearing, hybridization capture, library amplification, and index barcoding as outlined in the Custom SureSelect Target Enrichment protocol (Agilent). Genomic DNA was isolated from muscle tissue from one Tol2<flk1:RFP>is18/+ heterozygous adult from the F2, F3 and F4 generation of the is18 pedigree was isolated. Genomic DNA was also isolated from the tumor tissue from the F3 and F4 individuals. The genomic DNA was isolated with the Agilent SureSelect gDNA Extraction kit (Agilent). 3 µg of genomic DNA from each of the 5 samples was sheared to ~250 bp (Covaris, Inc., Woburn, MA). The SureSelect Captured Libraries were 75 bp paired end multiplex sequenced in one lane on an Illumina GA II instrument at the Iowa State University DNA Facility. The number of reads per sample was: Sample 1, 1519937; Sample 2, 4790568; Sample 3, 6396525; Sample 4, 10262541; Sample 5, 23644059. Each pair of PE fastq files was aligned using
GSNAP [37] to the *Danio rerio* reference genome (Zv9 64) amended with the *pTol2<flk1:RFP>* construct sequence as a separate scaffold. Reads were filtered to remove those that failed to map to both the transgene and a unique location in the zebrafish genome. The remaining reads were used to identify 1000 bp intervals in the genome that had a higher level of mapped reads than expected by random chance using a modification of a previously published bootstrap method written in the R programming language [38]. 4 locations were identified in which paired end sequences had one end mapped to the *pTol2<flk1:RFP>* construct and the other end mapped to a unique, non-repetitive sequence in the genome (S3 Table). Each site was tested for confirmation by PCR amplification of the predicted transgene-chromosome junction fragment with primers complementary to the *pTol2<flk1:RFP>* construct and the flanking genomic DNA (S4 Table). Only one of the 4 sites, chromosome 3:24,212,813–24,212,885, was validated by amplification in all 5 samples (S1 B, C Figure). A total of 81 sequences in the 5 samples mapped to the transgene and to genomic DNA flanking either the 5’ or 3’ sides of the integration site at 24,212,944 on chromosome 3 (S1 D Figure).

**Genomic Southern blot analyses**

Genomic DNA was isolated from adult zebrafish using a Qiagen midiprep kit and chemiluminescent non-radioactive Southern blot analyses performed as described previously [39]. Sequences of primers for probes for genomic Southern analyses: 450 bp probe complementary to chr 3 position 24,214,849–24,215,303; forward 5’ CTCATTCTGTCCATGTGTTCACAG 3’, reverse 5’ CTTCTTGCTTACATGAGGAGCCTAGCC 3’; 450 bp probe complementary to chr 3 positions 24,211,859–24,212,364; forward 5’ CTGACAAGCAGCTGACAGATTGG 3’, reverse 5’ GGAAGTTGCTCTCATAATTCACG 3’; 450 bp probe complementary to RFP cDNA; forward 5’ ATCAGTGAGGCACCTATCTCAGC 3’, reverse 5’ CATGGAGGGCACCGTGAACAA 3’; 477 bp probe complementary to βlactamase cDNA in pTol2 vector backbone; forward 5’ ATCAGTGAGGCACCTATCTCAGC 3’, reverse 5’ CATAACCAGTGATAACACTGC 3’.

