**Article**

**let-7 MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration**

**Graphical Abstract**

**Authors**
Simran Kaur, Shivangi Gupta, Mansi Chaudhary, Mohammad Anwar Khursheed, Soumitra Mitra, Akshai Janardhana Kurup, Rajesh Ramachandran

**Correspondence**
rajeshra@iisermohali.ac.in

**In Brief**
Kaur et al. demonstrate that microRNA let-7 in injured zebrafish retina regulates the translation of shha, shhb, smo, ptch1, and zic2b mRNAs. Further, Shh signaling is necessary during retina regeneration for inducing a pro-regenerative gene expression cascade involving several genes, including ascl1a, lin28a, mmp9, foxn4, and zic2b.

**Data and Software Availability**
GSE102063

**Highlights**
- Shh signaling is essential for MG dedifferentiation during retina regeneration
- Shh signaling components are regulated by let-7 microRNA in the zebrafish retina
- A regulatory feedback loop between Mmp9 and Shh signaling is active in the retina
- Shh signaling induced a gene-regulatory network involving mmp9, ascl1a, zic2b, and foxn4
**let-7 MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration**

Simran Kaur, Shivangi Gupta, Mansi Chaudhary, Mohammad Anwar Khursheed, Soumitra Mitra, Akshai Janardhana Kunup, and Rajesh Ramachandran1,3,*

1Department of Biological Sciences, Indian Institute of Science Education and Research, Mohali, Knowledge City, SAS Nagar, Sector 81, Manauli PO, 140306 Mohali, Punjab, India
2These authors contributed equally
3Lead Contact
*Correspondence: rajeshra@iisermohali.ac.in
https://doi.org/10.1016/j.celrep.2018.04.002

**SUMMARY**

Upon injury, Müller glia cells of the zebrafish retina reprogram themselves to progenitor cells with stem cell characteristics. This necessity for retina regeneration is often compromised in mammals. We explored the significance of developmentally inevitable Sonic hedgehog signaling and found its necessity in MG reprogramming during retina regeneration. We report on stringent translational regulation of sonic hedgehog, smoothened, and patched1 by let-7 microRNA, which is regulated by Lin28a, in Müller glia (MG)-derived progenitor cells (MGPCs). We also show Shh-signaling-mediated induction of Ascl1 in mouse and zebrafish retina. Moreover, Shh-signaling-dependent regulation of matrix metalloproteinase9, in turn, regulates Shha levels and genes essential for retina regeneration, such as lin28a, zic2b, and foxn4. These observations were further confirmed through whole-retina RNA-sequencing (RNA-seq) analysis. This mechanistic gene expression network could lead to a better understanding of retina regeneration and, consequently, aid in designing strategies for therapeutic intervention in human retinal diseases.

**INTRODUCTION**

In contrast to mammals, zebrafish retina possesses remarkable regenerative capacity after an acute injury, leading to functional restoration of vision (Sherpa et al., 2008). The Müller glia (MG) cells in zebrafish retina reprogram themselves to MG-derived progenitor cells (MGPCs) that systematically differentiate into all retinal neurons, namely rods, cones, horizontal, amacrine, ganglion, bipolar cells, and MG itself (Ramachandran et al., 2010b). Although induction of MGPCs immensely contributes to the successful regeneration of zebrafish retina, the complete mechanism remains elusive. While the mechanism of retina regeneration is histologically well described, only a subset of the involved genes/proteins has been identified and characterized functionally (Goldman, 2014; Wan and Goldman, 2016). Therefore, we attempted to identify previously uncharacterized regulators of zebrafish retina regeneration using the needle-poke method of injury, which reflects the situation of mechanical damage that occurs in nature.

Even though several studies have elucidated the importance of Delta-Notch, Wnt, and Fgf signaling during retina regeneration in zebrafish, the roles of developmentally important Shh signaling remain largely underexplored (Goldman, 2014; Sun et al., 2014; Wan and Goldman, 2016). Recent studies have revealed the potential roles of Shh signaling during tissue regeneration (Ando et al., 2017; Dunaeva and Waltenberger, 2017; Thomas et al., 2018; Todd and Fischer, 2015). Therefore, we investigated the mechanistic involvement of Shh signaling during zebrafish retina regeneration. Subsequently, we hypothesized that MG dedifferentiation may depend on Shh signaling and have some similarities to the reprogramming of somatic cells by pluripotency-inducing factors (Hochedlinger and Plath, 2009; van den Hurk et al., 2016). Since we were interested in the possible involvement of Shh signaling during the early regenerative response of MG to injury, we analyzed the retina within the first few days after blockade of Shh signaling. We identified expression pattern of several important genes induced by Shh signaling and vice versa that reveal the robust regulatory network associated with retina regeneration. These include the interplay of Shh/Notch signaling components, transcription factors (namely, Ascl1a, Zic2b, Foxn4, and Insm1a), the matrix metalloproteinase Mmp9, the RNA-binding protein Lin28a, and microRNA let-7. Complete retina regeneration in zebrafish has provided valuable clues as to why their mammalian counterparts often fail (Goldman, 2014; Wan and Goldman, 2016). The findings from this study add clarity to the enigmatic process of retina regeneration lacking in mammals.

**RESULTS**

**Injury-Dependent Induction of Shh Signaling Is Essential for Regeneration**

We explored the temporal expression pattern of Shh signaling component genes such as sonic hedgehog (shha, shhb), smoothened ( smo), patched1 (ptch1), patched2 (ptch2), dispatched1 (disp1), dispatched2 (disp2), and glioma-associated
oncogene (gli1, gli2a, and gli3) in total retina. We found that most of these genes were upregulated after retinal injury, except gli3, which showed a downregulation (Figures 1A and 1B). Moreover, the Shh signaling components Shh, Ptch1, Smo, and Gli3 showed co-localization with bromodeoxyuridine (BrdU)+ MGPCs (Figures 1C, S1A, S1B, and S7A). Western blot analysis revealed a temporal upregulation of Shh protein with a peak of expression at 4 days post-injury (dpi) (Figures S1C and S7A). The Shh protein is expressed in MG cells of wild-type (WT) injured retina marked by glutamine synthetase (GS) at 4 dpi (Figure S1D).

Using tuba1a1016:GFP transgenic zebraphish (Faussett and Goldman, 2006), we showed the expression of Shh and its signaling components in proliferating MGPCs marked by GFP. Immunofluorescence (IF) studies and cell sorting revealed a relative abundance of Shh protein and its signaling components in GFP+ MGPCs compared with the rest of the cells of tuba1a1016: GFP transgenic retina at 4 dpi (Figures 1D and 1E). We confirmed the secretion of Shh and its probable autocrine action in MG using brefeldin A, a protein transport inhibitor, (Miller et al., 1992) and observed an increased expect in intracellular Shha and a decline in BrdU+ cells (Figures S1E and S1F).

To decipher the influence of Shh signaling on retina regeneration, we used the pharmacological agent cyclopamine (Incardona et al., 1998), a potent inhibitor of Smo (Chen et al., 2002). We found that at 30 μM concentration, 90% of zebraphish embryos exhibited cyclopia, a hallmark of impaired Shh signaling, which also impacted developmentally important genes (Figures 1F, 1G, and S1G). We then explored the impact of continuous cyclopamine exposure on MGPC induction and regeneration in WT and tuba1a1016:GFP transgenic retina at 4 dpi. Interestingly, 10 μM and 30 μM concentrations significantly inhibited MGPC induction (Figures 1H–1J, S1H, and S1I), which was not the result of enhanced apoptosis (Figure S1J). A similar reduction in fin blastema was also seen with cyclopamine treatment on the 6th day post-amputation (Figure 1K), suggesting a conserved Shh signaling mechanism across tissues during regeneration. The few residual BrdU+ MGPCs in cyclopamine-treated retina failed to form any retinal cell types (Figure S1K). Moreover, morpholino (MO)-based targeted gene knockdown of Shh signaling component genes such as shha, shhb, ptch1, ptch2, and gli2a caused progenitor reduction, and that of negative regulators sufu (suppressor of fused) (Figures 1L and 1M and S2A–S2C) and gli3 (Figures S1I, S6A, and S6B; Table S1) enhanced MGPC induction as compared with control retina at 4 dpi. These increased MGPCs when traced until 20 dpi revealed the formation of amacrine, bipolar, and MG cells, indicating their functional potential to give rise to different retinal cell types (Figures S2D and S2E). These results emphasize the importance of Shh signaling during retina regeneration.

We also performed whole-retina RNA sequencing (RNA-seq) at 12 hr post-injury (hpi), 4 dpi, and 4 dpi with cyclopamine treatment compared with uninjured controls to get a holistic view of the blockade of Shh signaling. We found that several transcription factor genes, including ascl1a, zic2b, foxn4, and matrix metalloproteinase mmp9, are regulated with cyclopamine treatment (Table S3; Figures S1L and S1M; GEO: GSE102603).

**Shh Signaling Affects Expression of Repressor Genes**

We then explored the impact of compromised Shh signaling in the expression pattern of well-known regeneration-associated repressor genes such as her4.1 and insm1a (Goldman, 2014). RT-PCR and qPCR analysis in cyclopamine-treated retina revealed that the pivotal regeneration-associated genes are downregulated, with the exception of insm1a and a few Notch signaling genes (Figures 1N, 1O, and S2F). Insma1, a known transcriptional repressor in MGPC induction and cell-cycle exit (Ramachandran et al., 2012; Zhang et al., 2009), showed upregulation, whereas levels of her4.1, one of the effectors of Notch signaling (Casini et al., 2004; Wilson et al., 2016), showed down-regulation, which was confirmed by mRNA in situ hybridization (ISH) and luciferase assays (Figures 1P and 1Q). Upregulation of insm1a and downregulation of her4.1 with blocked Shh signaling in post-injured retina led us to hypothesize the involvement of a well-known transcription factor such as Ascl1a in this regulatory loop. Insma1, a known transcriptional repressor of ascl1a (Ramachandran et al., 2012), could influence its...
Figure 2. Shh-Signaling-Dependent ascl1a Regulation in the Injured Retina

(A and B) RT-PCR (A) and qPCR (B) analysis of ascl1a in the post-injured retina; n = 6 biological replicates.

