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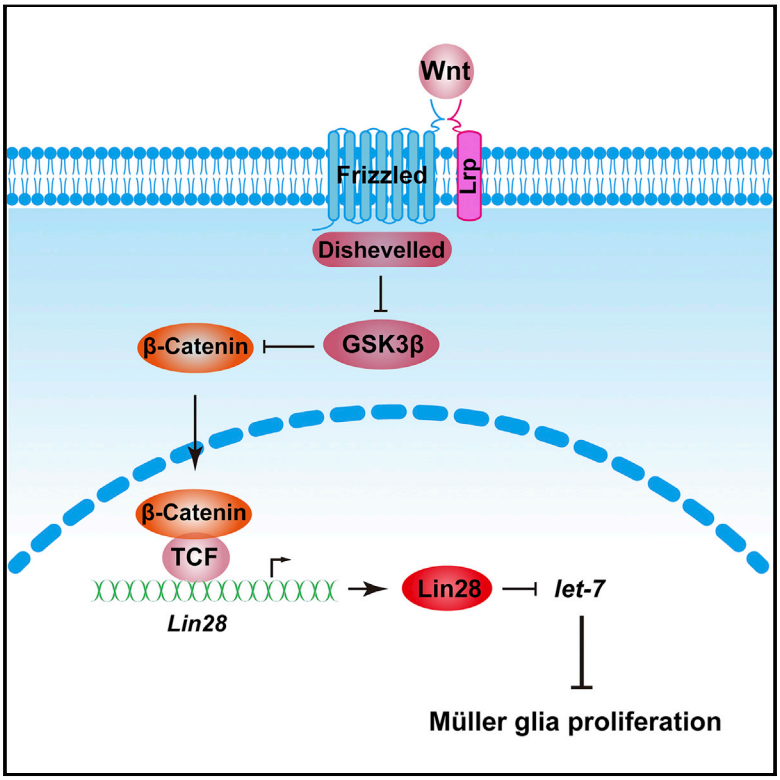
Graphical Abstract



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In Brief

Müller glial cells (MGs) are a source of retinal stem cells. To overcome proliferation quiescence of MGs in the adult mammalian retina, Yao et al. report that modulation of Wnt/Lin28/*let-7* miRNA signaling stimulates MG proliferation without retinal injury. A subset of cell-cycle-reactivated MGs expresses markers for retinal interneurons.

Highlights

- Retinal injury activates Wnt signaling in MGs
- Wnt-Lin28-*let-7* miRNA signaling regulates MG proliferation
- Gene transfer of β-catenin or Lin28 stimulates MG proliferation without injury
- Cell-cycle-reactivated MGs show neurogenic potential

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Wnt Regulates Proliferation and Neurogenic Potential of Müller Glial Cells via a Lin28/*let-7* miRNA-Dependent Pathway in Adult Mammalian Retinas

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SUMMARY

In cold-blooded vertebrates such as zebrafish, Müller glial cells (MGs) readily proliferate to replenish lost retinal neurons. In mammals, however, MGs lack regenerative capability as they do not spontaneously re-enter the cell cycle unless the retina is injured. Here, we show that gene transfer of β-catenin in adult mouse retinas activates Wnt signaling and MG proliferation without retinal injury. Upstream of Wnt, deletion of *GSK3β* stabilizes β-catenin and activates MG proliferation. Downstream of Wnt, β-catenin binds to the *Lin28* promoter and activates transcription. Deletion of *Lin28* abolishes β-catenin-mediated effects on MG proliferation, and *Lin28* gene transfer stimulates MG proliferation. We further demonstrate that *let-7* miRNAs are critically involved in Wnt/Lin28-regulated MG proliferation. Intriguingly, a subset of cell-cycle-reactivated MGs express markers for amacrine cells. Together, these results reveal a key role of Wnt-Lin28-*let7* miRNA signaling in regulating proliferation and neurogenic potential of MGs in the adult mammalian retina.

INTRODUCTION

Müller glial cells (MGs) are the primary glial cell type in the vertebrate retina, serving to provide structural support and maintain homeostasis for retinal neurons (Vecino et al., 2016). In cold-blooded vertebrates such as zebrafish, MGs are a source of retinal stem cells to replenish lost retinal neurons (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007; Qin et al., 2009; Ramachandran et al., 2010b; Thummel et al., 2008). In mammals, however, MGs do not spontaneously re-enter the cell cycle, and therefore they lack regenerative capability (Sahel et al., 1991). Recent studies suggest that the regenerative machinery exists in the adult mammalian retina, but injury is required to restore the stem cell status of MGs (Close et al., 2006; Dyer and Cepko, 2000; Karl et al., 2008; Ooto et al., 2004), which is counterproductive for regeneration as it massively kills retinal neurons (Dyer and Cepko, 2000; Karl et al., 2008; Ooto et al., 2004). The molecular nature of injury-induced signals that stimulates MG proliferation in mammals remains poorly understood. We hypothesized that retinal injury may induce signaling events to stimulate MG proliferation and that direct activation of these pathways could allow MGs to re-enter the cell cycle in the absence of injury. Wnt signaling regulates proliferation of adult hippocampal stem cells (Lie et al., 2005). In the adult mammalian retina, injury enhances Wnt signaling and Wnt activation promotes injury-induced MG proliferation (Das et al., 2006; Liu et al., 2013). Canonical Wnt signaling involves the binding of Wnt proteins to Frizzled receptors and activation of Dishevelled, leading to the stabilization and nuclear accumulation of β-catenin, a key effector of Wnt signaling that regulates gene transcription (Logan and Nusse, 2004).

The serine/threonine kinase GSK3 (glycogen synthase kinase 3) regulates Wnt signaling as inhibition of GSK3 leads to increased β-catenin levels (Doble and Woodgett, 2003). Pharmacological studies have implicated GSK3 in the regulation of self-renewal of embryonic stem cells (Sato et al., 2004; Ying et al., 2008). In the developing nervous system, deletion of *GSK3* causes excessive proliferation of early neural progenitors while the generation of intermediate neural progenitors and postmitotic neurons is largely suppressed (Kim et al., 2009). Genetic evidence is needed to examine the role of GSK3 in regulating the proliferation of MGs in the adult mammalian retina.

Lin28, a RNA-binding protein consisting of Lin28a and Lin28b, has emerged as a master regulator for cell proliferation through inhibition of the biogenesis of *let-7* miRNA (microRNA) in embryonic stem cells and cancer cells (Shyh-Chang and Daley, 2013).

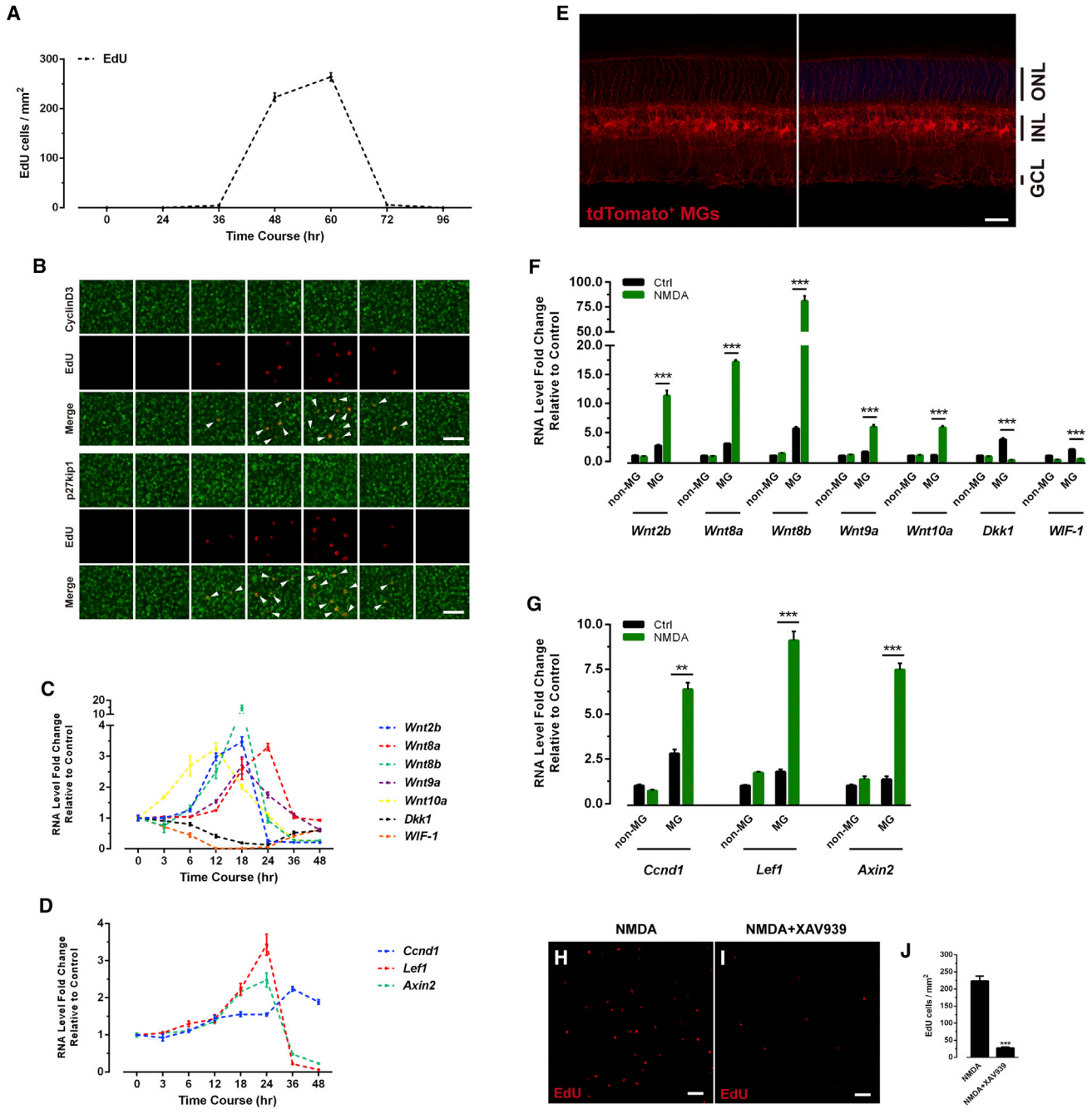

Figure 1. Neurotoxic Injury Activates Wnt Signaling and MG Proliferation

(A) A time course analysis of MG proliferation by scoring the number of EdU$^+$ cells/mm^2 on retinal flatmount preparations following NMDA-induced neurotoxic injury. Data are presented as mean ± SEM, n = 4.

(B) Representative images of EdU detection/anti-CyclinD3 or EdU detection/anti-p27^{kip1} immunohistochemistry at each time point. Arrowheads, EdU$^+$ cells were double positive for CyclinD3 or p27^{kip1} immunoreactivity. Scale bar, 25 μm.

