Development 140, 1445-1456 (2013) doi:10.1242/dev.071878

SOX2 maintains the quiescent progenitor cell state of postnatal retinal Müller glia

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SUMMARY

Within discrete regions of the developing mammalian central nervous system, small subsets of glia become specialized to function as neural stem cells. As a result of their self-renewal and neurogenic capacity, these cells later serve to replenish neurons and glia during persistent or injury-induced adult neurogenesis. SOX2, an HMG box transcription factor, plays an essential role in the maintenance of both embryonic and adult neural progenitors. It is unclear, however, which biological mechanisms regulated by SOX2 are required for neural stem cell maintenance. In this study, we address this question through genetic analysis of SOX2 function in differentiating postnatal Müller glia, a cell type that maintains neurogenic capacity in the adult retina. By utilizing molecular analysis and real-time imaging, we show that two progenitor characteristics of nascent Müller glia – their radial morphology and cell cycle quiescence – are disrupted following conditional genetic ablation of *Sox2* in the mouse postnatal retina, leading to Müller cell depletion and retinal degeneration. Moreover, we demonstrate that genetic induction of the Notch signaling pathway restores Müller glial cell identity to *Sox2* mutant cells, but does not secure their quiescent state. Collectively, these results uncouple the roles of SOX2 and the Notch signaling pathway in the postnatal retina, and uncover a novel role for SOX2 in preventing the depletion of postnatal Müller glia through terminal cell division.

KEY WORDS: SOX2, Quiescence, Retina, Müller glia

INTRODUCTION

During central nervous system (CNS) development, both neurons and glia derive from a population of radial neural progenitors. SOX2, a member of the HMG box transcriptional activators (of the SOXB1 subfamily), plays an essential role in progenitor cell maintenance in the developing and adult CNS (Favaro et al., 2009; Ferri et al., 2004; Pevny and Lovell-Badge, 1997; Wegner and Stolt, 2005). Disruption of SOX2 function in the embryonic and adult CNS results in both a loss of neural progenitor cells (NPCs) and a decrease in neurogenesis. To date, the key biological function of SOX2 required for NPC maintenance has not been elucidated. In this study, we address this question by genetically dissecting the role of SOX2 in the postnatal mouse retina.

In the developing retina, six neuronal and one glial cell type are produced by retinal progenitor cells (RPCs) in a distinct temporal order that is conserved among vertebrates (Livesey and Cepko, 2001). To ensure the correct final composition of the retina with respect to the ratios of neuronal and glial cell types, a delicate balance between RPC maintenance and differentiation must be preserved throughout the course of retinogenesis. This balance is achieved through the coordinated actions of transcription factors regulating neuronal cell fate specification, such as those of the basic helix-loop-helix (bHLH) and homeobox families, and the molecular mechanisms acting to maintain the undifferentiated state of RPCs (Marquardt, 2003; Ohsawa and Kageyama, 2008). We previously demonstrated that SOX2 is essential for the maintenance of both the RPC undifferentiated state and their neurogenic potential (Matsushima et al., 2011; Taranova et al., 2006). Ablation of *Sox2* in embryonic RPCs abrogates neurogenesis through the loss of key RPC characteristics: their molecular identity, morphology and proliferative and differentiation capacity. In this study we dissect the mechanism of SOX2 function in the maintenance of NPC identity.

During the postnatal period, RPCs primarily give rise to rod photoreceptors, bipolar cells and Müller glia (MG) (Marquardt and Gruss, 2002; Ohsawa and Kageyama, 2008). Among these late-born cell types, MG are the only cells to maintain both an RPC-like radial morphology and gene expression profile, including expression of SOX2 (Nelson et al., 2011; Roesch et al., 2008; Taranova et al., 2006). Under homeostatic conditions, MG support neuronal function by maintaining retinal architecture and providing trophic support. However, recent evidence suggests that MG do not irreversibly exit the progenitor state, as they can re-enter the cell cycle in response to retinal injury and maintain the potential to give rise to neurons (Del Debbio et al., 2010; Fischer and Reh, 2001; Hitchcock et al., 2004; Karl et al., 2008; Ooto et al., 2004). Thus, MG are considered a presumptive neural stem cell population in the retina and a key target of potential retinal regeneration therapies.