(C) Fluorescence ISH (FISH) and IF microscopy images of a 0.5-μm-thick optical section of retina showing co-localization of ascl1a with ptch1 in BrdU+ MGPCs at 4 dpi. Arrowheads mark co-expression of genes in BrdU+ cells.

(D–F) BF microscopy images of ascl1a mRNA ISH in retina at 4 dpi with cyclopamine treatment, shha or gli1 knockdowns (D), and gli3 or sufu knockdowns (E). The number of ascl1a+ cells from (E) is quantified in (F).

(G) qPCR analysis of ascl1a mRNA with cyclopamine treatment and shha or sufu knockdown in 2 dpi retina.

(legend continued on next page)
expression in a Shh-signaling-dependent manner. Moreover, Ascl1a could impact the expression of delta genes (Henke et al., 2009; Nelson et al., 2009), the ligand of Notch signaling, capable of inducing her4.1 expression in Notch-expressing cells (Takke et al., 1999). Thus, the Shh-signaling-dependent increase in Insm1a could cause a downregulation of ascl1a, which in turn reduces her4.1 levels in injured retina. These results suggest possible crosstalk between Shh and Notch signaling, contributing to retina regeneration.

**Shh Signaling Induces ascl1a during Retina Regeneration**

Apart from the potential involvement of Insm1a in repressing ascl1a levels, we also speculated its direct regulation mediated through Shh signaling. This is presumably true, as the temporal expression pattern of ascl1a by RT-PCR and qPCR matched that of Shh signaling components (Figures 1A, 1B, 2A, and 2B). We found the co-expression of *ptch1*, a bona fide marker of active Shh signaling (Jeong and McMahon, 2005), with ascl1a mRNA in retina at 4 dpi (Figure 2C). This suggests the potential involvement of Shh signaling in ascl1a induction and vice versa. Inhibition of Shh signaling, by cyclopamine treatment or knockdown of *gli1* or *shha*, significantly downregulated ascl1a expression (Figures 1N and S2G), which was also confirmed by mRNA ISH and qPCR in retina (Figures 2D and 2G). Conversely, knockdown of negative regulators of Shh signaling, *gli3* and *sufu*, caused an upregulation of ascl1a (Figures 2E–2G), suggesting its possible direct regulation. This is supported by the presence of several Gli-binding sites on the ascl1a promoter, revealed by in silico analysis (Figure 2H). Further, we performed a post-injured retinal chromatin immunoprecipitation (ChIP) assay using antibodies against the Shh signaling effector proteins Gli1 and Gli3 separately to examine whether these Gli-binding sites (Gli-BSs) are functional. Interestingly, both antibodies could separately precipitate Gli-bound chromatin, supporting the direct physical interaction of Gli1/Gli3 on the ascl1a promoter (Figures 2I and S2K). Furthermore, a luciferase assay performed in zebralfish embryos confirmed the effect of stimulators and inhibitors of Shh signaling on ascl1a expression (Figure 2J). The Gli-BS mutations in the ascl1a promoter almost completely abolished the effect of inhibitors and stimulators as revealed by the luciferase assay (Table S2; Figure 2K). These results suggest that Shh signaling regulates the important gene ascl1a.

**Shh Signaling/in28a/let-7 Regulatory Loop Is Essential for MGPC Induction**

We then explored whether the RNA-binding protein and pluripotency-inducing factor lin28a, a necessary and well-known target of Ascl1a during retina regeneration, is regulated directly through Shh signaling (Ramachandran et al., 2010a). This was supported by the co-expression of *ptch1* and lin28a in 4 dpi retinal sections (Figure 3A), suggesting the possible interdependency or hierarchical regulation. We further evaluated the expression pattern of lin28a that goes down with inhibited Shh signaling in retinal cross sections (Figure 3B). This was also proven by qPCR (Figure 3C). The opposite expression pattern of lin28a was found with *sufu* knockdown, as expected (Figures 3B and 3C). Evaluation of the lin28a promoter revealed putative Gli-BSs (Figure 3D) located as clusters, which were probed using Gli1 and Gli3 antibodies for a ChIP assay in the post-injured retina. Interestingly, both Gli1 and Gli3 bind to one of these Gli-BS clusters (Figures 3E and S2K), suggesting direct regulation of lin28a by Gli proteins. These results were further confirmed by luciferase assay performed in zebralfish embryos co-injected with *lin28a:GFP-luciferase* vector along with MOs against positive and negative regulators of Shh signaling (Figure 3F). The introduction of Gli-BS mutations in the lin28a promoter alleviated the impact of inhibitors and stimulators as revealed by a luciferase assay (Table S2; Figure 3G). Furthermore, let-7 microRNA, which is downregulated by Lin28a (Ramachandran et al., 2010a), was abundant in the uninjured inner nuclear layer (INL) in BrdU+ MGPCs at 4 dpi (Figure 3H). This let-7 downregulation in MGPCs is opposite to the IF pattern of Shh (Figures 3H and S1), which suggested possible regulation of *shha* mRNA by let-7 microRNA. The mRNA ISH of *shha* and *ptch1* also revealed a diffused expression pattern in both uninjured and 4 dpi retina (Figures S2H–S2J). In silico analysis predicted several let-7 microRNA-binding sites present in *shha*, *shhb*, *smo*, and *ptch1* genes (Table S4). We cloned these four genes in-frame with GFP reporter regulated by the cytomegalovirus (CMV) promoter and transfected these constructs with increasing concentrations of *let-7a* and let-7f microRNA expression plasmid (Ramachandran et al., 2010a) in HEK293T cells (Figure S5F). The results showed a dose-dependent decline in GFP expression (Figure 3J), which was quantified (Figures S6C–S6F). The knockdown of *lin28a* led to an expected decline in Shha protein at 4 dpi (Figure 3K). These findings suggest that *lin28a*-mediated suppression of *let-7* is required for the translational regulation of Shh signaling components in MGPCs as a part of positive feedback loop mediated through the Ascl1a-lin28a axis.

**Mmp9 Regulates ascl1a through Shh Signaling**

We also investigated the involvement of *mmp9*, a gene highly induced in regenerating MG cells, as revealed in microarray analysis (Ramachandran et al., 2012) and whole-retina RNA-seq done in the present study. Mmp9 is not only an important enzyme prerequisite for proliferative and pro-differentiative roles (Mannello et al., 2006), but also essential during fin regeneration (LeBert et al., 2015; Yoshinari et al., 2009). We found that *mmp9* is rapidly induced in the injured retina, with a peak expression at...
24 hpi (Figures 4A and S3A), and later (at 4 dpi), mmp9 levels were restricted to the neighboring cells of BrdU+ MGPCs (Figures S3B and S3C). Interestingly, inhibition of Shh signaling caused a significant upregulation of mmp9, and an opposite effect was seen with sufu knockdown (Figures 4B and S3D–S3F), which was confirmed by qPCR (Figure 4C) and a luciferase assay performed in zebrafish embryos injected with mmp9:GFP-luciferase vector (Figure 4D). These results suggest a negative correlation between mmp9 and active cell proliferation. However, upon inhibition of Mmp9 using pharmaceutical agents such as salvianolic acid B and SB-3CT, or by mmp9 targeting MO (Figures S6A, and S6B; Table S1), we found a drastic decline in BrdU+ cells in WT or GFP+ cells in tuba1016 transgenic retina (Figures 4E–4G and S3G). Interestingly, no impact was seen with mmp9 blockade after 2 dpi (Figure S3H), suggesting that its role preludes cell proliferation. To evaluate this further, we analyzed the expression pattern of an important gene, ascl1a, in mmp9-expressing cells in 4 dpi retina. We found significant co-localization of ascl1a+ cells with mmp9 expression (Figures 4H and S4A). Moreover, mmp9 knockdown caused a decline in ascl1a expression, whereas ascl1a knockdown caused an upregulation of mmp9 in 4 dpi retina (Figures 4I and S3I). Since the regulation of ascl1a is established through Shh signaling, we further explored whether Mmp9-mediated regulation of ascl1a was through Shh. Knockdown of mmp9 abolished the expression of Shh, as found with cyclopamine treatment (Figures 4J, S3J, and S7B). We also found an Shh-signaling-dependent regulation of Ascl1a protein with both shha or sufu knockdowns in 2 dpi retina (Figures 4K and S7C). Reombinant-SHH could induce Ascl1a expression and cell proliferation in zebrafish retina, similar to sufu knockdown (Figures 4L, S3K–S3M, and S7D). Interestingly, we also found a drastic increase in mRNA levels of Ascl1, Lin28a, and ASCL1 protein in injured mouse retina treated with recombinant-SHH (Figures 4M, S3N, and S7E).

Inhibition of Notch signaling through N-[3,5-difluorophenylacetyl]-L-alanyl-S-phenylglycine t-buty1 ester (DAPT) treatment, which causes a decline in Her4.1 levels and enhancement of MGPCs during retina regeneration (Conner et al., 2014; Wan et al., 2012), increased mmp9, ascl1a mRNA, and Shh protein levels (Figures S4B, S4C, 4N, and S7F). We further explored whether ascl1a upregulation seen with DAPT treatment is mediated through the Mmp9/Shh axis. Interestingly, we found that in the DAPT-treated retina, ascl1a translation was nullified with mmp9 knockdown (Figures 4O, 4P, and S7G). We speculated that upregulation of mmp9 with blockade of Notch signaling is possibly due to a lack of Her4.1-mediated transcriptional repression. Expression of mmp9 and her4.1 showed co-labeling in a few and co-exclusion in the majority of retinal cells (Figure 4Q). In silico analysis of the mmp9 promoter revealed several hair enhancer of split (Hes/Her)-binding N-boxes (Kageyama et al., 2007), suggesting its potential regulation through Notch signaling (Figure 4R). We performed a luciferase assay in zebrafish embryos co-injected with notch intracellular domain (nicd) mRNA along with mmp9:GFP-luciferase vector. nicd mRNA could cause an upregulation of Her4.1 (Nakahara et al., 2016; Wilson et al., 2016), and the luciferase assay showed dose-dependent downregulation of mmp9 promoter activity (Figure 4S), while mutations in Her4-binding sites abolished this impact (Figure S4D; Table S2). In summary, these results suggest that active Notch-signaling-mediated induction of her4.1 restricts the span of mmp9 expression at the site of injury. Further, Mmp9 coaxes MG to regenerate through Shh signaling and Ascl1a induction during retina regeneration.