(C and D) A time course analysis of the RNA levels for *Wnt* genes, Wnt antagonists *Dkk1* and *WIF-1* (C), and Wnt target genes following NMDA-induced neurotoxic injury (D). Data are presented as mean ± SEM, n = 4.

(E) MGs are visualized as tdTomato$^+$ cells in Rosa26-tdTomato reporter mice. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

(F) The RNA levels for *Wnt* genes and Wnt antagonists *Dkk1* and *WIF-1* in FACS-purified MGs and non-MGs at 18 hr after neurotoxic injury. Data are presented as mean ± SEM, n = 3. ***p < 0.001, Student's t test.

(legend continued on next page)

Several signals upstream of Lin28 have been discovered, including regulation of *Lin28a* expression by Sox2 based on single-cell expression data analysis during cellular reprogramming (Buganim et al., 2012) and transactivation of *Lin28b* by c-Myc and NF-κB in transformed cancer cells (Chang et al., 2009; Iliopoulos et al., 2009). Interestingly, a recent study showed that β-catenin activates the transcription of *Lin28a*, but not *Lin28b*, by directly binding to the *Lin28a* promoter in breast cancer cells (Cai et al., 2013), providing evidence that Wnt signaling may directly regulate *Lin28a* expression to control cancer cell proliferation. Beyond the studies in cancer cells, how Wnt/β-catenin signaling might interact with Lin28/*let-7* to regulate cell proliferation in progenitor or stem cells is largely unknown.

In the present study, we characterized Wnt as an injury-induced signaling event for stimulating the proliferative response of MGs in the adult mammalian retina. Cell-type-specific gene transfer of β-catenin is sufficient to stimulate MG proliferation in vivo without retinal injury. GSK3β regulates Wnt signaling through phosphorylation of β-catenin, targeting it for proteasome degradation (Cohen and Frame, 2001). Deletion of *GSK3β* resulted in β-catenin stabilization and MG proliferation without retinal injury. Importantly, we found that β-catenin activates the transcription of *Lin28a* and *Lin28b* by binding to the *cis*-regulatory elements of their promoters and that Lin28/*let-7* miRNAs play an important role downstream of Wnt in regulating MG proliferation. Intriguingly, after gene transfer of β-catenin or *Lin28*, a subset of cell-cycle-reactivated MGs express markers for amacrine cells, a type of retinal interneurons.

RESULTS

Neurotoxic Injury Leads to Transient Proliferation of MGs in the Adult Mouse Retina

To examine MG proliferation induced by neurotoxic injury in the adult mammalian retina, we injected 200 nmol NMDA into the vitreous chamber of the mouse retina at 4 weeks of age. This NMDA dose results in retinal injury and MG proliferation in adult rat and mouse retina (Karl et al., 2008; Ooto et al., 2004). We used anti-HuC/D immunohistochemistry to assay the time course of NMDA-induced cell death in the ganglion cell layer (GCL). Retinal ganglion and amacrine cells were progressively lost with only ~45% of HuC/D$^+$ cells remaining 36 hr post-injury (Figure S1). To assay cell proliferation, we injected 1μL of 1 mg/mL of EdU into the vitreous chamber 5 hr before tissue collection at 24, 36, 48, 60, 72, and 96 hr post-NMDA injection and quantified the number of EdU$^+$ cells/mm^2 in retinal flatmount preparations. No EdU incorporation was detected at 24 hr until a small number (4.7 ± 0.8) of EdU$^+$ cells appeared 36 hr post-NMDA injection. This number continued to increase, reaching 264.5 ± 8.4 at 60 hr, before it was reduced to 5.8 ± 1.1 at 72 hr post-NMDA injection (Figure 1A). The EdU$^+$ cells were also CyclinD3 and p27^{kip1} positive (Figure 1B), MG-specific nuclear antigens (Dyer and Cepko, 2000). Our results indicate that NMDA-induced neurotoxic injury transiently stimulated MG proliferation in a time window of 36–72 hr after injury.

Neurotoxic Injury Activates Wnt Signaling Leading to MG Proliferation

To examine whether Wnt signaling is activated by NMDA-induced neurotoxic injury, we performed qPCR to assay the RNA levels of Wnt genes at 3, 6, 12, 18, 24, 36, and 48 hr post-NMDA injection, relative to the PBS injected retina as a control. Several Wnt genes, including *Wnt2b*, *Wnt8a*, *Wnt8b*, *Wnt9a*, and *Wnt10a*, were upregulated at least 3-fold between 6 and 24 hr post-injury (Figure 1C). *Dkk1*, a Wnt signaling antagonist, was downregulated to 13.2% ± 0.6% of the control 24 hr post-injury (Figure 1C). The RNA levels for WIF-1, a secreted protein that binds to Wnt proteins and inhibits their activities (Hsieh et al., 1999), were also reduced to 1.6% ± 0.2% of the control 12 hr post-injury (Figure 1C). The upregulation of Wnt genes and concomitant downregulation of *WIF-1* and *Dkk1* would predict activation of the canonical Wnt/β-catenin signaling pathway. Indeed, 24 hr post-injury, we detected a 3.4-fold upregulation of *CyclinD1* (*Ccnd1*; Figure 1D), a target gene of Wnt signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999) and key regulator of the cell-cycle progression from the G1 to S phase (Resnitzky et al., 1994). We also detected significant upregulation of *Lef1* and *Axin2* (Figure 1D), two other Wnt target genes (Filali et al., 2002; Hovanes et al., 2001). Our results indicate that NMDA-induced neurotoxic injury led to activation of Wnt signaling, which preceded MG proliferation.

To examine whether NMDA-induced Wnt activation is a cell-autonomous response of MGs, we generated a MG-specific reporter mouse line by crossing the GFAP-Cre line to the Rosa26-tdTomato reporter line (Kuzmanovic et al., 2003; Madisen et al., 2010), resulting in cell-type-specific labeling of MGs with tdTomato (Figure 1E), and used fluorescence-activated cell sorting (FACS) to isolate tdTomato$^+$ MGs after NMDA treatment. The RNA levels for Wnt genes and Wnt antagonists were assayed in tdTomato$^+$ MGs and the non-MG population at 18 hr post-injury. While the RNAs levels for Wnt genes (*Wnt2b*, *Wnt8a*, *Wnt8b*, *Wnt9a*, and *Wnt10a*) and Wnt antagonists (*Dkk1* and *WIF-1*) remained largely unchanged in the non-MG group, even greater fold changes were detected for these genes in MGs (Figure 1F). Robust activation of Wnt target genes (*CyclinD1*, *Lef1*, and *Axin2*) was also detected in MGs (Figure 1G).

We next examined whether inhibition of Wnt signaling could suppress NMDA-induced MG proliferation. We co-injected NMDA with XAV939, a selective Wnt signaling antagonist (Huang et al., 2009), and quantified the number of EdU$^+$ cells per mm^2 in retinal flatmount preparations 60 hr post-injection, when the maximum number of EdU$^+$ cells were detected (Figure 1A). The number of EdU$^+$ cells was significantly reduced when Wnt signaling was inhibited by XAV939 relative to NMDA injection

(G) The RNA levels for Wnt target genes in FACS-purified MGs and non-MGs at 18 hr after neurotoxic injury. Data are presented as mean ± SEM, n = 3. **p < 0.01, ***p < 0.001, Student's t test.

(H and I) Inhibition of Wnt signaling suppresses neurotoxic injury-induced MG proliferation. In comparison to NMDA treatment alone, Wnt inhibitor XAV939 treatment significantly reduced the number of EdU$^+$ 60 hr after NMDA injection. Scale bar, 50 μm.

(J) Quantification of EdU$^+$ cells after XAV939 treatment. Data are presented as mean ± SEM, n = 4. ***p < 0.001, Student's t test.

alone (Figures 1H–1J). Taken together, our results indicate that Wnt is an injury-induced signal to stimulate MG proliferation.

Gene Transfer of β-Catenin Stimulates MG Proliferation without Retinal Injury

To develop a MG-specific gene transfer method, we modified an adeno-associated virus (AAV) variant, known as ShH10 (Klimczak et al., 2009; Koerber et al., 2009), by replacing the ubiquitous CAG promoter with the MG-specific promoter GFAP (Kuzmanovic et al., 2003). ShH10-CAG-mediated gene transfer of GFP, via intravitreal injections in adult mice, resulted in transgene expression predominantly in MGs as well as a subset of retinal ganglion cells and amacrine cells labeled by NeuN immunoreactivity (Figures S2A–S2C). By contrast, ShH10-GFAP-mediated gene transfer was highly specific for MGs as GFP expression was not detected in retinal ganglion cells or amacrine cells (Figures S2D–S2F). In addition, ShH10-GFAP-mediated gene transfer was highly efficient to transduce many MGs (Figures S2G–S2O). We further dissociated ShH10-GFAP-GFP infected retinas into single cells and analyzed the percentage of GFP^+ cells that express MG-specific nuclear antigens CyclinD3 and $p27^{kip1}$. Out of 524 GFP^+ cells analyzed from 6 retinas, all of them (100%) were immunoreactive for CyclinD3 or $p27^{kip1}$ (data not shown), indicating that ShH10-GFAP may only transduce MGs.

Previous reports indicate that retinal injury is a prerequisite to stimulate the proliferative response of MGs in the adult mammalian retina. We next examined whether activation of Wnt signaling, without retinal injury, is sufficient to stimulate MG proliferation using ShH10-GFAP-mediated gene transfer of wild-type β-catenin. To assess the activation of the canonical Wnt pathway, we made a ShH10-Wnt reporter in which the GFP reporter gene is driven by Tcf/Lef-mediated transcriptional activation. 2 weeks after viral infection in the adult mouse retina, significant activation of the reporter gene was observed in MGs co-infected with ShH10-GFAP-β-catenin, in comparison to those infected with the ShH10-Wnt reporter alone (Figures S3A and S3B). A time course study showed that Wnt target genes were activated following gene transfer of β-catenin in MGs in comparison to ShH10-GFAP-GFP infected retinas as a control (Figure S3C). To examine whether Wnt activation leads to MG proliferation, we analyzed EdU incorporation following ShH10-GFAP-mediated gene transfer of wild-type β-catenin in adult mouse retina at 4 weeks of age. Intravitreal injection of EdU was performed 10 days post-viral infection, and the treated retinas were collected 4 days later for analysis. EdU^+ cells were detected in retinal sections co-labeled by immunohistochemistry for MG-specific antigens, including glutamine synthetase (Figures 2A–2F), CyclinD3 (Figures 2G–2L), and $p27^{kip1}$ (Figures 2M–2R). Many EdU^+ cells were detected following β-catenin gene transfer, indicating that these cells re-entered the cell cycle. The nuclear-localizing EdU signals were surrounded by the MG cytoplasm labeled by glutamine synthetase immunoreactivity (Figure 2F) and were co-localized with MG nuclear antigens CyclinD3 (Figure 2L) or $p27^{kip1}$ (Figure 2R). In these co-labeling experiments, all EdU^+ cells were positive for MG-specific antigens, indicating that the proliferating cells were MGs. Noticeably, when MGs re-entered the cell cycle, their somas migrated along their radial processes instead of being localized in the middle of the inner nuclear layer (INL) as they normally do. A quantitative analysis (Figure 2Y) showed that the EdU^+ cells were unevenly distributed in retinal layers, with a majority of them (66.2% ± 2.1%) migrating to the outer nuclear layer (ONL) where photoreceptors are localized. The migration of cell-cycle-reactivated MGs to the ONL was further confirmed by ShH10-GFAP-β-catenin infection in the MG-specific reporter mice (Figure 1E), where EdU signals were detected in the $tdTomato^+$ MGs in the ONL (Figures 2S–2X). As a result of Wnt-induced cell proliferation, more $CyclinD3^+$ MGs were present relative to the untreated retina (Figure 2Z).