**Imaging, histopathology and immunohistochemistry**

Living 3 dpf embryos were anesthetized in Tricaine, mounted in 1% low melt agarose, and imaged on a Zeiss LSM700 Confocal microscope. Whole heads dissected from adult zebrafish were fixed in 10% Formalin (Fisher) or Davidson’s fixative (2:3:1:3 Formalin:Ethanol:Glacial Acetic Acid:Water) for 16 hr at 4°C, decalcified in Cal-Ex (Fisher) for 2 days at 4°C, then processed and embedded in paraffin at the Iowa State University Clinical Histopathology Laboratory. Paraffin blocks of head tissue were serial sectioned at 6um on a Shandon Finesse 325 microtome, stained with Hematoxylin 7211 Richard-Allan Scientific (Fisher) and 3% Eosin Y (Argos Organics), and mounted with Permount (Fisher). Sections were imaged on a Zeiss Axioskop II using Nikon Coolpix and Nikon Rebel cameras.
For immunohistochemistry heads were removed from anesthetized adults and fixed in 4% paraformaldehyde overnight at 4°C, decalcified in Cal-Ex for 2 days at 4°C, then processed for embedding in optimal cutting temperature (OCT) medium (Fisher). Heads were serial sectioned at 12 µm on a Microm HM 550 cryotstat at 25°C. For BrdU labeling experiments, adults were incubated in 5 µM BrdU (Sigma) in fish water (60.5 mg ocean salts/l) for 2 hours, placed in fresh fish water for 4 hours, then sacrificed and processed as above for immunohistochemical labeling experiments. Antibody labeling of cryosectioned tissue was as described previously [9, 40]. Dilutions and primary antibodies used for labeling sectioned tissue were as follows: rabbit polyclonal anti-recoverin 1:1000 (Millipore); mouse monoclonal anti-glial fibrillary acid protein GFAP 1:1000 obtained from the Zebrafish International Research Center (ZIRC); rabbit polyclonal anti-Brain Lipid Binding Protein BLBP 1:200 (Abcam); mouse monoclonal anti-SV2 1:100 (Developmental Studies Hybridoma Bank); mouse monoclonal anti-RT97 1:250 (Developmental Studies Hybridoma Bank); anti-BrdU 1:500 (Bio-Rad). Alexa-594 and Alexa-697 conjugated secondary antibodies (Invitrogen) and Cy3 and Cy5 conjugated secondary antibodies (Jackson Immunoresearch Labs) were used at a dilution of 1:500. Tissues were counterstained with 5 µg/ml DAPI (Sigma) and mounted in Fluorogel (EMS). To aid antigen retrieval tissues were pretreated with 2 M HCl (for anti-BrdU labeling). Immunofluorescent labeling of cryosections was imaged on a Nikon Microphot-FXA microscope and captured using a QImaging Retiga 2000R Fast 1394 camera and QCapture software. All images were edited and assembled in Adobe Photoshop CS2.

RT-PCR and in situ hybridization

Total RNA for staged developmental series, tissue panel, and analysis of homozygous embryos, was isolated with a Qiagen RNeasy Isolation Kit. RT-PCR was carried out using a One-Step RT-PCR Kit (Invitrogen). cDNA for lincRNAis18 was amplified by RT-PCR out of total RNA isolated from wild type 48 hpf embryos or adult retina. cDNAs were cloned into pBluescript or the pCR4-TOPO vector (Invitrogen). Primers for amplification were as follows: lincRNAis18 forward1 5’TCACTGTCTGCTGAGATC 3’; lincRNAis18 nested forward 5’ GACAGACTCTGGCACAATCTCTG 3’; lincRNAis18 forward2 5’CAACAGTTCCTGAACACGC 3’; lincRNAis18 reverse 5’GACAGACTCTGGCACAATCTCTG 3’; lincRNAis18 nested reverse 5’TGACATACATCAAATACTCCAGC 3’; cbx1a forward 5’TGGTGACATGAGCCAACC 3’; cbx1a reverse 5’CCTGAGACGAGCTTGCATCCTTC 3’.

Primers complementary to the ribosomal protein S6 kinase b, rps6kb1 gene were used as control for RT-PCR reactions. forward rps6kb1 forward 5’CATGGGCAGCTGCTGTTTCAT 3’; rps6kb1 reverse 5’AGCTTGGCCGCGGCT-CTGAAA 3’. Digoxygenin-labeled antisense and sense probes for in situ hybridization were synthesized using the T3 Dig labeled RNA synthesis kit (Roche). In situ hybridization on 12–16 µm cryosections of head and eye tissue
was performed as described [41]. Probes were purified over Biorad Biospin or Qiagen RNeasy MinElute Cleanup Kit columns following the manufacturers instructions and stored in 50% formamide at −20°C.