**Shh Signaling Regulates zic2b Expression during Regeneration**

We explored a zinc-finger transcription factor, Zic2, essential for normal brain patterning during development (Elms et al., 2003), which upon mutation shows holoprosencephaly (HPE) or cyclopia (Brown et al., 2001; Tesiia et al., 2013), a phenotype similar to cyclopaamine treatment. Zic2 is also known to collaborate with Gli proteins (Koyabu et al., 2001). Therefore, we investigated whether a relationship exists between Gli proteins and Zic2 during retina regeneration, because both proteins occupy the same DNA sequence of the target genes’ promoters (Vokes et al., 2007). zic2b, orthologous to the mammalian Zic2 gene, showed upregulation in the retina microarray (Ramachandran et al., 2012) and our RNA-seq analysis. zic2b is also expressed in fin blastema (Figure S4E). The temporal expression pattern of zic2b in post-injured retina showed a peak expression at 4 dpi, a time when cell proliferation is at the maximum level (Figure 5A). Pulse labeling of MGPCs with BrdU also revealed its co-localization with zic2b (Figure 5B). Co-expression of ptc1 in
with zic2b in BrdU+ cells suggests their interaction during regeneration (Figure 5C). The zic2b showed downregulation with blockade of Shh signaling and an upregulation with sufu knockdown (Figures 5D and 5E). These results were also confirmed by a luciferase assay done in zebrafish embryos injected with zic2b:GFP-luciferase construct along with MOs against shha and sufu and also exposed to cyclopamine (Figure 5F). Analysis of the zic2b promoter revealed a cluster of Gli-BSs (Figure 5G), and spanning chromatin was pulled down using both Gli1 and Gli3 antibodies separately (Figures 5H and S2K). Gene knockdowns of gli1, gli3, and zic2b significantly influenced MGPCs proliferation in 4 dpi retina (Figures 5I, 5J, S6A, and S6B; Table S1). The luciferase assay revealed that Shh signaling inhibitors and stimulators had a small impact on zic2b promoter activity with mutated Gli-BSs (Table S2; Figure S4F). Early or late knockdowns of gli1/zic2b caused a decline in the number of BrdU+ cells in the retina, but the opposite was seen with gli3 knockdown (Figures 5I, 5J, S4G, and S4H). zic2b showed a pan retinal expression pattern with DAPT treatment, and the same was seen with gli3/sufu knockdowns (Figures S4I–S4K). Interestingly, zic2b knockdown nullified the enhancement of MGPCs with gli3 knockdown (Figures 5I and 5J). Moreover, the induction of Gli3 seems to block the responsiveness of MGPCs to Gli1, as the late knockdowns and double knockdown of gli1 and gli3 also caused a drastic decline in cell proliferation (Figures 5I, 5J, S4G, and S4H). The gli1 knockdown significantly impacted several regeneration-associated genes as the possible cause of the lack of MGPC induction (Figure S4L). These results suggest that the induction of zic2b in MGPCs largely triggers a proliferative phase mediated through Shh signaling, and it may collaborate with or outcompete Gli proteins in targeting a proliferative phase mediated through Shh signaling, and it suggests that the induction of foxn4. We also examined whether zic2b expression depends on the mmp9:shha-ascl1a signaling axis, because a substantial proportion of BrdU+ MGPCs co-expressed ascl1a and zic2b (Figure 5K). We probed for zic2b expression in 4 dpi retina elec-troporated with mmp9 and ascl1a MOs separately and found that zic2b levels declined drastically, as found with blockade of Shh signaling (Figures 5D and 5L). We further speculated that apart from its transcriptional control, zic2b might be regulated at translational levels. This speculation is mainly because of the presence of bona fide microRNA-binding sites in the zic2b coding region (Figure 5S). Surprisingly, we found a downregulation in the translation of GFP protein from an expression cassette appended with zic2b in HEK293T cells (Figure 5M), which was quantified (Figure 5G). These results suggest that zic2b is an essential regeneration-associated gene in zebrafish retina that is regulated through the mmp9-shha-ascl1a-lin28a-let-7 pathway.

The Foxn4/Ascl1a/Shh/Zic2b Regulatory Loop Is Associated with Regeneration

Foxn4, a member of the forkhead box family of proteins and discovered in retina microarray (Ramachandran et al., 2012) and RNA-seq analyses performed in the present study, showed an upregulation, with a peak expression at 4 dpi (Figures 6A and 6B). Foxn4 expression was restricted to BrdU+ MGPCs at 4 dpi (Figure 6C). Furthermore, we explored the significance of foxn4 induction during retina regeneration. Interestingly, MO-mediated gene knockdown of foxn4 inhibited MGPC induction up to 90% (Figures 6D, 6E, and S5A). To ascertain whether foxn4 is regulated through Shh signaling or its downstream effector genes, we adopted a pharmacological inhibition or gene-knockdown approach. Blockade of Shh signaling with cyclopamine or MOs against shha or gli1 significantly abolished foxn4 expression in the retina (Figures 6F, 6G, and S5B), whereas the opposite was seen with sufu knockdown (Figures S5C and S5D). Analysis of the foxn4 promoter revealed 2 putative Gli-BS clusters (Figure 6G) that were strongly bound by Gli1 and Gli3, as revealed by a ChIP assay (Figures 6H and S2K), suggesting a direct involvement of Shh signaling in its expression. As discussed earlier, the influence of Mmp9 on expression levels of Shha led us to suspect its involvement in the regulation of foxn4. Knockdown of mmp9 in 4 dpi retina caused a significant downregulation of foxn4 (Figures 6I and S5E).

Figure 4. Shh-Mmp9-Ascl1a Interplay is Necessary during MG Reprogramming

(A) RT-PCR (top) and qPCR (bottom) analysis of injury-dependent mmp9 expression in the retina; n = 6 biological replicates. (B–D) BF microscopy images of mmp9 mRNA ISH in the retina at 4 dpi with cyclopamine treatment and shha or sufu knockdown (B), as quantified by qPCR (C), and a luciferase assay in 24 hpf embryos injected with mmp9:GFP-luciferase vector (D). (E–G) IF microscopy images of 4 dpi retina with Mmp9 blockade using drugs (E) and MO against mmp9 (F). The number of BrdU+ MGPCs is quantified in (G). (H) FISH and IF microscopy images of a 0.5-μm-thick optical section of retina showing co-localization of mmp9 and ascl1a in BrdU+ MGPCs at 4 dpi. Arrowheads mark co-expression of genes in BrdU+ cells. (I) BF microscopy images of ascl1a and mmp9 mRNA ISH in ascl1a and mmp9 knockdowns in 4 dpi retina. (J) Western blotting experiment showing Shh levels in 2 dpi retina with the mmp9 knockdown. (K) Western blotting assay of Ascl1a in 2 dpi retina with shha or sufu knockdowns. (L) Western blotting assay of Ascl1a in 2 dpi zebrafish retina injected with recombinant SHH protein. (M) Western blotting assay of ASCL1 in 6 dpi mouse retina injected with recombinant SHH protein. (N) Western blotting assay of Shha in DAPT-treated retina at 1 dpi. (O and P) RT-PCR (top) and qPCR (bottom) analysis of ascl1a and mmp9 in DAPT-treated retina, with or without ascl1a or mmp9 knockdown (O), and confirmed by western blotting assay (P). (Q) FISH and IF microscopy images of a 0.5-μm-thick optical section of retina showed substantial co-exclusion and marginal co-localization of mmp9 with her4.1 at 4 dpi. Arrowheads mark co-expression of the gene, and arrows mark her4.1+ cells. (R and S) Schematic of the mmp9 promoter with potential Hes/Her-BS binding sites (inside box), and luciferase assay in 24 hpf embryos co-injected with mmp9:GFP-luciferase construct and notch intracellular domain (nicd) mRNA (S).

Scale bars represent 10 μm (H and Q) and 20 μm (B, E, F, and I). Asterisk indicates the injury site (B, E, F, H, I, and Q). Error bars represent SD. *p < 0.001 (C, D, G, and S). Biological replicates n = 6 in (O) and (Q), and n = 3 in (D) and (B). See also Figures S3, S4, S6, and S7.
The temporal gene expression pattern and co-localization of foxn4 with MGPCs prompted us to investigate its potential parallels with ascl1a gene. Fluorescence ISH (FISH) analysis showed co-expression of ascl1a and foxn4 in BrdU⁺ MGPCs (Figure 6J). We then explored the possibility of a hierarchical regulation between ascl1a and foxn4 during retina regeneration, as there is already a reported role for Foxn4 in the regulation of Ascl1 expression in mouse and chick (Del Barrio et al., 2007). We found significant downregulation of foxn4 expression in retinal sections with knockdown of ascl1a (Figure 6I). foxn4 promoter analysis predicted several Ascl1a-binding E-boxes (Bertrand et al., 2002; Li et al., 2006; Ramachandran et al., 2010a, 2011), and binding was confirmed by a ChIP assay (Figures 6K, 6L, and S5G). The transactivation of the foxn4 promoter by Ascl1a was confirmed with a luciferase assay, which was done by co-injection of ascl1a mRNA or MO against it, along with the promoter of foxn4 driving the GFP-luciferase fusion construct in zebrafish embryos (Figure 6M). The mutation of Ascl1a-BS in the foxn4 promoter had a negligible effect on its promoter activity both by ascl1a mRNA or by MO co-injections in zebrafish embryos (Figure S5H; Table S2).