Characterization of Cell-Cycle Progression, Distribution, and Efficiency of MG Proliferation following β-Catenin Gene Transfer

EdU is incorporated into the newly synthesized DNA during the S phase of the cell cycle. To examine the progression of EdU-labeled MGs through other active phases of the cell cycle, we performed co-labeling experiments (Figure S4) for EdU detection and immunohistochemical analysis of cell proliferation antigens Ki67 (Figures S4A–S4D) and phospho-histone H3 (PH3) (Figures S4E–S4H), expressed in the active phases (G_1, S, G_2, and Mitosis) of the cell cycle for Ki67 (Scholzen and Gerdes, 2000), and late G_2 and the actual phases of mitosis for PH3 (Hendzel et al., 1997). We quantified the percentage of Ki67 or PH3 positive cells that were also EdU positive, and vice versa (Figure S4I). While the vast majority of Ki67 or PH3 positive cells were EdU positive, only a small portion of EdU positive cells were positive for Ki67 or PH3, indicating that most EdU^+ MGs did not proceed to the later phases of the cell cycle in the 4-day time frame between EdU injection and tissue collection.

To further analyze the efficiency and distribution pattern of MG proliferation following β-catenin gene transfer, we examined EdU-labeled cells in retinal flatmount preparations. In the ShH10-GFAP-GFP infected retina as a control, neither the viral infection nor the injection procedure itself led to cell proliferation (Figures 3A and 3B). By contrast, many MGs re-entered the cell cycle in the β-catenin treated retina, labeled by ShH10-GFAP-GFP co-infection (Figures 3C and 3D). We quantified the number of EdU^+ cells/mm^2 in four retinal quadrants (dorsal, ventral, temporal, and nasal) at three distances (700, 1,400, and 2,100 μm) from the center of the retina (Figure 3E). Although the number of EdU^+ cells in each of the four quadrants was not significantly different from one another as long as their distance from the center remained the same, it appeared that there was a slight gradient of an increasing number of EdU^+ cells from the center to the periphery (Figure 3F). Our results show that Wnt activation was highly efficient to stimulate MG proliferation in an uninjured retina.

EdU incorporation assay can only label a small fraction of cell-cycle-reactivated MGs when they are proceeding through the S phase of the cell cycle at the time of EdU injection. After retinal dissociation, we quantified the number of EdU^+ cells as a percentage of all Wnt-activated MGs labeled by ShH10-GFAP-GFP co-infection (Figures 3G–3I). As expected, all EdU^+ cells were GFP^+. However, only ~8% of GFP^+ cells were labeled by EdU, indicating that the majority of Wnt-activated MGs were not at the S phase of the cell cycle at the time of EdU injection.

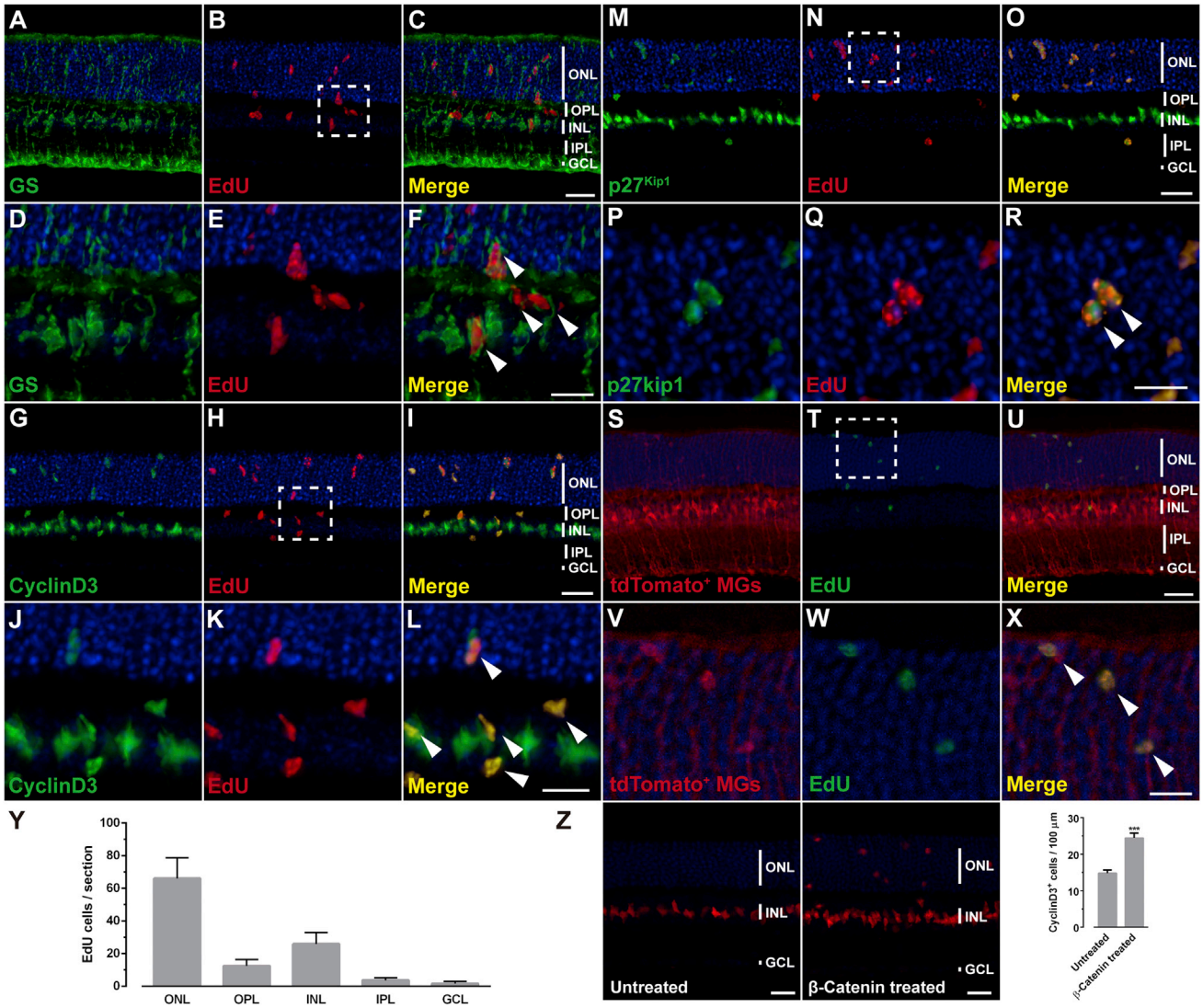

Figure 2. MGs Re-enter the Cell Cycle following ShH10-GFAP-Mediated Gene Transfer of β-Catenin without Retinal Injury

(A–R) Analysis of EdU incorporation by immunohistochemistry co-labeling for MG-specific antigens glutamine synthase (GS in A–F), CyclinD3 (G–L), and $p27^{kip1}$ (M–R). The boxed areas in (B), (H), and (N) are enlarged in (D)–(F), (J)–(L), and (P)–(R), respectively. Arrowheads in (F), (L), and (R) show that EdU signals were detected specifically in MGs.

(S–X) EdU incorporation in the MG-specific reporter mice. The boxed area in (T) is enlarged in (V)–(X). Arrowheads in (X) show that the EdU signals were detected in the tdTomato-labeled MGs in the ONL.

(Y) Distribution of EdU^+ cells in retinal layers. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Data are presented as mean ± SEM, n = 38.

(Z) Anti-CyclinD3 immunohistochemistry in untreated and β-catenin treated retinas at 2 weeks after viral infection. The number of $CyclinD3^+$ cells was quantified per 100 μm in retinal sections. Data are presented as mean ± SEM, n = 4.

Scale bars in (C), (O), (I), and (U), 25 μm. Scale bars in (F), (R), (L), and (X), 10 μm. Scale bars in (Z), 20 μm.

Interestingly, gene transfer of a dominant active form of β-catenin did not result in a significant increase in MG proliferation relative to wild-type β-catenin (data not shown).

GSK3β Deletion Stimulates MG Proliferation without Retinal Injury

GSK3β regulates Wnt signaling by phosphorylation of β-catenin, resulting in its degradation by the ubiquitin-proteasome system (Logan and Nusse, 2004). To investigate whether GSK3β plays a role in regulating Wnt signaling and MG proliferation in the adult mammalian retina, we deleted *GSk3β* by infecting $GSK3\beta^{loxp/loxp}$ mice at 4 weeks of age with ShH10-GFAP-Cre. In comparison to $GSK3\beta^{loxp/loxp}$ mice infected with ShH10-GFAP-tdTomato as a control (Figures S5A–S5F), deletion of *GSK3β* resulted in stabilization and nuclear accumulation of β-catenin in MGs, labeled by ShH10-GFAP-tdTomato co-infection (Figures S5G–S5L). To

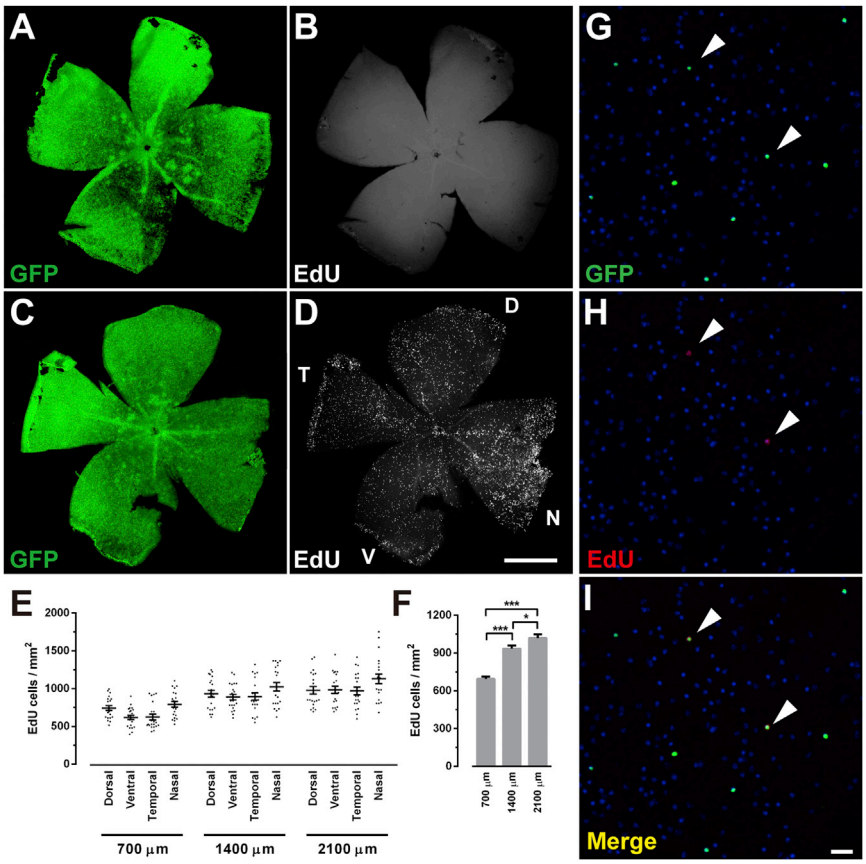

Figure 3. ShH10-GFAP-Mediated Gene Transfer of β-Catenin Is Highly Efficient to Stimulate MG Proliferation in Adult Mouse Retina

(A and B) ShH10-GFAP-GFP infection itself did not lead to detection of EdU$^+$ cells in the whole retina.