TALEN directed isolation of lincRNAis18 deletion allele

TALEN pairs targeting sequences in exon 2 and exon 5 of lincRNAis18 were designed using TAL Effector-Nucleotide Targeter 2.0 [42] and synthesized using the modified GoldyTALEN scaffold [43]. 1-cell stage WIK embryos were co-injected with 30 or 60 pg in vitro transcribed TALEN mRNA targeting sequences in exons two and exons five of lincRNAis18. Individual injected embryos were assayed for mutation efficiency by disruption of restriction enzyme sites in a amplicon spanning the targeted region. Genomic DNA was extracted from embryos and adult fin clips by placing tissue in 50 μl 50 mM NaOH and heating at 95°C for 10 minutes. Primers to amplify lincRNAis18 exon two: exon2F 5’ GGTCATGTCCCTGTGTTTTG 3’; exon2R’ CTCCAGCTCCTGTGTATTGATTG 3’. Primers to amplify lincRNAis18 exon five: exon5F 5’ CACACAGTTTCATGTGGCTCT 3’; exon5R 5’ TGGATTACTCGTAACTGAGGAAAAC 3’. Founders were raised to adulthood and screened for germline transmission of the deletion allele. 20 individual embryos from 27 F0 adults were tested by PCR amplification across the exon two – exon five junction using primers exon2F 5’ GGTCATGTCCCTGTGTTTTG 3’ and exon5R 5’ TGGATTACTCGTAACTGAGGAAAAC 3’.

RNA-Seq, real time PCR and differential gene expression analyses

RNA-Seq data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2886. Tissues for isolation of total RNA for RNA-Seq libraries were dissected from three age-matched genotypes. Total RNA was isolated using Qiagen RNeasy RNA Isolation Kit (Qiagen). The first sample, “Wild Type”, contained three pooled retinas from 6-month-old wild type adults. The second sample, “Pretumor”, consisted of three pooled retinas from age-matched heterozygous Tg(flk1:RFP)is18/+ adults that did not show obvious gross ocular tumors. The third sample, “Tumor”, consisted of tumor tissue dissected from the eyes of two age-matched heterozygous Tg(flk1:RFP)is18/+ adults that had advanced, large tumors that filled the vitreous and distorted the ocular cavity. RNA-Seq libraries from each sample were 100 bp paired-end sequenced in individual lanes on an Illumina HiSeq 2000 instrument at the Genome Sequencing and Analysis Core Resource, Duke Institute for Genome Sciences and Policy, Duke University. Mapping of the RNA-Seq data was performed at the Genome Informatics Facility, Iowa State University. To map the RNA-Seq data the Danio rerio reference genome v9 and gene models (release 71) were downloaded from ensemble. Sequences were mapped using GSNAP version 2012-07-20 [37]. Raw read counts per gene were determined using the program
HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/) [44] to identify unique reads that mapped within a gene model. Upper quartile normalization was applied to the raw reads across the three samples. The Fisher’s exact test was performed to determine differential expression of a gene between samples. Q-value estimation for false discovery rate was performed in R using open source software qvalue at bioconductor (http://bioconductor.org/biocLite.R; Alan Dabney, John D. Storey and with assistance from Gregory R. Warnes. qvalue: Q-value estimation for false discovery rate control. R package version 1.32.0). Heat maps representing log2(fold change) in gene expression were created in Excel. GO Term analysis was done at the Gene Ontology (GO) Tools website http://go.princeton.edu.

Two-Step real time PCR was performed on a Roche LightCycler 480 instrument using SYBR green reaction mix (Fisher). RNA isolation from Wild Type, Pre-tumor, and Tumor tissues was as described above. cDNA template was synthesized with SuperScript II (Invitrogen). qRT-PCR reactions were run in triplicate for each tissue template and primer pair. Primers for qRT-PCR are listed in S4 Table.