We then explored, using a knockdown approach in the retina, whether Foxn4 impacted ascl1a or other regeneration-associated genes such as zic2b and mmp9. We found that both ascl1a and zic2b were downregulated, which also explained the downregulation of foxn4 itself, whereas no appreciable change was seen in mmp9 levels (Figure 6N). A luciferase assay confirmed transactivation of the ascl1a promoter by Foxn4, which was done by co-injection of foxn4 mRNA or MO against it, along with the promoter of ascl1a driving the GFP-luciferase fusion construct in zebrafish embryos (Figure 6O). Both the ascl1a and zic2b promoters harbor 2 potential Foxn4-binding E-boxes (Luo et al., 2012) (Figure 6P), and this was confirmed by a ChIP assay, which was done using an antibody targeting Foxn4 (Figure S6O). Mutated Foxn4-BS on the ascl1a promoter caused an almost complete alleviation of upregulated luciferase activity, as seen by its overexpression (Figures S6O and S6I; Table S2). These results suggest that foxn4 expression is dependent on Shh signaling directly as well as through other genes such as ascl1a, which in turn regulates another regeneration-associated gene such as zic2b in a feedback loop. The findings from this study are summarized in a model (Figures 7A and 7B).

**DISCUSSION**

In this study, we explored the significance and potential regulators of Shh signaling during zebrafish retina regeneration. Our findings unravel mechanisms through which Shh signaling contributes to retina regeneration. We propose that Shh-dependent induction of Ascl1a and Lin28a contributes to Müller glia dedifferentiation through let-7 microRNA-mediated translational downregulation of shha, shhb, smo, ptch1, and zic2b from respective mRNAs. Such stringent translational regulation probably accounts for the lack of an immature regenerative response despite the marginal expression of Shh signaling components such as shha, shhb, smo, and ptch in the uninjured retina. Cyclopamine-mediated repression of MGPCs might result from a decline in the regeneration-specific genes ascl1a and lin28a. This situation could be further exacerbated by upregulation of the repressor insm1a and the lack of the Delta-Notch signaling effector her.1. These observations suggest the ability of Shh signaling to impinge upon various other signaling pathways important for regeneration.

Our results also show that Shh signaling impacted regeneration not only through transcription factors but also through negative regulation of enzymes such as Mmp9. Moreover, Mmp9-dependent expression of Shha causes the induction of Ascl1a as a prelude to MG dedifferentiation and MGPC induction. The increased expression of Mmp9 in a regeneration-compromised scenario like cyclopamine treatment (shha or ascl1a knockout retina) suggests the existence of a feedback loop between Mmp9 and Shh signaling. The abundance of Mmp9 is probably due to the lack of Shha protein to give a feedback response for a decrease in its expression in MG to induce MGPCs. This observation is also supported by the sufu knockout-mediated decline in mmp9 expression. Co-labeling of ascl1a and mmp9, which was seen in a good number of cells, may appear paradoxical, but they all need not be Shh-positive or BrdU⁺. Only a subset of ascl1a-positive cells is ptch1 positive and can have active Shh signaling.

**Figure 5.** The Shh-Mediated Zic2b Axis Is Necessary during Retina Regeneration

(A) RT-PCR (top) and qPCR (bottom) analysis of injury-dependent zic2b expression in the retina; n = 6 biological replicates.
(B) ISH and IF microscopy revealed co-localization of zic2b mRNA with BrdU⁺ MGPCs in 4 dpi retina.
(C) FISH and IF microscopy images of a 0.5-μm-thick optical section of retina showing co-localization of zic2b with ptch1 in BrdU⁺ MGPCs at 4 dpi.
(D and E) BF microscopy images of zic2b mRNA ISH in 4 dpi retina, with cyclopamine treatment, MO mediated shha or sufu knockout done separately (D), which is quantified in (E).
(F) Luciferase assay in 24 hpf embryos injected with zic2b:GFP-luciferase vector with cyclopamine treatment and shha or sufu knockdowns.
(G) Schematic of the zic2b promoter with a putative Gli-BS. Arrows mark ChIP primers. N.S marks negative control devoid of Gli-BSs, and capital letters mark consensus of Gli-BSs.
(H) Retinal ChIP assay at 4 dpi showing both Gli1 and Gli3 bound to the zic2b promoter.
(I) IF microscopy images of BrdU⁺ cells in the regenerating retina with zic2b, gli1, and gli3 knockdowns in isolation or combination, delivered at the time of injury, compared with control MO.
(J) BrdU⁺ cells are quantified in the indicated knockdowns.
(K) FISH and IF microscopy images of a 0.5-μm-thick optical section of retina showing co-localization of zic2b with ascl1a in BrdU⁺ MGPCs at 4 dpi. Arrowheads indicate ascl1a and zic2b co-expression, whereas arrows indicate ascl1a but zic2b⁻ cells.
(L) ISH microscopy retinal images of zic2b mRNA with mmp9 or ascl1a knockdown at 4 dpi.
(M) let-7 microRNA downregulated translation of the GFP construct appended with zic2b harboring microRNA responsive regions in a dose-dependent manner in HEK293T cells.

Scale bars represent 10 μm (B, C, and K) and 20 μm (D, I, and L). Error bars represent SD. *p < 0.001 (E, F, and J). n = 6 biological replicates (E and J); n = 3 (F). See also Figures S4–S7.
Figure 6. Expression Dynamics and Necessity of Foxn4 during Regeneration
(A and B) RT-PCR (A) and qPCR (B) analysis of injury-dependent foxn4 expression in the retina; n = 6 biological replicates. (C) IF microscopy of a 0.5-μm-thick optical section of retina revealing co-localization of Foxn4 with BrdU+ MGPCs in 4 dpi retina.

(legend continued on next page)
signaling and downregulated mmp9. The remainder of the ascl1a positive cells can have upregulated mmp9 due to the lack of Shh signaling. Moreover, the Mmp9 expression is necessary for normal cycling of MGPCs during regeneration, and the repression of mmp9 by Her4.1 could enable its expression restricted to the injury site at a later time. We anticipate a much wider role for the Shh-Mmp9-Ascl1a-Lin28a-let-7 regulatory loop during retinal regeneration.

The induction of repressor Gli3 might cause the exit of MGPCs from the cell cycle to restrict the impact of a transcriptional activator, Gli1. This is evident from the knockdown results of gli1 and gli3 either in isolation or in combination. The gli1* knockdown indicated a decline in the number of MGPCs, whereas gli3 inhibition caused an expansion of MGPCs. Interestingly, double knockdown of gli1 and gli3 resulted in significant decline in MGPCs, suggesting that the Gli3 is necessary to quit the cell cycle as a prelude to differentiation. Similar results were seen with zic2b knockdown or cyclopamine treatment. This could be due to the impact of Shh signaling on the expression of downstream genes through Zic2b, although both Gli and Zic2b may compete for the central pillars of regeneration.

Taken together, our study sheds light on the mechanisms of MGPC induction in zebrafish retina in response to injury in an Shh-signaling-dependent manner and the significance of its downstream effector genes such as ascl1a, lin28a, zic2b, foxn4, and mmp9. These findings also suggest ways to coax mammalian MG dedifferentiation that may enable us to find ample solutions to intervene therapeutically for an efficient regenerative response.}

**EXPERIMENTAL PROCEDURES**

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

**Animals and Retinal Injury**

Zebrafish were maintained at 26–28 °C on a 14 hr/10 hr light/dark cycle for all experiments unless otherwise specified. The retinal injury was performed on 53x226 days post-fertile maturation (dpf) animals with a 6° optical section of retina showing co-localization of foxn4 and ascl1a in BrdU+ MGPCs at 4 dpi. Arrowheads mark co-expression of genes in BrdU+ cells.

(D and E) IF microscopy images of the retina with foxn4 knockdown at 4 dpi (D). The number of PCNA* MGPCs is quantified in (E).

(F) BF microscopy images of foxn4 mRNA ISH in retinal sections with cyclopamine treatment and shha or gli1 knockdowns.

(G and H) Schematic of foxn4 promoter with a putative Gli-BS cluster, where arrows mark ChIP primers, N.S marks negative control, and capital letters mark putative Gli-BSs (G). A retinal ChIP assay at 4 dpi showing both Gli1 and Gli3 bound to the foxn4 promoter (H).

(I) BF microscopy images of foxn4 mRNA ISH in retinal sections with mmp9 or ascl1a knockdowns.

(J) FISH and IF microscopy images of a 0.5-μm-thick optical section of retina showing co-localization of foxn4 and ascl1a in BrdU+ MGPCs at 4 dpi. Arrowheads mark co-expression of genes in BrdU+ cells.

(K and L) Schematic of the foxn4 promoter with a putative Ascl1a-binding site cluster, where arrows mark ChIP primers, N.S marks negative control, and capital letters mark putative Ascl1a-BS (K). A retinal ChIP assay at 4 dpi showing Ascl1a bound to the foxn4 promoter (L).

(M) Luciferase assay showing foxn4 promoter activity with overexpression or knockdown of ascl1a in 24 hpf embryos.

(N) BF microscopy images of mRNA ISH in retinal sections with foxn4 knockdown showing levels of genes (namely, ascl1a, zic2b, mmp9, and foxn4) at 4 dpi.

(O) Luciferase assay showing ascl1a promoter activity with overexpression or knockdown of foxn4 in 24 hpf embryos.

(P and Q) Schematic of ascl1a and zic2b promoter with a putative Foxn4-binding site cluster, where arrows mark ChIP primers, N.S marks negative control, and capital letters mark putative Foxn4-BS (P). A retinal ChIP assay at 4 dpi showing Foxn4 bound to both the ascl1a and zic2b promoters (Q).

Scale bars represent 10 μm (C, D, F, I, J, and N). Error bars represent SD. *p < 0.001 (M); **p < 0.04 (O). Biological replicates n = 6 in (M) and O, and n = 3 in (B).

Asterisk marks injury spots in (O), and N. See also Figures S5–S7.