(C and D) Many MGs re-entered the cell cycle 2 weeks after ShH10-GFAP-mediated gene transfer of β-catenin and GFP (co-infection marker).

(E) The number of EdU$^+$ cells/mm^2 in each of the four retinal quadrants (Dorsal, Ventral, Nasal, and Temporal) was quantified at 700 μm, 1,400 μm, and 2,100 μm from the center of the retina. Data are presented as mean ± SEM, n = 21.

(F) Quantification of the number of EdU$^+$ cells/mm^2 at different distances from the center of the retina. Data are presented as mean ± SEM, n = 21. *p <0.05, ***p <0.001, Student's t test. Scale bar, 1 mm.

(G–I) 2 weeks after ShH10-GFAP-mediated gene transfer of β-catenin and GFP (co-infection marker), retinas were dissociated for EdU incorporation analysis. Arrowheads, GFP$^+$ cells that were also EdU$^+$. Scale bar, 20 μm.

further assess whether *GSK3β* deletion activates Wnt signaling, we infected *GSK3β*$^{loxp/loxp}$ mouse retina with ShH10-GFAP-Cre and the ShH10-Wnt reporter. In comparison to *GSK3β*$^{loxp/loxp}$ mice infected with the ShH10-Wnt reporter alone (Figure S5M), significant activation of the reporter gene was observed 2 weeks after ShH10-GFAP-Cre co-injection (Figure S5N).

In zebrafish, pharmacological inhibition of GSK3β leads to β-catenin stabilization and MG proliferation (Ramachandran et al., 2011). In cultured mouse retinal explants, GSK3 inhibitor treatment enhances MG proliferation in SvJ129 mice (Suga et al., 2014). To investigate whether *GSK3β* deletion is sufficient to stimulate MG proliferation without retinal injury, we assayed EdU incorporation in the *GSK3β*$^{loxp/loxp}$ mouse retina infected with ShH10-GFAP-Cre at 4 weeks of age. While no EdU signal was detected in the *GSK3β*$^{loxp/loxp}$ mouse retina with sham injection (Figures S6A–S6C and S6G–S6I), many EdU$^+$ cells were detected 2 weeks after ShH10-GFAP-Cre injection, co-labeled by immunohistochemistry for CyclinD3 (Figures S6D–S6F) and p27^{kip1} (Figures S6J–S6L). Our results demonstrate that *GSK3β* deletion is sufficient to stimulate MG proliferation without retinal injury.

Wnt Regulates Lin28 Expression through Transcriptional Activation in MGs

The pluripotency factor Lin28 is highly expressed in mammalian embryonic stem cells and cancer cells (Moss and Tang, 2003). Lin28 was used together with Oct4, Sox2, and Nanog to reprogram human somatic fibroblasts to pluripotent stem cells (Yu et al., 2007). In zebrafish, Lin28 regulates MG proliferation in response to retinal injury (Ramachandran et al., 2010a). To determine the role of Lin28 in MG proliferation in mammals, we first examined whether Wnt signaling regulates *Lin28* expression by analyzing *Lin28* RNA levels following gene transfer of β-catenin in adult mouse retina. In comparison to ShH10-GFAP-GFP infection as a control, the RNA levels for both *Lin28a* and *Lin28b* were significantly upregulated at 2 weeks after ShH10-GFAP-β-catenin infection (Figure S7A). We further assessed Lin28 protein levels using immunohistochemistry in retinal sections. In the ShH10-GFAP-GFP infected retina as a control, Lin28a (Figures S7B–S7D) or Lin28b (Figures S7H–S7J) was not detected. By contrast, ShH10-GFAP-mediated gene transfer of β-catenin resulted in detection of Lin28a (Figures S7E–S7G) and Lin28b (Figures S7K–S7M) in MGs, labeled by ShH10-GFAP-GFP co-infection. Our results indicate that Wnt activation induces the expression of both *Lin28a* and *Lin28b* in adult mouse MGs.

To determine whether Lin28 is a direct transcriptional target of Wnt/β-catenin signaling, we cloned a 3-kb promoter sequence of the mouse *Lin28* to drive the expression of a GFP reporter, namely Lin28a-GFP and Lin28b-GFP. We first tested the promoter activity in HEK293T cells transfected with Lin28a-GFP or Lin28b-GFP and CAG-tdTomato (a transfection marker), with or without a Flag-tagged β-catenin. In the absence of β-catenin, the reporter gene expression was barely detectable for either Lin28a-GFP (Figures S8A–S8C) or Lin28b-GFP (Figures S8J–S8L). In the presence of β-catenin, however, the reporter gene expression markedly increased for both *Lin28a* (Figures S8D–S8F) and *Lin28b* (Figures

S8M–S8O). To further examine whether β-Catenin activates *Lin28a* and *Lin28b* transcription by direct binding to their promoters as a co-activator of LEF/TCF in the canonical Wnt/β-Catenin pathway, we performed chromatin immunoprecipitation (ChIP) assays in HEK293T cells transfected with an expression vector encoding a Flag-tagged β-catenin or a Flag-tagged GFP as a control, together with the *Lin28* promoter construct. Analysis of multiple independent clones from the ChIP assay using an anti-Flag antibody revealed β-catenin-specific binding to both *Lin28a* and *Lin28b* promoters, proximally located within 500 bp upstream of the ATG start codon. Examination of the proximal promoter sequences using MatInspector (Cartharius et al., 2005) led to the identification of two putative β-catenin binding sites for *Lin28a* (Figure S8S) and *Lin28b* (Figure S8T), which were confirmed by additional ChIP assay experiments using primers flanking the putative binding sites for *Lin28a* (Figure S8U) and *Lin28b* (Figure S8V). To further validate these binding sites, we generated Lin28-GFP reporter constructs with the binding sites mutated (Figures S8S and S8T), dubbed Lin28amut-GFP and Lin28bmut-GFP. Mutation of these binding sites abolished β-catenin-activated reporter gene expression for *Lin28a* (Figures S8G–S8I and S8W) and *Lin28b* (Figures S8P–S8R and S8X). Taken together, our results demonstrate that β-catenin activates the transcription of *Lin28a* and *Lin28b* through binding to the *cis*-regulatory elements of their promoters.

To further test whether β-catenin activates the promoters of *Lin28a* and *Lin28b* in MGs, we infected adult mouse retinas at 4 weeks of age with ShH10-Lin28a-GFP or ShH10-Lin28b-GFP, in the presence or absence of ShH10-GFAP-β-catenin co-infection. 2 weeks after viral infection, the reporter gene expression was detected only in the presence of β-catenin for *Lin28a* (Figures 4A–4F) and *Lin28b* (Figures 4J–4O). Mutation of the β-catenin binding sites abolished the reporter gene expression for *Lin28a* (Figures 4G–4I) and *Lin28b* (Figures 4P–4R). The binding of β-catenin to the *Lin28* promoters was further confirmed by ChIP assays using viral infected retinal tissues for *Lin28a* (Figure 4S) and *Lin28b* (Figure 4T).

Lin28 Plays an Essential Role in Controlling MG Proliferation via *let-7* miRNAs

As a direct transcriptional target of Wnt/β-catenin signaling, we decided to examine the role of Lin28 in MG proliferation. To determine whether gene transfer of *Lin28* is sufficient to stimulate MG proliferation without retinal injury, we performed EdU incorporation analysis of adult mouse retinas infected with ShH10-GFAP-Lin28a (Figure 5A) or ShH10-GFAP-Lin28b (Figure 5B). 2 weeks after viral infection, many MGs re-entered the cell cycle with greater than 1,000 EdU^+ cells per mm^2 at 1,400 μm from the retinal center in *Lin28a* or *Lin28b* infected retinas, a robust effect relative to β-catenin gene transfer or GSK3β deletion (Figure 5C). We next investigated whether Lin28 is required for β-catenin-induced MG proliferation by co-deletion of *Lin28a* and *Lin28b* in $Lin28a^{loxp/loxp}$; $Lin28b^{loxp/loxp}$ double floxed mice. MG proliferation induced by gene transfer of β-catenin was largely neutralized in ShH10-GFAP-Cre infected retinas (Figures 5D–5F). We next examined whether Lin28 plays a role in NMDA-induced MG proliferation. $Lin28a^{loxp/loxp}$;$Lin28b^{loxp/loxp}$ double floxed mice were infected with ShH10-GFAP-Cre 2 weeks before NMDA injection. EdU was administered 5 hr before tissue collection at 60 hr post-NMDA injection. The number of EdU^+ MGs induced by NMDA damage was largely reduced when *Lin28a* and *Lin28b* were co-deleted (Figures 5G–5I). These results demonstrate that Lin28 serves as a key molecular switch downstream of the canonical Wnt pathway to regulate MG proliferation in both injured and uninjured retinas.