Supporting Information

S1 Figure. Molecular mapping of the Tol2<flk1:RFP> concatemer transgene in zebrafish line Tg(flk1:RFP)is18 to chromosome 3. (A) Schematic of Agilent Sure Select Target Enrichment mapping technique. Tg(flk1:RFP)is18 and flanking genomic sequences were captured with complementary biotin-RNA probes followed by Illumina GAIIx sequencing of barcoded libraries and mapping to the zebrafish genome (B) Snapshot of alignment to chromosome 3 in the zebrafish genome of genomic DNA-transgene junction fragments captured with a custom SureSelect Target Enrichment kit. (C) Diagram illustrating integration site of the Tol2<flk1:RFP> concatemer at position 24, 212, 944 on chromosome 3. The sequence flanking the transgene, containing an 8 bp duplication at the integration site, is shown below. Primers used for PCR amplification of the junction fragments at the integration site are shown. Chr3F and chr3R, position of primers on chromosome 3. Tol2-5’R and Tol2-3’F sit within the left and right inverted repeats of the Tol2<flk1:RFP> transposon. (D) PCR products verify the location of the transgene integration in the 5 genomic samples used for SureSelect Target Enrichment. *, amplification of the 350 bp 5’ genomic-transgene junction fragment. **, amplification of the 200 bp 3’ genomic-transgene junction fragment.
doi:10.1371/journal.pone.0114888.S001 (TIF)

S2 Figure. RFLP caused by integration of Tol2<flk1:RFP> concatemer at position 24, 212, 944 on chromosome 3. (A) Diagram of Tol2<flk1:RFP> transposon construct with position of probe complementary to RFP cDNA (red box). (B) BamHI/BglII restriction map of region surrounding Tol2<flk1:RFP> concatemer integration on chromosome 3. Blue box shows position of probe

Genomic Southern blots of BamH1/BglII double digested genomic DNA from wild type WIK, 6th generation Tg(ﬂk1:RFP)is18, and 6th generation non-transgenic +/+ siblings. BamHI cuts once within the Tol2<ﬂk1:RFP> transposon, releasing each copy from the concatemer. Left panel shows chromosome 3 RFLP due to transgene integration (blue asterisk) present only in Tg(ﬂk1:RFP)is18 transgenic fish. Right panel shows an intense band at the expected size for the Tol2<ﬂk1:RFP> transposon construct and many other bands of varying sizes. This confirms the identity of the Tg(ﬂk1:RFP)is18 transgenic fish and reveals the complex nature and disorganization of the transgenes in the high copy number array.

doi:10.1371/journal.pone.0114888.S002 (TIF)

S3 Figure. RFLP caused by integration of Tol2<ﬂk1:RFP> concatemer at position 24, 212, 944 on chromosome 3. (A) Diagram of Tol2<ﬂk1:RFP> transposon construct with position of probe complementary to β-lactamase cDNA (orange box) in the vector backbone. (B) BstEII restriction map of region surrounding Tol2<ﬂk1:RFP> concatemer integration on chromosome 3. BstEIII does not cut in the Tol2<ﬂk1:RFP> concatemer. Blue box shows position of probe complementary to region on chromosome 3 just 5’ to integration site. (C) Genomic Southern blots of BstEII digested genomic DNA from wild type WIK, 5th generation Tg(ﬂk1:RFP)is18, 6th generation Tg(ﬂk1:RFP)is18, and 6th generation non-transgenic +/+ siblings. Left panel shows blot of linear digested plasmids of known size for comparison. Panel second from left shows high molecular weight band (blue asterisk) corresponding to chromosome 3 RFLP caused by concatemer integration. Right panels show blots hybridized with a probe specific to the transgene construct in the concatemer. The intense band (orange asterisk) corresponds to the concatemer integrated in chromosome 3. The band runs at the same position as the band recognized by the chromosome 3 probe. Far right panel represents longer exposure of blot shown in second panel from right.