**Figure 7. Schematic Representation of the Gene Regulatory Network during Retina Regeneration**

(A and B) Genetic interrelationships in uninjured (A) and injured (B) retina. Faded arrows and gene names show absence and bold shows presence. See also Figures S1–S7.
using a 30G needle as described previously (Fausett and Goldman, 2006). The C57BL/6 mice used in this study were maintained on a 12 hr/12 hr light/dark cycle with continuous access to food and water.

**RNA-Seq Analysis**

The RNA-seq analysis of the total RNA of the retina at different time points post-injury and with cyclopamine treatment was performed as described previously (Brooks et al., 2012).

**Statistical Analysis**

Observed data were analyzed for statistical significance by comparisons done using a two-tailed unpaired Student’s t test to analyze data from all experiments. Error bars represent SD in all histograms.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the RNA-seq data reported in this paper is GEO: GSE102063.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.04.002.

**ACKNOWLEDGMENTS**

This work was supported by the Department of Biotechnology (DBT) (S.K.), the Indian Council of Medical Research (S.G.), IISER Mohali (S.M., M.A.K., and M.C.), the Wellcome Trust/DBT India Alliance (Intermediate Fellowship IA/12/2/500630, to R.R.), extramural research funding from DBT India (102/IFD/SAN/39/5/2015-2016 and 102/IFD/SAN/22/55/2017-2018, to R.R.), and intramural funding support from IISER Mohali. The authors would like to express their gratitude to Daniel Goldman (Molecular and Behavioral Neuroscience Institute, University of Michigan) for sharing promoter clones of *Ins1, Ins2a, Ascl1, and Ins3m1a* genes. The authors also thank Dr. Kuljeet Singh Sandhu and Mr. Arashdeep Singh (IISER Mohali) for their help predicting Gli-Bs, Dr. Mahak Sharma’s laboratory (IISER Mohali) for cell transfection experiments, and Dr. Adrene Freedia D’cruz and Dr. Rayman Matharu (IISER Mohali) for critical reading of the manuscript.

**AUTHOR CONTRIBUTIONS**

R.R. conceived the study and designed experiments. S.K. performed the majority of experiments. S.G. and M.C. contributed to western blotting assays. M.A.K. conducted the fin experiments. S.M. performed RNA-seq Venn diagrams, and A.J.K helped with cell sorting. R.R., S.K., and S.M. analyzed the experimental data. R.R. wrote the manuscript with critical input from S.K., S.G., M.C., and S.M.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: October 13, 2017

Revised: February 3, 2018

Accepted: March 30, 2018

Published: May 1, 2018

**REFERENCES**

Ando, K., Shibata, E., Hans, S., Brand, M., and Kawakami, A. (2017). Osteoblast proliferation by reserved progenitor cells in zebrafish bone regeneration and maintenance. Dev. Cell 43, 643-650.

Bertrand, N., Castro, D.S., and Guillomet, F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. 3, 517-530.

Brooks, M.J., Rajasimha, H.K., and Swaroop, A. (2012). Retinal transcriptome profiling by directional next-generation sequencing using 100 ng of total RNA. Methods Mol. Biol. 884, 319-334.

Brown, L.Y., Odent, S., David, V., Blayau, M., Dubourg, C., Apacik, C., Delgado, M.A., Hall, B.D., Reynolds, J.F., Sommer, A., et al. (2001). Holoprosencephaly due to mutations in ZIC2: alanine tract expansion mutations may be caused by parental somatic recombination. Hum. Mol. Genet. 10, 791-796.

Chen, J.K., Taipele, J., Cooper, M.K., and Beachy, P.A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16, 2743-2748.

Conner, C., Ackerman, K.M., Lahme, M., Hobgood, J.S., and Hyde, D.R. (2014). Repressing notch signaling and expressing TNFα are sufficient to mimic retinal regeneration by inducing Müller glial proliferation to generate committed progenitor cells. J. Neurosci. 34, 14403–14419.

Danilova, N., Visel, A., Willett, C.E., and Steiner, L.A. (2004). Expression of the winged helix/forkhead gene, foxn4, during zebrafish development. Brain Res. Dev. Brain Res. 153, 115–119.

Del Barrio, M.G., Taveira-Marques, R., Muroyama, Y., Yuk, D.I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H.K., Xiang, M., Rowitch, D., and Richardson, W.D. (2007). A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. Development 134, 3427–3436.

Dunaeva, M., and Walterberger, J. (2017). HH signaling in regeneration of the ischemic heart. Cell. Mol. Life Sci. 74, 3481–3490.

Elms, P., Siggers, P., Napper, D., Greenfield, A., and Arkell, R. (2003). Zic2 is required for neural crest formation and hindbrain patterning during mouse development. Dev. Biol. 264, 391–406.

Fausett, B.V., and Goldman, D. (2006). A role for alpha1 tubulin-expressing Müller glia in regeneration of the injured zebrafish retina. J. Neurosci. 26, 6303–6313.

Goldman, D. (2014). Müller glial cell reprogramming and retina regeneration. Nat. Rev. Neurosci. 15, 431–442.

Henke, R.M., Meredith, D.M., Borromeo, M.D., Savage, T.K., and Johnson, J.E. (2009). Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. Dev. Biol. 328, 529–540.

Hochedlinger, K., and Plath, K. (2009). Epigenetic reprogramming and induced pluripotency. Development 136, 509–523.

Incardona, J.P., Gaffield, W., Kapur, R.P., and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. Development 125, 3553–3562.

Jeong, J., and McMahon, A.P. (2005). Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. Development 132, 143–154.

Kageyama, R., Ohtsuka, T., and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 134, 1243–1251.

Koyabu, Y., Nakata, K., Mizugishi, K., Aruga, J., and Mikoshiba, K. (2001). Physical and functional interactions between Zic and Gli proteins. J. Biol. Chem. 276, 6889–6892.

LeBert, D.C., Squirrell, J.M., Rindy, J., Broadbridge, E., Lui, Y., Zakrzewska, A., Eliceiri, K.W., Meijer, A.H., and Huttenlocher, A. (2015). Matrix metalloproteinase 9 modulates collagen matrices and wound repair. Development 142, 2136–2146.

Li, J., Liu, Q., Qiu, M., Pan, Y., Li, Y., and Shi, T. (2006). Identification and analysis of the mouse basic/Helix-Loop-Helix transcription factor family. Biochem. Biophys. Res. Commun. 350, 648–656.

Luo, H., Jin, K., Xie, Z., Qiu, F., Li, S., Zou, M., Cai, L., Hozumi, K., Shima, D.T., and Xiang, M. (2012). Forkhead box N4 (Foxn4) activates Dll4-Notch signaling to suppress photoreceptor cell fates of early retinal progenitors. Proc. Natl. Acad. Sci. USA 109, E553–E562.

Mannello, F., Pinti, G.A., Bagnara, G.P., and Papa, S. (2006). Role and function of matrix metalloproteinases in the differentiation and biological characterization of mesenchymal stem cells. Stem Cells 24, 475–481.
Supplemental Information

*let-7 MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration*

Simran Kaur, Shivangi Gupta, Mansi Chaudhary, Mohammad Anwar Khursheed, Soumitra Mitra, Akshai Janardhana Kurup, and Rajesh Ramachandran
Experimental Procedures

Animals, fin cut, retinal injury and drugs.
Zebrafish were maintained at 26-28 °C on a 14 h:10 h light/dark cycle for all experiments unless specified. The 1016 tuba1a:GFP transgenic fish used in this study have been characterized previously (Fausett and Goldman, 2006). Tricaine methanesulfonate is used as anesthetic. Fish embryos for all assays were obtained by natural breeding in laboratory. The Shh signaling inhibitor, cyclopamine; Mmp blockers, Salvianolic acid B and SB-3CT; protein transport inhibitor, Brefeldin A; Notch signaling blocker, N-[N-(3,5-difluorophenylacetyl)-L-alanyl]-S-phenylglycine β-butyl ester (DAPT), were made to a stock of 1mM, in DMSO for all experiments (all drugs were from Sigma-Aldrich). Drugs were delivered either through dipping or administration to the eye using a Hamilton syringe equipped with a 30-gauge needle. Retinal injury performed were previously described (Fausett and Goldman, 2006). All experiments were done to a minimum of six times for consistency and s.d.

C57BL/6 mice were used in this study. They were maintained at a cycle of 12 h light and 12 h dark cycle with continuous food and water accessibility. Animals were anaesthetized using isoflurane and eyes were injured or injected with a 30-gauge needle. Before harvesting the eyes animals were exposed to CO2 for euthanasia. The animal ethical committee at IISER Mohali approved these experiments.

RNAseq and analysis.
RNA was obtained from total retina from uninjured (control), 12 hours post injury and 4dpi, as previously described (Ramachandran et al., 2011), with or without cyclopamine treatment. The RNAseq was performed as shown previously (Brooks et al., 2012). The post sequence analysis were performed using TopHat and Cufflinks as reported earlier (Trapnell et al., 2012). The Supplementary Table S1 was created using a code developed in Python and the RNAseq data with special reference to transcription factors, obtained from the database AnimalTFDB2.0 (Zhang et al., 2015), were analysed using it. The Venn diagrams in Figures S1J and S1K are created using FunRich (Functional Enrichment Analysis Tool; version 3.0) software (Pathan et al., 2015). The RNAseq data is deposited in repository at GEO Submission with ID of GSE102063.

Primers and plasmid construction
All primers are listed in Supplementary Table S5. The promoter of her4.1 was amplified from zebrafish genomic DNA using primer pairs Xho-her4.1pro-F and Bam-her4.1 pro-R (~4 kb). The digested PCR amplicon was cloned into a pEL luciferase expression vector to create her4.1:GFP-luciferase constructs. The ascl1a:GFP-luciferase, lin28a:GFP-luciferase, insm1a:GFP-luciferase constructs were described previously (Ramachandran et al., 2010a; Ramachandran et al., 2012).