Lin28 plays a central role in regulating proliferative growth of cancer cells and embryonic stem cells through inhibition of post-transcriptional maturation of *let-7* miRNAs (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). *let-7* miRNAs suppress cell proliferation pathways through mRNA degradation or translation inhibition of a network of cell-cycle regulators (Shyh-Chang and Daley, 2013). Therefore, Lin28 promotes cell proliferation through *let-7* repression (Viswanathan et al., 2009). As a further downstream effector of Wnt signaling, we first examined whether Wnt activation regulates *let-7* miRNA levels using qPCR assays. As expected, ShH10-GFAP-mediated gene transfer of *Lin28a* or *Lin28b* in the adult mouse retina, compared to ShH10-GFAP-GFP infection as a control, led to a significant reduction in *let-7a*, *7b*, and *7f* miRNA levels 2 weeks after viral infection (Figure 6A). Significantly, a marked decrease in *let-7a*, *7b*, and *7f* miRNA levels was also observed in retinas infected by ShH10-GFAP-β-catenin (Figure 6A). To determine whether β-catenin-induced downregulation of *let-7* miRNAs is mediated by Lin28, we co-deleted *Lin28a* and *Lin28b* by injection of ShH10-GFAP-Cre together with ShH10-GFAP-β-catenin in $Lin28a^{loxp/loxp}$;$Lin28b^{loxp/loxp}$ double floxed mice. Co-deletion of *Lin28a* and *Lin28b* largely neutralized β-catenin-mediated effects on *let-7* miRNA downregulation (Figure 6B), indicating that Wnt/β-catenin acts through Lin28 to regulate *let-7* miRNA expression. To determine whether Wnt/Lin28-regulated *let-7* miRNA expression occurs in MGs, we constructed a *let-7* miRNA-responsive GFP sensor, with three perfectly complementary *let-7* binding sites inserted in the 3′-UTR of the GFP sensor (Cimadamore et al., 2013; Rybak et al., 2008), and packaged it into ShH10-GFAP for MG-specific delivery. In the viral transduction area labeled by ShH10-GFAP-tdTomato co-infection, the GFP sensor expression was barely detectable, indicating a high level of *let-7* miRNAs in MGs to suppress proliferation under normal conditions (Figures S9A–S9C). As expected, co-infection with ShH10-GFAP-Lin28a (Figures S9D–S9F) or ShH10-GFAP-Lin28b (Figures S9G–S9I) activated the GFP sensor in MGs. Significantly, ShH10-GFAP-mediated gene transfer of β-catenin also activated the GFP senor in MGs (Figures S9J–S9L).

To examine whether *let-7* miRNA is critically involved in Wnt/β-catenin-mediated effects on MG proliferation, we co-expressed *let-7b* miRNA with β-catenin in MGs using ShH10-GFAP-mediated gene transfer in the adult mouse retina at 4 weeks of age and observed that β-catenin-induced MG proliferation was largely suppressed (Figures 6C–6E). Taken together, our results delineate a key role of the Wnt/Lin28/*let-7* signaling module in regulating the proliferative response of MGs in adult mouse retina.

Neurogenic Potential of Cell-Cycle-Reactivated MGs

To analyze the fates of cell-cycle-reactivated MGs, we injected EdU at 10 days after ShH10-GFAP-mediated gene transfer of

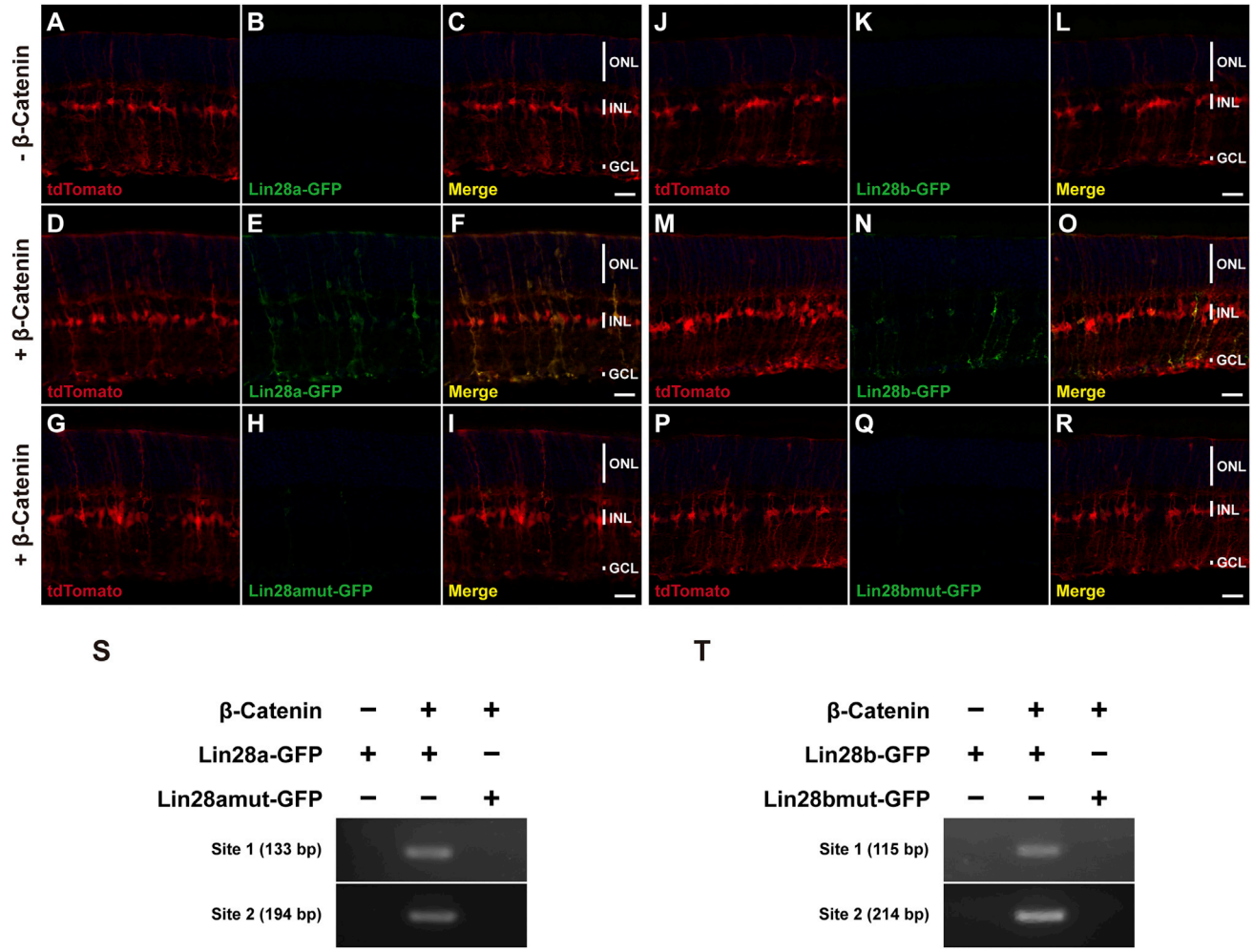

Figure 4. Wnt/β-Catenin Transactivates *Lin28a* and *Lin28b* in MGs in Adult Mouse Retina

(A–F) Lin28a-GFP reporter analysis in retinas infected with ShH10-GFAP-tdTomato (infection marker), in the absence (A–C) or presence (D–F) of ShH10-GFAP-β-catenin co-infection.

(G–I) Mutation of the β-catenin binding sites in the *Lin28a* promoter abolished the reporter activity.

(J–O) Lin28b-GFP reporter analysis in retinas infected with ShH10-GFAP-tdTomato (infection marker), in the absence (J–L) or presence (M–O) of ShH10-GFAP-β-catenin co-infection.

(P–R) Mutation of the β-catenin binding sites on the *Lin28b* promoter abolished the reporter activity. Scale bars, 20 μm.

(S and T) ChIP analysis reveals direct binding of β-catenin to the *Lin28a* (S) or the *Lin28b* (T) promoter. Adult mouse retinas were infected with ShH10-Lin28-GFP or Lin28mut-GFP, in the presence or absence of ShH10-GFAP-β-Catenin co-infection. Chromatin, immunoprecipitated with an antibody specific to β-Catenin, was assayed by PCR with primers flanking the putative β-catenin binding sites 1 and 2.

β-catenin, *Lin28a*, or *Lin28b* in the 4-week-old adult mouse retina, and quantified the number of EdU^+ at 4, 7, and 10 days after EdU injection. Although many EdU^+ cells were scored per mm^2 in retinas treated with β-catenin (970.3 ± 40), *Lin28a* (1,325.3 ± 64.4), or *Lin28b* (1,177.3 ± 94.2) at 4 days after EdU injection, the number of EdU^+ cells declined over time for all three treatment groups, with only a small number of EdU^+ cells remaining at 10 days after EdU injection (Figure S10). Our results are consistent with the previous report that the majority of cell-cycle-reactivated MGs die, resulting in a time-dependent decrease in the number of BrdU labeled cells (Ooto et al., 2004). To examine the neurogenic potential of cell-cycle-reactivated MGs, we analyzed whether EdU^+ cells express cell-type-specific markers after retinal dissociation at 4 days after EdU injection in retinas infected with ShH10-GFAP-β-catenin, *Lin28a*, or *Lin28b*. We did not detect the expression of markers for rods (rhodopsin), cones (red/green or blue cone opsins), bipolar cells (PKCα), and ganglion cells (Tuj1 or Brn3) in EdU^+ cells in the three treatment groups (data not shown). Intriguingly, a small number of EdU^+ cells were immunoreactive for amacrine cell markers (Figure 7). In β-catenin-treated retinas (Figures 7A–7C and 7J), 33.1% ± 4.3% of EdU^+ cells were positive for Pax6 (progenitors and amacrine cells), 6.5% ± 1.5% were positive for Syntaxin1 (amacrine cells), and 6.0% ± 0.9% were positive for NeuN (amacrine and

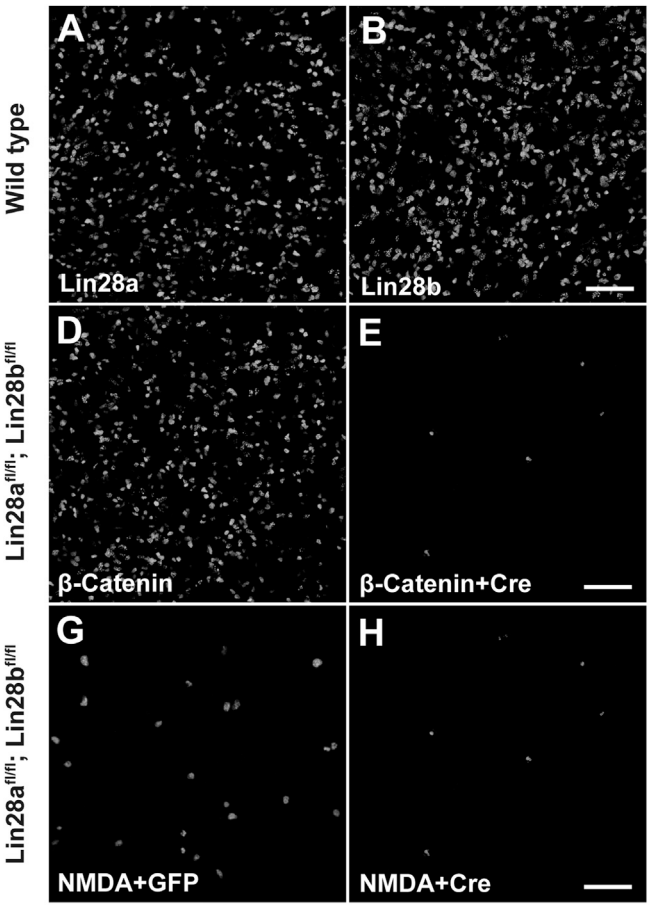

Figure 5. Lin28 Plays an Essential Role in MG Proliferation in the Adult Mouse Retina

(A–C) Lin28 is sufficient to stimulate MG proliferation without retinal injury. ShH10-GFAP-mediated gene transfer of *Lin28a* (A) or *Lin28b* (B) led to proliferative response of MGs, analyzed by EdU incorporation and quantified (C) in comparison to β-catenin gene transfer and *GSK3β* deletion.