doi:10.1371/journal.pone.0114888.S003 (TIF)

S4 Figure. STR marker linkage analysis of the Tol2<ﬂk1:RFP> concatemer in line Tg(ﬂk1:RFP)is18. Upper panel. Genomic position and name of Short Tandem Repeat markers in the region of the transgene integration site on chromosome 3. Representative images of marker PCR products show genotype of an F5 generation Tg(ﬂk1:RFP)is18 and a wild type WIK fish used for linkage analysis. Lower panel. Analysis in 20 offspring from a cross between the genotyped Tg(ﬂk1:RFP)is18 and wild type WIK adults shows linkage of the chromosome to the long allele of Z7419, the short allele of G3927, the long allele of Z5197, and the long allele of cbx1aSTS. Further analyses of 200 progeny (Table 1) confirmed tight linkage of the concatemer integration site to the Z7419, G3927, Z5197 and cbx1aSTS markers.

doi:10.1371/journal.pone.0114888.S004 (TIF)
S5 Figure. Examination of zebrafish lincRNAis18 expression in early development and adult tissues. (A) Nested RT-PCR showing expression of lincRNAis18 within the adult zebrafish retina. (B) In situ hybridization using non-radioactive DIG-labeled lincRNAis18 probes on adult zebrafish retina cryosections. lincRNAis18 expression is detected in the ganglion cell layer (GCL) and a subset of cells at the vitreal side of the inner nuclear layer (INL) (left, middle). Negative control, lincRNAis18 sense DIG-labeled probe (right). (C, D) RT-PCR showing relative levels of lincRNAis18 expression throughout development and in adult tissues. Control, expression of ribosomal protein S6 kinase b, polypeptide 1, rps6kb1. Blue bracket and asterisks indicate lincRNAis18 cloned and sequence confirmed products. Red bracket and asterisks indicate nonspecific products cloned and confirmed by sequencing. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. Scale bars 100 µm.
doi:10.1371/journal.pone.0114888.S005 (TIF)

S6 Figure. Genotyping of progeny from Tg(flk1:RFP)is18 incross. (A–C) Five individual larvae from each progeny class were genotype confirmed by PCR. Primer pair 1, chr3F and chr3R, amplify a fragment of the wild type chromosome 3 spanning the concatemer integration site. Primer pair 2, chr3F and Tol2R, amplify a genomic DNA-transgene junction fragment. Primer pair 3, control primers for amplification of a fragment of the flh gene. (A) Wild type +/+ sibling larvae. As expected primer pair 1 amplifies the wild type fragment of chromosome 3, while the concatemer genomic junction fragment that would be amplified by primer pair 2 is absent. (B) Homozygous mutant Tg(flk1:RFP)is18/ Tg(flk1:RFP)is18 larvae. As expected, the wild type fragment of chromosome 3 is absent, while the concatemer genomic DNA junction fragment is present. (C) Heterozygous Tg(flk1:RFP)is18/+ genotyped larvae. Both the wild type chromosome 3 fragment and the concatemer genomic DNA junction fragment amplify.
doi:10.1371/journal.pone.0114888.S006 (TIF)

S7 Figure. TALEN directed mutagenesis of lincRNAis18 exons two and five. (A) TALEN pairs targeting exon 2 and exon 5 of lincRNAis18. TALEN spacers are shown in red./marks location of FOK1 endonuclease cut site. HincII restriction enzyme site (exon 2) and MseI restriction enzyme site (exon 5) are underlined. (B) Individual embryos injected with TALENs targeting lincRNAis18 exon 2 (left panel) or exon 5 (right panel). The presence of HincII and MseI digestion resistant amplicons demonstrates mutation of the targeted site.
doi:10.1371/journal.pone.0114888.S007 (TIF)