Genes like ascl1a, insm1a, lin28a, and ncid were cloned from complementary DNA amplified from zebrafish retina RNA at 4 dpi using primer pairs Bam-Ascl1a FL-F and Xho-Ascl1a FL-R (~0.6 kb); Bam-insm1a F and Xho-insm1a-R (~1.1kb); Bam-lin28a FL-F and Xho-lin28a FL-R (~0.6 kb). Post-digested PCR amplicons were cloned into their respective enzyme sites in pCS2 plasmid to obtain cmv:ascl1a, cmv:insm1a and cmv:lin28a. The ncid mRNA was prepared from PCR amplification using primer pairs T7-HSP M-F and S v40-R (~2kb) from a clone of ncid driven by Hsp70 promoter, which in turn was made in pTAL plasmid vector by digesting an amplicon of ncid obtained using PCR primers Hind2X-flag-nicd-F and MluI NICD-R.

Micro-RNA response elements (MRE) sequences of shhb, pitch1 and smo were cloned in pEGFP-C1 vector using BamHI and MfeI restriction sites, and shha and zic2b were cloned using BamHI and XhoI restriction sites. Site directed mutagenesis of various constructs were performed as described previously (Ramachandran et al., 2012).

For the confirmation of MO activity, an adaptor having respective MO targeted region for gli1, gli2a, gli3, mmp9 or pitch1 was cloned in pEGFP-N1 in XhoI and BamHI restriction sites, and shha and zic2b were cloned using BamHI and XhoI restriction sites. Site directed mutagenesis of various constructs were performed as described previously (Ramachandran et al., 2012).

Total RNA isolation, RT-PCR and qPCR analysis.
Total RNA was isolated from dark-adapted zebrafish retinae of control, injured and drug treated/MO electroporated group using TRIzol (Invitrogen). A combination of oligo-dT and random hexamers were used to reverse transcribe approximately 5 μg of RNA using Superscript II reverse transcriptase.
methane sulphonate and eyes were dissected, lens removed, fixed in 4% paraformaldehyde and retina dissection, unless mentioned specifically. Some animals required for long periods before euthanasia. BrdU labeling was performed by single i.p. injection of 20 μl of BrdU (20 mM) 3 h before euthanasia. The relative expression of mRNAs in control and injured retinae was deciphered using the ΔΔCT method and normalized to ribosomal protein l-24 or β-actin mRNA levels.

mRNA synthesis, embryo micro-injection and ChIP assay.
Gene clone of ncid cDNA in pCS2+ plasmid was linearized and capped mRNAs were synthesized using the mMESSAGE mMACHINE (Ambion) in vitro transcription system. For luciferase assay experiments, single-cell zebrafish embryos were injected with a total volume of ~1nl solution containing 0.02 pg of Renilla luciferase mRNA (normalization), 5 pg of promoter:GFP-luciferase vector and 0-6 pg of ncid mRNA or 0.1 to 0.5mM shha/sufu MOs. To assure consistency of results, a master mix was made for daily injections and ~300 embryos were injected at single cell stage. 24 hours later, embryos were divided into 3 groups (~ 70 embryos/group) and lysed for dual luciferase reporter assays (Promega, catalogue number E1910).

Chromatin immunoprecipitation (ChiP) assays were done in adult retina at 4dpi using ~50 adult retinae after dark adaptation. Chromatin was isolated by sonication as described previously (Ramachandran et al., 2012). Primers used for ChiP assays are described in Supplementary Table S5.

Morpholino (MO) electroporation.
MOs tagged with lissamine (Gene Tools) of approximately 0.5 μl (0.5 to 1.0 mM) were injected at the time of injury using a Hamilton syringe of 2 µl volume capacity. MO delivery to cells was accomplished by electroporation as previously described (Fausett et al., 2008). The control and ascl1a targeting MOs have been previously described (Ramachandran et al., 2012). Morpholinos targeting shha, shhb, sufu, gli1, gli2a, gli3, patched1, patched2, mmp9 and zic2b are: shha(1)-5’-GGGTTCCATGACAACTGGGCATTCC-3’; shha(2)-5’-CACGCTGTAAT CTGCCTCGGGTGTTTC-3’; shhb-5’-TCAGATGCAGCCTTACGTCCATGAC-3’; patched1-5’-AGGAGACATTACAGCCGGGCACTTT3’; patched2-5’-CGGGGTCT GTGGGATCCGAGGCCAT-3’; gli1-5’-CTCCATGATGAGACTTCTTGGATGA-3’; gli2a-5’-GGGTTCCATGACA CACTGGGAGGCGCAT-3’; gli3-5’-GTTCATGAGACTTTCTTGGAATGA-3’; mmp9-5’-GCTGCATATCCACTGGCATGAGAC-3’; zic2b(1)-5’-GTTGGCCGGC GTCCAGTAACATCACA-3’; zic2b(2)-5’-CAGCATTATTGACAAAGAATGC-3’

Cell culture and transfection and western blotting.
The HEK293T cells were grown in a 90 mm petriplate before seeding into 24-well plate at approximately 40% confluence and grown in Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) fetal bovine serum, with antibiotics and antymycotics in a 37 °C incubator with 5% CO2. Cells were then transfected after 24 h from time of plating. To examine involvement of let-7 microRNA in regulation of gene expression, cells were transfected with 50 ng of pEGFP-C1 vector harboring GFP-reporter tagged to shha, shhb, smo, ptc1h or zic2b cDNA, along with 0, 50, 200 or 500 ng of the ubC:let-7a/let-7f vector and 50 ng of the β-actin2:mCherry normalization vector. 48 h post-transfection, cells were harvested and protein expression was assayed by western blotting. For in vivo experiments, Western blotting was performed using whole retina tissue using 4 retinae per experimental sample, lysed in Laemmli buffer, size fractioned in 12% acrylamide gel with SDS at denaturing conditions before transferring on to Immun-Blot PVDF membrane (Biorad Catalogue number 162-0177), followed by probing with specific primary antibodies and HRP conjugated secondary for chemiluminescence assay using Clarity Western ECL (Biorad Catalogue number 170-5061).

BrdU labeling, Retina tissue preparation for mRNA in situ hybridization, immunofluorescence microscopy, and TUNEL Assay
BrdU labeling was performed by single i.p. injection of 20 μl of BrdU (20 mM) 3 h before euthanasia and retina dissection, unless mentioned specifically. Some animals required for long-term cell tracing experiments received more BrdU injections over multiple days. Fish were given higher dose of tricaine methane sulphonate and eyes were dissected, lens removed, fixed in 4% paraformaldehyde and
sectioned as described previously (Fausett and Goldman, 2006). The mRNA in situ hybridization (ISH) was performed on retinal sections with fluorescein or digoxigenin-labelled complementary RNA probes (FL/DIG RNA labeling kit, Roche Diagnostics) (Barthel and Raymond, 2000) The micro RNA let-7 ISH were done as described previously (Ramachandran et al., 2010a). Fluorescence ISH was performed according to the manufacturer’s directions (Thermo Fisher Scientific, catalogue numbers T20917, B40955, B40953). Sense probes were used in every ISH separately as control, to assess the potential of background signal. Immunofluorescence microscopy protocols and antibodies were previously described (Ramachandran et al., 2010b; Ramachandran et al., 2012). Immunofluorescence microscopy was performed rabbit polyclonal antibody against human ASCL1/MASH1 (Abcam, catalogue number ab74065); Rat monoclonal antibody against BrdU (Abcam, catalogue number ab6326); Mouse monoclonal antibody against human proliferating cell nuclear antigen, PCNA (Santa Cruz, catalogue number sc-25280); Rabbit polyclonal antibody against zebrafish Gli1 (Anaspec, catalogue number AS-55627); Rabbit polyclonal antibody against zebrafish Gli3 (Anaspec, catalogue number AS-55641); Rabbit polyclonal antibody against zebrafish Shh (Anaspec, catalogue number AS-55641); Rabbit polyclonal antibody against zebrafish Smo (Anaspec, catalogue number AS-55647); Mouse polyclonal antibody against GFP (Abcam, catalogue number ab-38689); Rabbit polyclonal antibody against zebrasfish mCherry (Abcam, catalogue number ab-183628); Mouse monoclonal antibody against Actin (Santa Cruz, catalogue number sc-81178); Rabbit polyclonal antibody against mouse glutamine synthetase (Abcam, catalogue number ab93439); Mouse polyclonal antibody against HuD (Santa Cruz, catalogue number sc-48421); Goat polyclonal antibody against protein kinase C β1 (PKCβ1) (Santa Cruz, catalogue number sc-209-G) at 1:500 dilution. Before BrdU immunofluorescence microscopy, retinal sections were treated with 2 N HCl at 37 °C for 20 min, equilibrated with 100mM sodium borate (pH 8.5) for 10 min, twice and then processed using standard procedures (Senut et al., 2004). BrdU labelled MGPC lineage-experiments were done in retinal sections from single eye sections of 8 µm thickness, distributed across five slides. Individual slide was first processed for immunofluorescence based detection of specific antigen or mRNA and then BrdU or PCNA staining was performed as mentioned above using respective antibodies (Powell et al., 2012; Ramachandran et al., 2012). The total number of BrdU+ cells and the number of co-labelled BrdU+ cells that also stained with a specific ISH probe and subsequent enzymatic reaction, were quantified on each slide. TUNEL assay was performed on retinal sections using In Situ Cell death Detection Fluorescein kit (Roche, Ref no:11684795910) as per manufacturer recommended protocol.

Fluorescence and confocal microscopy, cell counting and statistical analysis.

After the completion of staining experiments, the slides were examined with a Nikon Ni-E fluorescence microscope equipped with fluorescence optics and Nikon A1 confocal imaging system. The PCNA+ and BrdU+ cells were counted by observation of their fluorescence in retinal sections, ISH+ cells through bright field, visualized in the same microscope and quantified. Every sections of the stained retina were mounted, observed and analysed, and at least three retinae from separate fish were used. Observed data were analysed for statistical significance by comparisons done using a two-tailed unpaired Student’s t-test to analyse data from all experiments. For all other comparisons, analysis of variance (ANOVA) was performed and subsequently a Bonferroni/Dunn post hoc t-test was done using Stat View software. Error bars represent s.d in all histograms.