(D–F) Co-deletion of *Lin28a* and *Lin28b* abolishes β-catenin-induced MG proliferation, and *Lin28a*$^{loxp/loxp}$;*Lin28b*$^{loxp/loxp}$ double floxed mice were infected with ShH10-GFAP-β-catenin, in the absence (D) or presence (E) of ShH10-GFAP-Cre co-infection. MG proliferation was analyzed and quantified (F) by EdU incorporation.

(G–I) Co-deletion of *Lin28a* and *Lin28b* largely suppresses NMDA-induced MG proliferation. *Lin28a*$^{loxp/loxp}$;*Lin28b*$^{loxp/loxp}$ double floxed mice were infected with ShH10-GFAP-GFP (G) or ShH10-GFAP-Cre (H) 2 weeks before NMDA damage. MG proliferation was analyzed and quantified (I) by EdU incorporation.

Scale bars, 50 μm. Data are presented as mean ± SEM, n = 4. ***p < 0.001, Student's t test.

ganglion cells). In *Lin28a*-treated retinas (Figures 7D–7F and 7J), 51.0% ± 4.0% of EdU$^+$ cells were positive for Pax6, 6.1% ± 1.1% were positive for Syntaxin1, and 5.1% ± 1.0% were positive for NeuN. In *Lin28b*-treated retinas (Figures 7G–7J), 49.5% ± 2.6% of EdU$^+$ cells were positive for Pax6, 5.5% ± 0.9% were positive for Syntaxin1, and 5.9% ± 2.0% were positive for NeuN. Using confocal microscopy in intact retina, we further confirmed the expression of amacrine cell markers in a small subset of cell-cycle-reactivated MGs located in the lower part of the inner nuclear layer where amacrine cell somas are located, following ShH10-GFAP-mediated gene transfer of β-catenin, *Lin28a*, or *Lin28b* (Figure S11). Our results signify neurogenic potential of Wnt/Lin28-activated MGs in the adult mammalian retina.

DISCUSSION

Previous studies have indicated that retinal injury is required for restoring the stem or progenitor cell status of adult mammalian MGs, evidenced by cell-cycle re-entry (Close et al., 2006; Dyer and Cepko, 2000; Karl et al., 2008; Osakada et al., 2007; Takeda et al., 2008; Wan et al., 2007, 2008). However, neurotoxin-induced retinal injury inevitably causes death of amacrine and ganglion cells and therefore is counterproductive to the aim of restoring regenerative capabilities in the mammalian retina. An injury-free strategy that would not necessitate inflicting further damage to a diseased retina is highly desirable. We found that retinal injury elicited a transient activation of Wnt signaling, prior to the proliferative response of MGs. Activation of Wnt signaling was manifested by an upregulation of multiple Wnt genes, downregulation of Wnt antagonists *Dkk1* and *WIF-1*, and transcriptional activation of Wnt target genes (*Ccnd1*, *Lef1*, and *Axin2*). Furthermore, pharmacological inhibition of Wnt signaling by XAV939 significantly reduced injury-induced MG proliferation. Consistent with studies in zebrafish that demonstrate that Wnt plays an indispensable role in injury-induced proliferation of MG-derived retinal progenitors (Ramachandran et al., 2011; Wan et al., 2014), our results indicate that Wnt is a conserved pathway for MG proliferation in mammals.

To directly activate Wnt signaling without retinal injury, we developed a MG-specific gene transfer tool (ShH10-GFAP) to deliver wild-type β-catenin and observed a robust proliferative response of MGs that was equivalent to retinal injury in combination with mitogenic growth factor treatment (Karl et al., 2008). In addition to the high efficiency in stimulating MG proliferation, targeting the intracellular signaling pathway(s) of MGs also eliminates the need for a global treatment of the entire retina with growth factors, which could cause undesirable side effects from untargeted cells. GSK3 regulates Wnt signaling by promoting β-catenin degradation. GSK3 plays an important role in the maintenance and self-renewal of human and mouse embryonic stem cells (Bone et al., 2009; Sato et al., 2004; Ying et al., 2008). Genetic evidence is required to determine whether GSK3 participates in regulating the progenitor or stem cell status of MGs in mammals. Our study showed that deletion of *GSK3β*

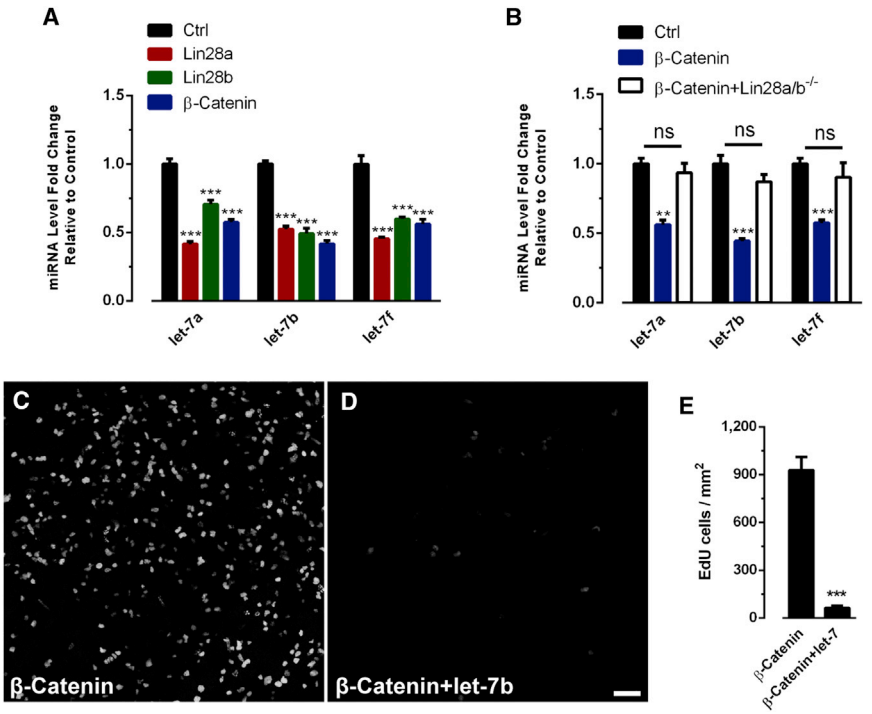

Figure 6. Wnt/β-Catenin Acts through *let-7* miRNAs to Regulate MG Proliferation

(A) ShH10-GFAP-mediated gene transfer of β-catenin downregulates *let-7a*, *let-7b*, and *let-7f* miRNA levels in the adult mouse retina. Data are presented as mean ± SEM, n = 3. ***p < 0.001, Student's t test.

(B) Co-deletion of Lin28a and Lin28b largely neutralizes β-catenin-induced suppression of *let-7a*, *let-7b*, and *let-7f* miRNAs. Data are presented as mean ± SEM, n = 3. ***p < 0.001, one-way ANOVA analysis.

(C–E) Co-expression of *let-7b* miRNA abolishes β-Catenin-mediated effects on MG proliferation, analyzed and quantified (E) by EdU incorporation. Scale bar, 25 μm. Data are presented as mean ± SEM, n = 4. ***p < 0.001, Student's t test.

resulted in β-catenin stabilization and MG proliferation without injury. Controlling GSK3β kinase activity appears to be an additional regulatory mechanism that tunes the proliferative response of MGs in the adult mammalian retina.

Lin28 has emerged as a master control gene that defines "stemness" in multiple tissue lineages (Shyh-Chang and Daley, 2013). As an RNA-binding protein, Lin28 represses *let-7* miRNA biogenesis and thus regulates the self-renewal of mammalian embryonic stem cells. Upstream factors regulating Lin28 remain largely unexplored relative to the effectors and targets downstream of Lin28/*let-7* miRNAs. In breast cancer cells, Wnt/β-catenin binds to the promoter of *Lin28a*, but not *Lin28b*, and activates its transcription (Cai et al., 2013). We found that gene transfer of β-catenin in MGs induced the expression of both Lin28a and Lin28b. Using ChIP assays, we identified distinct β-catenin binding sites in the proximal region of *Lin28a* and *Lin28b* promoters that are critical for transcriptional activation. Mutation of these binding sites abolished β-catenin-induced *Lin28a* and *Lin28b* promoter activity. Interestingly, the two β-catenin binding sites in the *Lin28a* promoter we identified are different from the one reported for augmenting cancer cell expansion (Cai et al., 2013), suggesting that distinct β-catenin binding sites may be used to drive *Lin28a* transcription in progenitor or stem cells. We further demonstrate that Lin28a and Lin28b are essential factors in regulating MG proliferation in vivo, as gene transfer of *Lin28a* or *Lin28b* was sufficient to stimulate MG proliferation, and co-deletion of *Lin28a* and *Lin28b* abolished β-catenin-induced MG proliferation. Navigating downstream, β-catenin suppressed *let-7* miRNA expression, and co-deletion of *Lin28a* and *Lin28b* neutralized β-catenin-mediated effects on *let-7* miRNA expression, indicating that Wnt/β-catenin acts through Lin28 to suppress *let-7* miRNA biogenesis. Importantly, *let-7* miRNA regulation is functionally relevant for Wnt-activated MG proliferation as β-catenin-mediated proliferation effects were suppressed by *let-7b* miRNA co-expression.

Previous studies in mice used retinal injury, in combination with growth factor treatment (Karl et al., 2008) or transgenic expression of the proneural transcription factor Ascl1 (Ueki et al., 2015), to stimulate MG proliferation, reprogramming these cells to a neurogenic competent state. The neurogenic competence of cell-cycle-reactivated MGs after injury decreases in an age-dependent manner (Löffler et al., 2015). Without retinal injury, a subset of MGs migrated to the lower part of the inner nuclear layer and expressed amacrine cell markers after gene transfer of β-catenin, *Lin28a*, or *Lin28b*. Taken together, our results provide compelling evidence that GSK3-Wnt-Lin28-*let-7* miRNA constitutes a central signaling axis in regulating proliferation and neurogenic potential of MGs in the adult mammalian retina.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving the use of animals in this study were performed in accordance with NIH guidelines implemented by the Yale University Animal Care and Use Committee. Wild-type mice (strain C57BL/6J), Rosa26-tdTomato reporter mice (strain B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J), and *Lin28a*$^{flox/flox}$; *Lin28b*$^{flox/flox}$ mice (strain Lin28a$^{tm2.1Gqda}$ Lin28b$^{tm2.1Gqda}$/J) were obtained from The Jackson Laboratory. *GSK3β*$^{flox/flox}$ mice were kindly provided by Dr. James R Woodgett (McMaster University, Ontario, Canada).