S8 Figure. The lincRNAis18 e2e5del deletion allele is homozygous viable. (A) Diagram of lincRNAis18 gene structure and exon 2- exon 5 deletion allele. Primers e2F and e2R flank exon 2; primers e5F and e5R amplify exon 5. The genetic marker G38247 is located between exons 2 and 3. The genetic marker Z7419 is located 1 Mb downstream of the 3′ end of lincRNAis18. (B) Genotyping of fin clips from 10 adult progeny of a lincRNAis18 e2e5del/+ incross. Genomic DNA was amplified with primer pairs listed. The exon 2- exon 5 fusion amplicon was
detected in 4/10 adults, indicating they were homozygous for the lincRNAis18e2e5del chromosome (top panel). As expected these 4 adults lacked the G39247 marker (4th panel).
doi:10.1371/journal.pone.0114888.S008 (TIF)

**S9 Figure. Loss of lincRNAis18 mRNA at polyA selection step during RNA-Seq library preparation.** RT-PCR shows presence of cbx1a and lincRNAis18 in the total RNA from wild type retina sample used for RNA-Seq cDNA library preparation. The cbx1a transcript was detected following a single round of RT-PCR amplification in the total RNA sample (T) and in the Illumina RNA-Seq cDNA library sample (L). The lincRNAis18.v1 and.v2 transcripts were present in the total RNA sample (blue asterisks). However, only nonspecific products amplified from the cDNA library sample after one round of RT-PCR (red asterisks). Nested PCR resulted in amplification of multiple alternatively spliced transcripts from both samples (Right panel, blue asterisks). Bands marked with asterisks were cloned and sequence verified.
doi:10.1371/journal.pone.0114888.S009 (TIF)

**S1 Table.** SureSelect Target Enrichment Antisense 120nt Bait Library tiled across pTol2<flk1:RFP> plasmid vector.
doi:10.1371/journal.pone.0114888.S010 (XLS)

**S2 Table.** Illumina Paired End Reads from Custom SureSelect Target Enrichment of Tol2<flk1:RFP>is18 transgene integration site.
doi:10.1371/journal.pone.0114888.S011 (XLSX)

**S3 Table.** Candidate Tol2<flk1:RFP>is18 transgene integration locations.
doi:10.1371/journal.pone.0114888.S012 (XLSX)

**S4 Table.** Primers for genotyping and qRT-PCR.
doi:10.1371/journal.pone.0114888.S013 (XLSX)

**S5 Table.** Alternatively spliced isoforms of lincRNAis18 RT-PCR products.
doi:10.1371/journal.pone.0114888.S014 (XLSX)

**S6 Table.** Illumina Hi-Seq RNA-Seq data from zebrafish wild type retina, Tg(flk1:RFP)is18/+ pretumor retina and Tg(flk1:RFP)is18/+ tumor tissue.
doi:10.1371/journal.pone.0114888.S015 (XLSX)

**S7 Table.** All mapped reads from RNA-Seq of wild type, Tg(flk1:RFP)is18/+ pretumor and Tg(flk1:RFP)is18/+ tumor retinal tissue.
doi:10.1371/journal.pone.0114888.S016 (XLSX)

**S8 Table.** Differential expression of genes required for photoreception and neurotransmission in Tg(flk1:RFP)is18/+ pre-tumor retina and tumor tissue.
doi:10.1371/journal.pone.0114888.S017 (XLSX)

**S9 Table.** Differential Expression of lncRNAs in Tg(flk1:RFP)is18/+ pre-tumor retina and tumor tissues.
doi:10.1371/journal.pone.0114888.S018 (XLSX)
Acknowledgments
The authors thank Kristin Watt, Jennifer Groeltz-Thrush and Danhua Zhang for technical assistance, and the IGSP sequencing core at Duke University for construction and Illumina sequencing of RNA-Seq libraries. The RT97 and SV2 monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242. The authors thank Drs. Drena Dobbs, Phil Becraft, and Ann Morris for discussion of the work.