In this study, we utilize the transition of postnatal RPCs toward MG cell fate as an experimental premise to address the role of SOX2 in NPC maintenance. We combine mouse genetics and real-time imaging approaches to demonstrate that SOX2 is required to maintain the progenitor characteristic of MG in the early postnatal period. Ablation of *Sox2* causes nascent MG to lose their radial morphology, resulting in the disruption of retinal cellular organization. Real-time imaging of early postnatal MG reveals that, in contrast to *Sox2*-expressing quiescent MG, *Sox2* mutant MG undergo ectopic cell division.

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Accepted 26 January 2013

The re-entry of MG into the cell cycle in response to retinal injury is tightly associated with their neurogenic capacity (Fischer and Reh, 2001; Jadhav et al., 2009; Karl et al., 2008). Several pathways that regulate embryonic NPC identity have been implicated in this process (Jadhav et al., 2009; Roesch et al., 2008). One of the key regulators of both neural progenitor identity and gliogenesis is the Notch signaling pathway (Jadhav et al., 2006a; Jadhav et al., 2009). Components of the Notch pathway, including its transcriptional downstream regulators HES1/5, not only serve as markers of MG during postnatal development but also play a crucial role in establishing their cell identity (Hojo et al., 2000; Takatsuka et al., 2004; Tomita et al., 1996). Furthermore, HES1 regulates the quiescent state of neural stem cells (NSCs) through its sustained non-oscillatory expression, and can act as a safeguard against irreversible cell cycle exit during quiescence (Sang and Coller, 2009; Sang et al., 2008; Shimojo et al., 2011). We previously demonstrated that *Notch1* is a direct transcriptional target of SOX2 in the developing CNS (Matsushima et al., 2011; Taranova et al., 2006). Here we show that expression of both *Notch1* and *Hes5* is lost following ablation of *Sox2* in the postnatal retina. Moreover, we demonstrate that genetic induction of Notch signaling restores MG identity to *Sox2* mutant cells. However, Notch is insufficient to re-establish the quiescent state of nascent *Sox2* mutant MG.

Collectively, these results reveal a new role for SOX2 in the maintenance of the progenitor characteristics of postnatal MG, and demonstrate that sustained SOX2 expression in the postnatal retina is required to maintain MG quiescence.

MATERIALS AND METHODS

Mouse breeding

All animal work was carried out in accordance with the University of North Carolina IACUC and DLAM approval. The mouse lines described in this study were maintained on a C57BL/6J background. Generation of the *Sox2^{COND}* mouse line was described previously (Taranova et al., 2006). *CAGG-CreER^{TM}* transgenic mice were a gift from Dr A. P. McMahon (Hayashi and McMahon, 2002). *Rosa26* reporter (*R26R*) (Soriano, 1999) and *CALSL-NICD* [*Tg(ACTB-Notch1)1Shn*] (Yang et al., 2004) transgenic mice were obtained from Jackson Laboratories. Genotyping primers are listed in supplementary material Table S1.

Retinal explant culture

Retinal explants were prepared essentially as described (Hatakeyama and Kageyama, 2002). Briefly, P0-P1 retinas were cultured on 30-mm filter inserts (Millicell), two retinas per filter, with the retinal ganglion cell layer facing upward. The culture medium consisted of 50% MEM with Hepes and L-glutamine (Gibco), 25% 1× HBSS (Gibco), 25% heat-inactivated horse serum (Gibco), 5.75 mg/ml glucose and penicillin/streptomycin (Gibco). Each well was filled with 1 ml medium; 0.5 ml of medium was replaced daily. 4-hydroxytamoxifen (Sigma) was dissolved in 100% ethanol and added to the culture medium at 1 μM for the first 24 hours. BrdU (Sigma) was added to the culture medium at 1 μM for 2 hours. Retinas were cultured at 34°C in 5% CO_2.