AAV Production and Intravitreal Injection

Adeno-associated virus (AAV) was produced by plasmid co-transfection and iodixanol gradient ultracentrifugation. Purified AAVs were concentrated with Amicon Ultra-15 Filter Units (Millipore) to a final titer of 1.0–5.0 × 10^{13} genome copies/mL. Intravitreal injection was performed using a microsyringe equipped with a 33-gauge needle. The tip of the needle was passed through the sclera, at the equator and next to the dorsal limbus of the eye, into the vitreous cavity. Injection volume was 1 μL per eye for AAVs or other chemicals including NMDA (Acros Organics, 100 mM) and XAV939 (Sigma, 10 μM).

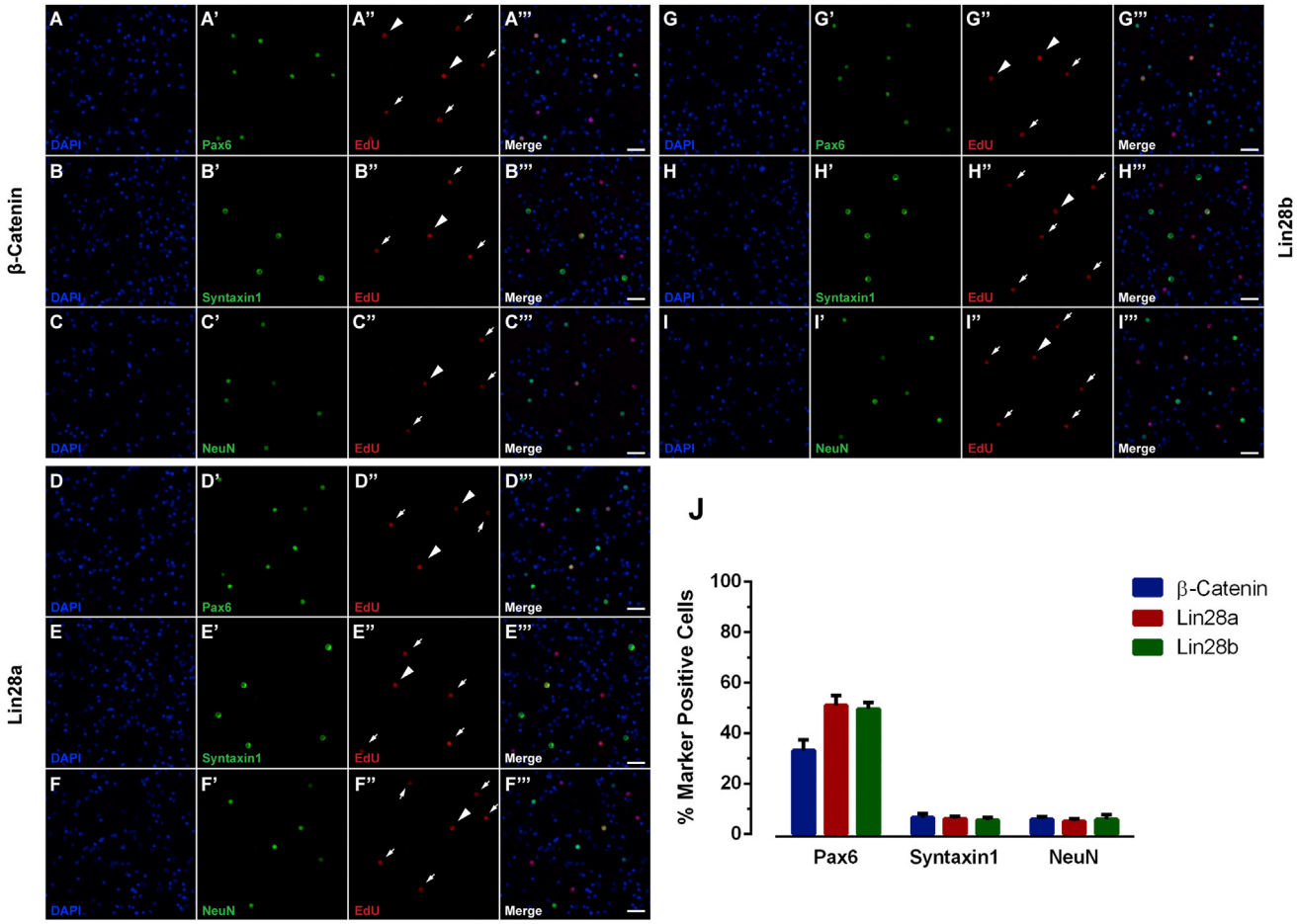

Figure 7. A Subset of Cell-Cycle-Reactivated MGs Express Markers for Amacrine Cells

Adult mouse retinas were treated with EdU 10 days after infection with ShH10-GFAP-β-Catenin, *Lin28a*, or *Lin28b*. Treated retinas were dissociated 4 days after EdU treatment and analyzed for EdU incorporation and immunohistochemistry for amacrine cell markers Pax6, Syntaxin1, and NeuN.

(A–C) Co-detection of EdU and the expression of amacrine cell markers in ShH10-GFAP-β-catenin infected retinas.

(D–F) Co-detection of EdU and the expression of amacrine cell markers in ShH10-GFAP-Lin28a infected retinas.

(G–I) Co-detection of EdU and the expression of amacrine cell markers in ShH10-GFAP-Lin28b infected retinas. Arrows, EdU positive but marker negative cells. Arrowheads, cells double positive for EdU and marker. Scale bars, 40 μm.

(J) Statistical analysis of the percentage of EdU^+ cells that were also $Pax6^+$, $Syntaxin1^+$, or $NeuN^+$. Data are presented as mean ± SEM, n = 4.

Retinal Cell Dissociation and Fluorescence-Activated Cell Sorting

Retinal dissection was performed in Hank's balanced salt solution (HBSS). Dissected retinas were incubated at 37°C for 20 min in the activated Papain mix composed of 40 μL Papain (Worthington, 500 U/mL), 40 μL Cysteine/EDTA mix (25 mM cysteine + 5 mM EDTA [pH 7.0]), and 320 μL HBSS/HEPES (normal HBSS + 10 mM HEPES). Cell pellet was collected after centrifugation for 3 min at 3,000 rpm and treated with 10 μL DNase I (Roche, 10 U/μL) in 400 μL HBSS at room temperature for 3 min with gentle trituration. Dissociated cells were resuspended in an appropriate volume of HBSS for further experiments. To purify $tdTomato^+$ MGs, retinas were isolated from MG-specific reporter mice. After retinal dissociation, cells were washed in DPBS before cell sorting using a BD FACS Aria cell sorter. After cell sorting, both $tdTomato^+$ MGs and non-MGs were subject to RNA isolation and reverse transcription.

Immunohistochemistry and Imaging

Retinas were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and sectioned at the thickness of 20 μm. Sample slides were washed with PBS before incubation with a blocking buffer containing 5% normal donkey serum, 0.1% Triton X-100, and 0.1% NaN_3 in PBS for 2 hr at room temperature. Primary antibodies were added for overnight incubation at 4°C. Primary antibodies used were CyclinD3 (Thermo Scientific), Rhodopsin (Thermo Scientific), NeuN (Millipore), $p27^{kip1}$ (BD Transduction Laboratories), GS (Millipore), Ki67 (Thermo Scientific), phospho-histone H3 (PH3) (Millipore), HuC/D (Thermo Scientific), Pax6 (BioLegend), and Syntaxin1 (Santa Cruz). Sections were washed with PBS and incubated with secondary antibodies (Jackson ImmunoResearch) for 2 hr at room temperature. Cell nuclei were counterstained with DAPI (Sigma). Confocal images were acquired using a Zeiss LSM 510 EXCITER microscope.

RNA Isolation, RT-PCR, and Real-Time qPCR

Total RNA was extracted from retinas using TRIzol (Invitrogen) according to manufacturer's instructions. Reverse transcription (RT) was performed at 42°C for 2 hr in a volume of 20 μL containing 0.5 μg of oligo(dT), 1 × RT buffer, 0.5 mM each deoxyribonucleotide triphosphate, 0.1 mM DTT, and 200 U of Superscript II reverse transcriptase (Invitrogen). Real-time qPCR was performed in triplicate with SsoFast EvaGreen supermix (Bio-Rad) using an iCycler real-time PCR detection system (Bio-Rad). The amplification protocol was 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, with a signal detection period of 7 s

at 80°C. A melt curve analysis was performed at the end of the reaction to check the reaction specificity. Results were obtained after normalization to the expression level of β-actin. All experiments were performed at least twice to ensure repeatable results.

EdU Injection and Incorporation Assay

EdU solution (1 uL) was intravitreally injected into the vitreous chamber at the concentration of 1 mg/mL. Analysis of EdU incorporation was performed using Click-iT EdU Kit (Invitrogen). In brief, retinal sections or flatmount preparations were washed in PBS for 10 min and then washed twice in PBS containing 3% BSA, followed by permeabilization in PBS containing 0.5% Triton X-100 for 20 min. EdU detection components were resuspended according to manufacturer's instructions and applied directly to the samples. The EdU reaction solution contains 215 μL 1X Click-iT reaction buffer, 10 μL CuSO4, 0.6 μL Alexa Fluor azide, and 25 μL 1X Reaction buffer additive. After incubation in the reaction solution for 30 min at room temperature, samples were washed with PBS and mounted for detection.

miRNA Analysis

Small RNAs were extracted with the *mir*Vana miRNA Isolation Kit and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Invitrogen) according to manufacturer's instructions. qRT-PCR was performed using specific primers and probes for snoRNA-202, has-let-7a, 7b, and 7f supplied in Taqman MicroRNA Assay Kits (Invitrogen). Fold changes were determined using the Δ(ΔCT) method after normalization to mouse snoRNA-202 endogenous controls.

Chromatin Immunoprecipitation Assays

HEK293T cells were transfected with the corresponding promoter and expression constructs using PEI (Polysciences). Cells were fixed 48 hr after transfection using 1% formaldehyde for 10 min at room temperature. Cross-linking reactions were terminated by adding glycine to a final concentration of 0.125 M. ChIP assays were performed according to manufacturer's instructions (Santa Cruz Biotechnology). In brief, cells were resuspended in 6 mL Lysis Buffer. Crude nuclear extract was collected after centrifugation and resuspended in 1.9 mL Lysis Buffer High Salt. After sonication, the supernatant was precleared by protein A/G PLUS-Agrose beads and then incubated with the primary antibody overnight at 4°C. After incubation with beads for 2 hr at 4°C, beads were washed twice with 1 mL Lysis Buffer High Salt and Wash Buffer and resuspended in 400 μl Elution Buffer, followed by incubation overnight at 65°C for reverse cross-linking. DNA was isolated using QIAGEN Qiaquick PCR purification Kit (QIAGEN) and analyzed by PCR.