Author Contributions
Conceived and designed the experiments: MM SLS YW JM LES DSS JJE. Performed the experiments: MM SLS YW JM LES DEL PAB CAJ JS-N JML. Analyzed the data: MM SLS YW DSS JJE JMH EMW JDC HHC AJS DSS. Contributed reagents/materials/analysis tools: MM HHC AJS. Wrote the paper: MM SLS YW JJE.

References
1. Gallo V, Deneen B (2014) Glial Development: The Crossroads of Regeneration and Repair in the CNS. Neuron 83: 283–308.
2. Gallina D, Todd L, Fischer AJ (2014) A comparative analysis of Muller glia-mediated regeneration in the vertebrate retina. Exp Eye Res 123: 121–130.
3. Stenkamp DL (2011) The rod photoreceptor lineage of teleost fish. Prog Retin Eye Res 30: 395–404.
4. Lenkowski JR, Raymond PA (2014) Muller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. Prog Retin Eye Res 40C: 94–123.
5. Goldman D (2014) Muller glial cell reprogramming and retina regeneration. Nat Rev Neurosci 15: 431–442.
6. Gorsuch RA, Hyde DR (2014) Regulation of Muller glial dependent neuronal regeneration in the damaged adult zebrafish retina. Exp Eye Res 123: 131–140.
7. Wan J, Ramachandran R, Goldman D (2012) HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell 22: 334–347.
8. Sun L, Li P, Carr AL, Gorsuch R, Yarka C, et al. (2014) Transcription of the SCL/TAL1 interrupting locus (Stil) is required for cell proliferation in adult zebrafish retinas. J Biol Chem.
9. Nelson CM, Gorsuch RA, Bailey TJ, Ackerman KM, Kassen SC, et al. (2012) Stat3 defines three populations of Muller glia and is required for initiating maximal muller glia proliferation in the regenerating zebrafish retina. J Comp Neurol 520: 4294–4311.
10. Kassen SC, Thummel R, Campochiaro LA, Harding MJ, Bennett NA, et al. (2009) CNTF induces photoreceptor neuroprotection and Muller glial cell proliferation through two different signaling pathways in the adult zebrafish retina. Exp Eye Res 88: 1051–1064.
11. Ramachandran R, Zhao XF, Goldman D (2011) Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proc Natl Acad Sci U S A 108: 15868–15863.
12. Ramachandran R, Zhao XF, Goldman D (2012) Insm1a-mediated gene repression is essential for the formation and differentiation of Muller glia-derived progenitors in the injured retina. Nat Cell Biol 14: 1013–1023.

13. Pauli A, Valen E, Lin MF, Garber M, Vastenhouw NL, et al. (2012) Systematic identification of long noncoding RNAs expressed during zebrafish embryogenesis. Genome Res 22: 577–591.

14. Ju B, Chen W, Spitsbergen JM, Lu J, Vogel P, et al. (2014) Activation of Sonic hedgehog signaling in neural progenitor cells promotes glioma development in the zebrafish optic pathway. Oncogenesis 3: e96.

15. Chew GL, Pauli A, Rinn JL, Regev A, Schier AF, et al. (2013) Ribosome profiling reveals resemblance between long non-coding RNAs and 5’ leaders of coding RNAs. Development 140: 2828–2834.

16. Gorsuch RA, Hyde DR (2013) Regulation of Muller glial dependent neuronal regeneration in the damaged adult zebrafish retina. Exp Eye Res.

17. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., et al. (2013) Cancer genome landscapes. Science 339: 1546–1558.

18. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, et al. (1998) Identification of c-MYC as a target of the APC pathway. Science 281: 1509–1512.

19. Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398: 422–426.