Fluorescence based cell sorting.

RNA was obtained from FACS purified MG and MG-derived progenitors at 4 dpi as previously described (Ramachandran et al., 2011, 2012). Briefly uninjured and injured retinas were isolated from 1016 tuba1a:GFP transgenic fish. GFP+ MGPCs from 1016 tuba1a:GFP retinas at 4 dpi were isolated by treating retinas with hyaluronidase and trypsin and then sorted on a BD FACs Aria Fusion high speed cell sorter. Approximately 40 injured retinas from 1016 tuba1a:GFP fish yielded 80,000 GFP positive and 170,000 GFP cells.

References
Barthel, L.K., and Raymond, P.A. (2000). In situ hybridization studies of retinal neurons. Methods Enzymol 316, 579-590.
Brooks, M.J., Rajasimha, H.K., and Swaroop, A. (2012). Retinal transcriptome profiling by directional next-generation sequencing using 100 ng of total RNA. Methods Mol Biol 884, 319-334.
Fausett, B.V., and Goldman, D. (2006). A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci 26, 6303-6313.
Fausett, B.V., Gumerson, J.D., and Goldman, D. (2008). The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci 28, 1109-1117.
Lindeman, L.C., Vogt-Kielland, L.T., Alestrom, P., and Collas, P. (2009). Fish’n ChiPs: chromatin immunoprecipitation in the zebrafish embryo. Methods Mol Biol 567, 75-86.
Pathan, M., Keerthikumar, S., Ang, C.S., Gangoda, L., Quek, C.Y., Williamson, N.A., Mouradov, D., Sieber, O.M., Simpson, R.J., Salim, A., et al. (2015). FunRich: An open access standalone functional enrichment and interaction network analysis tool. Proteomics 15, 2597-2601.
Powell, C., Elsaedli, F., and Goldman, D. (2012). Injury-dependent Muller glia and ganglion cell reprogramming during tissue regeneration requires Apobec2a and Apobec2b. J Neurosci 32, 1096-1109.
Ramachandran, R., Fausett, B.V., and Goldman, D. (2010a). Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12, 1101-1107.
Ramachandran, R., Reifler, A., Parent, J.M., and Goldman, D. (2010b). Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration. J Comp Neurol 518, 4196-4212.
Ramachandran, R., Zhao, X.F., and 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 USA 108, 15858-15863.
Ramachandran, R., Zhao, X.F., and 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.
Senut, M.C., Gulati-Leekha, A., and Goldman, D. (2004). An element in the alpha1-tubulin promoter is necessary for retinal expression during optic nerve regeneration but not after eye injury in the adult zebrafish. J Neurosci 24, 7663-7673.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7, 562-578.
Zhang, H.M., Liu, T., Liu, C.J., Song, S., Zhang, X., Liu, W., Jia, H., Xue, Y., and Guo, A.Y. (2015). AnimalTFDB 2.0: a resource for expression, prediction and functional study of animal transcription factors. Nucleic Acids Res 43, D76-81.
Supplementary Figure S1. Shh signaling mediated gene expression and lineage tracing of MGPCs in cyclopamine treatment.

(A,B) High magnification immunofluorescence microscopy (IF) images of 4dpi retinal sections showed co-localization of Shh signaling components with BrdU+ MGPCs (A), which is quantified in (B). (C) Western blotting assay showed regulation of Shha protein following injury at various time points. (D) IF microscopy images of wild type 4dpi retinal sections revealed significant co-localization of Shha with Glutamine Synthetase (GS), which marks all the Muller glia, at the injury site. (E) IF microscopy of Shha in BrdU+ MGPCs, in 4dpi retina, with Brefeldin A treatment, which is a protein transport inhibitor. (F) BrdU+ cells were quantified in Brefeldin A treatment. (G) RT-PCR analysis of indicated genes mRNA levels in DMSO and cyclopamine treated 24hpf embryos. (H,I) IF microscopy images showed a cyclopamine dose-dependent decline in PCNA+ MGPCs wild-type (H), retinae at 4 dpi, which is quantified in (I) (J) Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay done on DNase treated positive control, 4dpi and cyclopamine treated 4dpi retinal sections, showed presence of TUNEL+ cells only in positive control. (K) Cell-fate tracing experiment was done by injuring the fish followed by treating them with 5% (v/v) DMSO or 10µM cyclopamine for first four days followed by an i.p injection of BrdU and then were transferred to water for next 26 days until euthanasia. IF microscopy images of 30dpi retinal sections revealed co-localization of GS, which marks Muller glia, HuD which marks amacrine cells and PKC which marks horizontal cells, with BrdU+ cells in DMSO treated retina but not in cyclopamine treated retina. (L,M) Whole retina RNAseq analysis of DNA binding proteins and transcription factors at 12hpi, 4dpi and 4dpi cyclopamine treatment were compared with uninjured retina showed upregulated genes (L), and downregulated genes (M). Scale bars, 10 µm (A,E,H,J and K) and 20 µm (D). Error bars are SD.
Supplementary Figure S2: Shh signaling component genes’ knockdowns and lineage tracing of MGPCs in enhanced Shh signaling.

(A,B) IF microscopy images of 4dpi retina revealed decline in proliferation marked by reduction in BrdU+ cells in sshb, ptc1, ptc2 and gli2a knockdowns (A), which was quantified in (B). (C) Western blotting assay indicating shha knockdown caused downregulation in the expression of Shha protein. (D,E) The schematic of lineage tracing experiment, wherein control, sufu or gli3 MOs were injected and electroporated while injuring the retina, and an i.p. injection of BrdU was given on 4dpi and eyes were harvested at 20dpi (D), the increased number of BrdU+ cells could make retinal cell types (E). (F) RT-PCR analysis of indicated genes’ mRNA levels in DMSO and cyclopamine treated 2.5dpi retina. (G) RT-PCR analysis of ascl1a mRNA levels in uninjured retina, control knockdown and gli1 knockdown in 2.5dpi retina (H) Bright field (BF) and IF microscopy revealed the expression of ssha mRNA and BrdU in uninjured and 4dpi retina. (I,J) ISH and IF of ptc1 and BrdU respectively, in uninjured (I), 4dpi and 4dpi with cyclopamine treatment (J). (K) Relative abundance of ChIP DNA fragments obtained from Gli1 and Gli3 antibodies from various gene promoters, assayed by qPCR, which are normalized to control uninjured retina. Scale bars, 10 µm (A,E,H,I,J). Error bars are SD.
Supplementary Figure S3: mmp9 expression pattern and impact of SHH protein injection in regenerating retina.

(A-C) BF and IF microscopy images of mmp9 and BrdU+ cells at various time points post injury (A), and BrdU co-labeling with mmp9 at 5dpi (B), quantified in (C). (D,E) ISH microscopy revealed increased mmp9 expression in 4dpi retina with cycloamine treatment (D), mRNA levels quantified in (E). (F) Quantification of mmp9+ cells in 5% (v/v) DMSO control, 10µM cycloamine treatment and shha or sufu knockdowns in 4dpi retina. (G) IF microscopy images show Salvianolic acid B and SB-3CT dependent decline in GFP+ MGPCs in 1016 tuba:GFP transgenic zebrafish at 4dpi. (H) MOs against control and mmp9 were injected and electroporated at 2dpi, then an i.p. injection of BrdU was given on 4dpi, 3 hours before euthanasia, and no change in the number of BrdU+ cells was found in both knockdowns. (I) Quantification of ascl1a+ and mmp9+ cells in control and ascl1a knockdown retina at 4dpi. (J) Western blotting assay indicating cycloamine or mmp9 knockdown in 2.5dpi retina caused decline in Shha expression levels. (K) Zone of BrdU+ cells in the regenerating retina, increased upon injection of recombinant Shha protein (200ng) at 4dpi. (L,M) IF microscopy images reveal an increase in BrdU+ cell number in combined injection of recombinant SHH protein and shha MO, and isolated injection of SHH protein (200ng) in 4dpi retina, whereas BrdU+ cells declined in shha knockdown (L), which is quantified in (M), suggesting external SHH could impact retina regeneration even in absence of endogenous Shh protein. (N) RT-PCR analysis of Ascl1 and Lin28a genes in 6dpi mouse retina exposed to recombinant SHH protein at the time of injury until harvest. Scale bars, 10 µm (A,B,G,H,K,L). Error bars are SD.
Supplementary Figure S4: Impact of DAPT treatment or gli1/gli3 knockdowns in gene expression pattern and cell proliferation.