Statistical Analysis

Statistical differences between different experimental groups were analyzed by a Student's t test or one-way ANOVA test using Prism 6 (GraphPad Software). Data are presented as mean ± SEM. A value of $p < 0.05$ is considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes 11 figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.078.

AUTHOR CONTRIBUTIONS

B.C. conceived the project. K.Y. and S.Q. performed experiments and analyzed data. L.T., W.D.S., J.G.F., and D.V.S. provided important reagents and discussion. K.Y. and B.C. wrote the paper.

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REFERENCES

Bernardos, R.L., Barthel, L.K., Meyers, J.R., and Raymond, P.A. (2007). Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. J. Neurosci. *27*, 7028–7040.

Bone, H.K., Damiano, T., Bartlett, S., Perry, A., Letchford, J., Ripoll, Y.S., Nelson, A.S., and Welham, M.J. (2009). Involvement of GSK-3 in regulation of murine embryonic stem cell self-renewal revealed by a series of bisindolylmaleimides. Chem. Biol. *16*, 15–27.

Buganim, Y., Faddah, D.A., Cheng, A.W., Itskovich, E., Markoulaki, S., Ganz, K., Klemm, S.L., van Oudenaarden, A., and Jaenisch, R. (2012). Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. Cell *150*, 1209–1222.

Cai, W.Y., Wei, T.Z., Luo, Q.C., Wu, Q.W., Liu, Q.F., Yang, M., Ye, G.D., Wu, J.F., Chen, Y.Y., Sun, G.B., et al. (2013). The Wnt-β-catenin pathway represses let-7 microRNA expression through transactivation of Lin28 to augment breast cancer stem cell expansion. J. Cell Sci. *126*, 2877–2889.

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., and Werner, T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics *21*, 2933–2942.

Chang, T.C., Zeitels, L.R., Hwang, H.W., Chivukula, R.R., Wentzel, E.A., Dews, M., Jung, J., Gao, P., Dang, C.V., Beer, M.A., et al. (2009). Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc. Natl. Acad. Sci. USA *106*, 3384–3389.

Cimadamore, F., Amador-Arjona, A., Chen, C., Huang, C.T., and Terskikh, A.V. (2013). SOX2-LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors. Proc. Natl. Acad. Sci. USA *110*, E3017–E3026.

Close, J.L., Liu, J., Gumuscu, B., and Reh, T.A. (2006). Epidermal growth factor receptor expression regulates proliferation in the postnatal rat retina. Glia *54*, 94–104.

Cohen, P., and Frame, S. (2001). The renaissance of GSK3. Nat. Rev. Mol. Cell Biol. *2*, 769–776.

Das, A.V., Mallya, K.B., Zhao, X., Ahmad, F., Bhattacharya, S., Thoreson, W.B., Hegde, G.V., and Ahmad, I. (2006). Neural stem cell properties of Müller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev. Biol. *299*, 283–302.

Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. *116*, 1175–1186.

Dyer, M.A., and Cepko, C.L. (2000). Control of Müller glial cell proliferation and activation following retinal injury. Nat. Neurosci. *3*, 873–880.

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.

Filali, M., Cheng, N., Abbott, D., Leontiev, V., and Engelhardt, J.F. (2002). Wnt-3A/beta-catenin signaling induces transcription from the LEF-1 promoter. J. Biol. Chem. *277*, 33398–33410.

Fimbel, S.M., Montgomery, J.E., Burket, C.T., and Hyde, D.R. (2007). Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. J. Neurosci. *27*, 1712–1724.

Hendzel, M.J., Wei, Y., Mancini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett-Jones, D.P., and Allis, C.D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma *106*, 348–360.

Heo, I., Joo, C., Cho, J., Ha, M., Han, J., and Kim, V.N. (2008). Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol. Cell *32*, 276–284.

Hovanes, K., Li, T.W., Munguia, J.E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R.F., and Waterman, M.L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. Nat. Genet. *28*, 53–57.

Hsieh, J.C., Kodjabachian, L., Rebbert, M.L., Rattner, A., Smallwood, P.M., Samos, C.H., Nusse, R., Dawid, I.B., and Nathans, J. (1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature *398*, 431–436.

Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature *461*, 614–620.

Iliopoulos, D., Hirsch, H.A., and Struhl, K. (2009). An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell *139*, 693–706.

Karl, M.O., Hayes, S., Nelson, B.R., Tan, K., Buckingham, B., and Reh, T.A. (2008). Stimulation of neural regeneration in the mouse retina. Proc. Natl. Acad. Sci. USA *105*, 19508–19513.

Kim, W.Y., Wang, X., Wu, Y., Doble, B.W., Patel, S., Woodgett, J.R., and Snider, W.D. (2009). GSK-3 is a master regulator of neural progenitor homeostasis. Nat. Neurosci. *12*, 1390–1397.

Klimczak, R.R., Koerber, J.T., Dalkara, D., Flannery, J.G., and Schaffer, D.V. (2009). A novel adeno-associated viral variant for efficient and selective intravitreal transduction of rat Müller cells. PLoS ONE *4*, e7467.

Koerber, J.T., Klimczak, R., Jang, J.H., Dalkara, D., Flannery, J.G., and Schaffer, D.V. (2009). Molecular evolution of adeno-associated virus for enhanced glial gene delivery. Mol. Ther. *17*, 2088–2095.

Kuzmanovic, M., Dudley, V.J., and Sarthy, V.P. (2003). GFAP promoter drives Müller cell-specific expression in transgenic mice. Invest. Ophthalmol. Vis. Sci. *44*, 3606–3613.

Lie, D.C., Colamarino, S.A., Song, H.J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., and Gage, F.H. (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370–1375.

Liu, B., Hunter, D.J., Rooker, S., Chan, A., Paulus, Y.M., Leucht, P., Nusse, Y., Nomoto, H., and Helms, J.A. (2013). Wnt signaling promotes Müller cell proliferation and survival after injury. Invest. Ophthalmol. Vis. Sci. *54*, 444–453.

Löffler, K., Schäfer, P., Völkner, M., Holdt, T., and Karl, M.O. (2015). Age-dependent Müller glia neurogenic competence in the mouse retina. Glia *63*, 1809–1824.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. *13*, 133–140.

Moss, E.G., and Tang, L. (2003). Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. Dev. Biol. *258*, 432–442.

Newman, M.A., Thomson, J.M., and Hammond, S.M. (2008). Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. RNA *14*, 1539–1549.

Ooto, S., Akagi, T., Kageyama, R., Akita, J., Mandai, M., Honda, Y., and Takahashi, M. (2004). Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc. Natl. Acad. Sci. USA *101*, 13654–13659.

Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., and Takahashi, M. (2007). Wnt signaling promotes regeneration in the retina of adult mammals. J. Neurosci. *27*, 4210–4219.

Qin, Z., Barthel, L.K., and Raymond, P.A. (2009). Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish. Proc. Natl. Acad. Sci. USA *106*, 9310–9315.

Ramachandran, R., Fausett, B.V., and Goldman, D. (2010a). Ascl1a regulates Müller 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.

Resnitzky, D., Gossen, M., Bujard, H., and Reed, S.I. (1994). Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol. Cell. Biol. *14*, 1669–1679.

Rybak, A., Fuchs, H., Smirnova, L., Brandt, C., Pohl, E.E., Nitsch, R., and Wulczyn, F.G. (2008). A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat. Cell Biol. *10*, 987–993.

Sahel, J.A., Albert, D.M., Lessell, S., Adler, H., McGee, T.L., and Konrad-Rastegar, J. (1991). Mitogenic effects of excitatory amino acids in the adult rat retina. Exp. Eye Res. *53*, 657–664.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. *10*, 55–63.

Scholzen, T., and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J. Cell. Physiol. *182*, 311–322.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA *96*, 5522–5527.

Shyh-Chang, N., and Daley, G.Q. (2013). Lin28: primal regulator of growth and metabolism in stem cells. Cell Stem Cell *12*, 395–406.

Suga, A., Sadamoto, K., Fujii, M., Mandai, M., and Takahashi, M. (2014). Proliferation potential of Müller glia after retinal damage varies between mouse strains. PLoS ONE *9*, e94556.

Takeda, M., Takamiya, A., Jiao, J.W., Cho, K.S., Trevino, S.G., Matsuda, T., and Chen, D.F. (2008). alpha-Aminoadipate induces progenitor cell properties of Müller glia in adult mice. Invest. Ophthalmol. Vis. Sci. *49*, 1142–1150.

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

Thummel, R., Kassen, S.C., Montgomery, J.E., Enright, J.M., and Hyde, D.R. (2008). Inhibition of Müller glial cell division blocks regeneration of the light-damaged zebrafish retina. Dev. Neurobiol. *68*, 392–408.

Ueki, Y., Wilken, M.S., Cox, K.E., Chipman, L., Jorstad, N., Sternhagen, K., Simic, M., Ullom, K., Nakafuku, M., and Reh, T.A. (2015). Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. Proc. Natl. Acad. Sci. USA *112*, 13717–13722.

Vecino, E., Rodriguez, F.D., Ruzafa, N., Pereiro, X., and Sharma, S.C. (2016). Glia-neuron interactions in the mammalian retina. Prog. Retin. Eye Res. *51*, 1–40.

Viswanathan, S.R., Daley, G.Q., and Gregory, R.I. (2008). Selective blockade of microRNA processing by Lin28. Science *320*, 97–100.

Viswanathan, S.R., Powers, J.T., Einhorn, W., Hoshida, Y., Ng, T.L., Toffanin, S., O'Sullivan, M., Lu, J., Phillips, L.A., Lockhart, V.L., et al. (2009). Lin28 promotes transformation and is associated with advanced human malignancies. Nat. Genet. *41*, 843–848.

Wan, J., Zheng, H., Xiao, H.L., She, Z.J., and Zhou, G.M. (2007). Sonic hedgehog promotes stem-cell potential of Müller glia in the mammalian retina. Biochem. Biophys. Res. Commun. *363*, 347–354.

Wan, J., Zheng, H., Chen, Z.L., Xiao, H.L., Shen, Z.J., and Zhou, G.M. (2008). Preferential regeneration of photoreceptor from Müller glia after retinal degeneration in adult rat. Vision Res. *48*, 223–234.

Wan, J., Zhao, X.F., Vojtek, A., and Goldman, D. (2014). Retinal injury, growth factors, and cytokines converge on β-catenin and pStat3 signaling to stimulate retina regeneration. Cell Rep. *9*, 285–297.

Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519–523.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917–1920.