20. Banerjee S, Crouse NR, Emnett RJ, Gianino SM, Gutmann DH (2011) Neurofibromatosis-1 regulates mTOR-mediated astrocyte growth and glioma formation in a TSC/Rheb-independent manner. Proc Natl Acad Sci U S A 108: 15996–16001.

21. Kaul A, Chen YH, Emnett RJ, Dahiya S, Gutmann DH (2012) Pediatric glioma-associated KIAA1549:BRAF expression regulates neuroglial cell growth in a cell type-specific and mTOR-dependent manner. Genes Dev 26: 2561–2566.

22. Brennan CW, Verhaak RG, McKenna A, Campos B, Noughmehr H, et al. (2013) The somatic genomic landscape of glioblastoma. Cell 155: 462–477.

23. Rodriguez EF, Scheithauer BW, Giannini C, Rynearson A, Cen L, et al. (2011) PI3K/AKT pathway alterations are associated with clinically aggressive and histologically anaplastic subsets of pilocytic astrocytoma. Acta Neuropathol 121: 407–420.

24. Rodriguez FJ, Lim KS, Bowers D, Eberhart CG (2013) Pathological and molecular advances in pediatric low-grade astrocytoma. Annu Rev Pathol 8: 361–379.

25. Chen YH, Gutmann DH (2013) The molecular and cell biology of pediatric low-grade gliomas. Oncogene.

26. Lin N, Di C, Bortoff K, Fu J, Truszkowski P, et al. (2012) Deletion or epigenetic silencing of AJAP1 on 1p36 in glioblastoma. Mol Cancer Res 10: 208–217.

27. Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, et al. (1997) Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 17: 9212–9219.

28. Saul KE, Koke JR, Garcia DM (2010) Activating transcription factor 3 (ATF3) expression in the neural retina and optic nerve of zebrafish during optic nerve regeneration. Comp Biochem Physiol A Mol Integr Physiol 155: 172–182.

29. Neve LD, Savage AA, Koke JR, Garcia DM (2012) Activating transcription factor 3 and reactive astrocytes following optic nerve injury in zebrafish. Comp Biochem Physiol C Toxicol Pharmacol 155: 213–218.

30. Rowitch DH, Kriegstein AR (2010) Developmental genetics of vertebrate glial-cell specification. Nature 468: 214–222.

31. Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, et al. (1999) Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science 284: 479–482.

32. Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ (2006) Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC Dev Biol 6: 36.
33. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310.

34. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, et al. (2010) Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102: 1555–1577.

35. Balciunas D, Wangensteen KJ, Wilber A, Bell J, Geurts A, et al. (2006) Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. PLoS Genet 2: e169.

36. Liao HK, Wang Y, Noack Watt KE, Wen Q, Breitbach J, et al. (2012) Tol2 gene trap integrations in the zebrafish amyloid precursor protein genes appa and aplp2 reveal accumulation of secreted APP at the embryonic veins. Dev Dyn 241: 415–425.

37. Wu TD, Nacu S (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics 26: 873–881.

38. Severin AJ, Peiffer GA, Xu WW, Hyten DL, Bucciarelli B, et al. (2010) An integrative approach to genomic introgression mapping. Plant Physiol 154: 3–12.

39. McGrail M, Hatler JM, Kuang X, Liao HK, Nannapaneni K, et al. (2011) Somatic mutagenesis with a Sleeping Beauty transposon system leads to solid tumor formation in zebrafish. PLoS One 6: e18826.

40. Blong CC, Jeon CJ, Yeo JY, Ye EA, Oh J, et al. (2010) Differentiation and behavior of human neural progenitors on micropatterned substrates and in the developing retina. J Neurosci Res 88: 1445–1456.

41. Trimarchi JM, Stadler MB, Roska B, Billings N, Sun B, et al. (2007) Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J Comp Neurol 502: 1047–1065.

42. Doyle EL, Booher NJ, Standage DS, Voytas DF, Brendel VP, et al. (2012) TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res 40: W117–122.

43. Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, et al. (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491: 114–118.

44. Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11: R106.