(A) FISH microscopy of ascl1a and mmp9 in 4dpi retinal sections. (B-C) Low magnification BF microscopy images of mRNA in situ hybridization of mmp9 (B) and ascl1a (C), in DAPT treated retina at 12hpi, showed an increase in its expression as compared with control. (D) Mutated Her/Hes binding sites abolished the impact of niced over expression on mmp9 promoter, in zebrafish embryo luciferase assay. (E) BF image of mRNA in situ hybridization of zic2b in 4 days post amputated zebrafish fin. (F) Luciferase assay showed that mutations to the Gli-BS abolished the impact of Shh signaling in zic2b promoter. (G) Schematic describing experimental regime of MO injection at the time of injury and electroporation at 4dpi, followed by an i.p. injection of BrdU at 5dpi before euthanasia. (H) IF microscopy images revealed decrease and an increase in BrdU+ cells in gli1 and gli3 knockdowns respectively. (I) Low magnification BF microscopy images of mRNA in situ hybridization of zic2b in DAPT treated retina, at 12hpi. (J) BF images of mRNA in situ hybridization of zic2b in sufu or gli3 knockdown in 4dpi retina. (K) Quantification of ascl1a+, mmp9+ and zic2b+ cells in control and sufu knockdown. (L) RT-PCR analysis of indicated genes’ mRNA levels in uninjured retina, control and gli1 knockdown retina in 2.5dpi. Scale bars, 10 µm (A,B,C,H,I,J) and 500 µm (E). Error bars are SD.
Supplementary Figure S5: Expression of foxn4 in retina at various conditions

(A) Western blot analysis of Foxn4 in foxn4-MO electroporated retina, at 2.5dpi. (B) RT-PCR analysis of foxn4 in uninjured control, 2.5dpi DMSO-treated, and 2.5dpi cyclopaamine-treated whole retina. (C) RT-PCR analysis of foxn4 from sufu MO-electroporated retina compared with control MO, at 2dpi. (D) BF microscopy images of foxn4 mRNA ISH in retinal sections electroporated with control and sufu MOs at 4dpi. (E) RT-PCR (upper) and qPCR (lower) analysis of foxn4 in control MO, and mmp9 MO electroporated in 2.5dpi retina. *P<0.001 in E, and error bars are SD. (F) Schematic representation of DNA constructs used in transfection experiments for examining the impact of let-7 microRNA on various genes. (G)qPCR assay revealed the relative abundance of ChIP DNA fragments of foxn4 promoter obtained by Ascl1a antibody which are normalized to control uninjured retina. (H) Luciferase assay revealed that mutated Ascl1a-BS on foxn4 promoter had little effect on positive or negative regulation by ascl1a mRNA or MO respectively. (I) Luciferase assay revealed that mutated Foxn4-BS on ascl1a promoter had little effect on positive regulation by foxn4 mRNA. Scale bars, 10 µm (D).
Supplementary Figure S6: MO assay in embryos. (A,B) The fusion mRNA, prepared by *in vitro* transcription using the clone containing GFP coding sequence in pCS2+ plasmid appended with the morpholino binding region of the respective genes, was injected alone (A), and along with morpholinos (B) in zebrafish embryos at single cell stage and imaged for GFP and lissamine fluorescence in a fluorescence microscope, at 24hpf. (C-G) Densitometry plots showing the expression of various GFP fusion proteins in *let-7* micro RNA dependent manner in HEK293T cells, normalized to transfection control mCherry. *P<0.0001. Scale bars, 500 µm (A,B).
Supplementary Figure S7: Western blotting of various proteins in retinal tissue. 
(A) The western blotting performed using total retina lysate revealed the unique expected bands (marked by arrows) of various proteins used in this study. (B-G) Densitometry plots showing the expression of various proteins in retina, normalized to control beta actin or glutamine synthetase. *P<0.0003, **P<0.02.
Table S1

| Lissamine tagged MO | # of injected fish | # of GFP + fish |
|---------------------|--------------------|-----------------|
| gli1                | 63                 | 1               |
| gli2                | 72                 | 0               |
| gli3                | 61                 | 1               |
| mmp9               | 70                 | 0               |
| ptch1              | 67                 | 0               |
| ptch2              | 75                 | 4               |

Supplemental Table 1. Statistical analysis of MO injection data. Related to Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, and Figure S6. The MOs used in this study are validated in embryos co-injected with MO and the GFP construct with a prefixing of MO binding sequence in-frame with GFP. The survival statistics is given in tables.
| Gene          | Wild type                  | Mutated                  |
|--------------|----------------------------|--------------------------|
| 
| ascl1a (Mutated Gli BS) | GGGGC GTGGTCAGG          | GGGGC GTAAAAAG          |
| CCGGAGCCGCG CGGCG       | GGGC AA AAAAAG          |
| CCGAAGCCACCTCG          | CCGGAA AAAACGTG         |
| TGAAGCCACACGTG          | TGAAA AAAACGTG          |
| ACTGGGCAGTCCAA          | ACT AAAAAATCCAA         |
| 
| lin28 (Mutated Gli BS) | TTACACCA CAGAAA         | TTACAAA AAAAGAAA        |
| GCAGTGTGATCGCT          | GCAGTGT AAATAAT         |
| GATGTTGTAATTT           | GATA TAATATTT           |
| TTTAGGAGGTGTG           | TTTGA AAAATATGG         |
| GATGTTGTAATTT           | GATA TAATATTT           |
| 
| zic2b (Mutated Gli BS) | ATACAGAC GCACA          | ATAAAAAACGCACA          |
| AGAGAC CCCAGAGA         | AGAGAAAAAAAGAGA         |
| GTGGG GTGCCCTGG         | ATAGGGT GAAATGG         |
| GGGGT GGGGTGCCGGC       | AAAATAGGGTGAAA          |
| CCGTAGGGGGGGCGTG        | CCGTAGGGGAATG           |
| 
| foxn4 (Mutated Gli BS) | TTTTG AAGGGGTGT         | TTTTGA AAAATGT          |
| TAAGTC TACCAAGG         | TAAGTAAAAAAGG           |
| TTAGCCCACAGGTG          | TTAG AAAAATATG          |
| TGGGCCCTCCAGGG          | TAAAAAACGCAGG           |
| GTGGAGG GGTGTAC         | GTGA AAAAATGTAC         |
| 
| foxn4 (Mutated Ascl1a BS) | CACCTG              | AACCTG                  |
| CAGTTG                    | AAGTTG                  |
| 
| ascl1a (Mutated Foxn4 BS) | ATAAGCGTAAAA         | CCCCCGC GTAA           |
| 
| mmp9 (mutated for Her4 BS) | CACCAAG             | AAAAAG                  |
| CACCAAG                   | AAAAAG                  |
| CTGGTG                    | ATAAATG                |
| CTTGTG                    | CTTAAA                  |

**Micro RNA responsive elements**

| Gene | Wild type                  | Mutant                  |
|------|----------------------------|--------------------------|
| shha | GAGCT GTT GTATATTAC AC CACCCTCT | GAGCT GTT GTATATTAACA AAAT |
|      | CACGAC GCAGCGTGT TGGTTTCAGTCAT | CACGACGC AGCGTGTGTTAAAAAAA A |
|      | TGGGC TACCAGTTAACAA AAAAT T | TGGGC TACCAGTTAACCTG C CTT TT |
|      | ATATTCAA ACTGCT CCTTTT | ATATTCAA ACTG AAAAAAA |
| shhb | GGACGG GCAGTG GCACTACTAC CTCAG | GGACGG GCAGTG GAC ATC A C T AA |
|      | CACCAAGCTCACCCTC ACTGCGCGC GCAC | CACCAAGCTCACCCTC AAAAACGC GA C |
|      | TGCCGGGCAGCATTGTTCAGTG GAA ACTCTCCAG | TGCCGG GCAC CCTG TT C T GTG GAA CTA AA AAG |
| pitch1 | GAATATGCACAGTTTCCCTT C TCA | GAATATGCACAGTTTCCCTTAAAAAA A |
|      | GAGCCCATGCAATAGCAGTTT CCCTT ATACCTCAA | GAGCCCATGCAATAGCAGTTTCCCTT AAACCTCAA |
Supplemental Table 2. Wild type and mutated regions of various DNA constructs. Related to Figure 2, Figure 3, Figure 4, Figure 5, and Figure S6. The mutations, created on DNA sequences for disrupting transcription factor binding sites and let-7 micro RNA responsive elements of various constructs used in this study are highlighted in bold letters.

|        | Wild Type                                  | Mutated Region                           |
|--------|--------------------------------------------|------------------------------------------|
| smo    | CACTATGCGACTTGGAGAGCCATCA                  | CACTATGCGACTTGGAGAAAAAA                 |
|        | AGTACGGCCAGCGGCTCCTGAG                    | AGTACGGCCAGCGGTTAAAAA                   |
| zic2b  | CGGCGGCGCAGCTGCTCT                       | CGGCGGCGCACAAXAXAAAX                   |
|        | GAGCGCATGCGGCGGCACGC                       | GAGCGCATGCGGCGGCACAAAAAA                |
|        | TGCCTCT                                    | AAAAT                                   |
| ZF Gene | Ensembl ID | Position | Heteroduplex | let-7 miRNA |
|---------|------------|----------|--------------|-------------|
| Shha    | ENSDARG00000068567 | 750-772(CR) | GAGCUUGUUGAUUUCACUGCUU | let-7a, b, c, d, e, f, g, h, i |
|         |            | 1057-1083(CR) | CACGACCGGCCGUGUGCUUUUGGUACAU | let-7h, i |
|         |            | 2471-2494 (3’UTR) | UGGCCAUACCGUAAACCGCCU | let-7d |
|         |            | 2571-2589 (3’UTR) | AUUUUUCACGUCCUCU | let-7e |
| Shhb    | ENSDARG00000038867 | 649-676(CR) | GGACGGGCUACGCUACUCUAC | let-7e |
|         |            | 1005-1032(CR) | CACCACACACGGACCGGCAC | let-7c, d |
|         |            | 1023-1057(CR) | UGCCCCGCUCCCCGUUACUCCUG | let-7b |
| Ptch1   | ENSG00000185920 | 2590-2617(CR) | GAAUAUCCCAGCGAUUGAUGGACCAU | Let-7h, i |
|         |            | 2581-2618(CR) | GAGCCAUCUGAUAUGCAUGUUCCCCCUUCCUCUCAAC | Let-7b |
| Smo     | ENSDARG0000002952 | 1472-1497(CR) | CACUAUGCGACUUGGAGAGCCAUCAU | let-7a, b, c, d, e, f, g, h, i |
|         |            | 2612-2634(CR) | AGGAUAGGGUGUGUGAACGAG | let-7c, d |
| Zic2b   | ENSDARG00000037178 | 500-519(CR) | CGCGCCGCGACGCUGCCUCU | let-7h |
|         |            | 491-519(CR) | AGGCGCGCGGCACGCGCGCGCAG | let-7c, d |

Supplemental Table 4. List of genomic regions with let-7 micro RNA binding sites. Related to Figure 3 and Figure 5. The table shows a list of genomic regions of genes, mentioned in this study, with the micro RNA recognition elements (MREs) URL:https://bibiserv2.cebitec.unibielefeld.de/rnahybrid;jsessionid=be1041a93a76d436824f6e0f235b.