DNA constructs

The following DNA constructs were used: pCIG2 (Addgene), pCAG-H2BEGFP (Konno et al., 2008), GLASTp-dsRED2 [a gift from Dr N. Gaiano, The Johns Hopkins University School of Medicine, Baltimore, MD, USA (Mizutani et al., 2007)], pCRALBP-dsRED [Addgene (Matsuda and Cepko, 2007)], pCALNL-dsRED and pCALNL-EGFP (a gift from Dr C. L. Cepko, Harvard Medical School, Boston, MA, USA). To generate pCRALBP-CreEGFP-Nuc, the CreEGFP sequence was excised from pCAG-CreEGFP-Nuc (a gift from Dr Jaime Rivera, University of Massachusetts Medical School, Worcester, MA, USA) using *Eco*RI, blunt-ended and digested with *Not*I. The CreEGFP-Nuc fragment was inserted into pCRALBP-dsRED2 following the removal of the dsRED2 cassette using *Sma*I and *Not*I.

In vitro retina electroporation and time-lapse imaging

In transfection experiments, retinas were dissected at P0 and electroporated using an *in vitro* electroporation chamber (Nepagene) filled with 80 μl 2-5 μg/ml DNA in 1× HBSS, essentially as described (Donovan and Dyer, 2007). Five 30 V pulses of 50 msecond with 950 msecond interpulse durations were delivered to the chamber. For time-lapse imaging, retinas were subsequently embedded in 5% low melting point agarose/MEM blocks, sectioned vertically into 200 μm slices using a microtome (Leica) and cultured as described above. Retinal slices were imaged on days 3-4 of culture using an Olympus FV1000 laser-scanning microscope (inverted) equipped with an enclosure for environmental control (Precision Plastics); temperature and CO_2 were set to 34°C and 5%, respectively. *z*-stacks (1.5-2.5 μm apart) were collected at 800×800 resolution using a 40× dry objective every 30-50 minutes over the course of 12-24 hours. Collected image series were processed using ImageJ (NIH) and Adobe Photoshop software.

Immunohistochemistry, *in situ* hybridization and X-gal staining

Immunostaining was performed as described (Taranova et al., 2006). An antigen retrieval method was used for antibodies against PH3, PCNA and BrdU. Slides were heated to 60-80°C in a buffer containing 10 mM sodium citrate and 0.001% Tween 20, pH 6.0. The antibodies and dyes used in this study are listed in supplementary material Table S2. X-gal staining was performed as described (Matsushima et al., 2011). *In situ* hybridization was performed on 14 μm frozen retinal tissue sections using digoxigenin (DIG)-labeled antisense RNA probes followed by enzymatic detection according to manufacturer protocols (Roche). The following probes were used: *Chx10* (*Vsx2* – Mouse Genome Informatics) [a gift from Dr R. McInnes, The Hospital for Sick Children, Toronto, CA (Horsford et al., 2005)], *Hes5* (Chenn and Walsh, 2002) and *Notch1* (Lardelli and Lendahl, 1993).

Images were obtained on a Leica DMIRB inverted microscope equipped with either SPOT (RT Color Diagnostics) or Retiga (SRV-1394) cameras, or on a laser-scanning upright microscope (Zeiss LSM 510), and processed using Adobe Photoshop software.

Statistical analysis

Retinas from at least three pups of each genotype from independent litters were analyzed for every condition and molecular marker described in this study. Cells were counted on non-consecutive 14 μm sections through the central retinas of individual pups from separate litters. Unpaired two-tailed Student's *t*-test or one-way ANOVA and Tukey's multiple comparison test (Prism software, GraphPad) were used for analysis. Dividing GLAST-dsRED2-labeled cells were counted on *z*-stacks collected from retinal slices imaged for 12 hours. The number of cells counted ranged from 230 to 691 per genotype.

RESULTS

Sustained expression of SOX2 and *Notch1* defines MG in the postnatal and adult retina

During the first postnatal week, the pool of RPCs rapidly decreases as they undergo terminal cell divisions (Young, 1985b). We analyzed the dynamics of SOX2 expression in the developing retina between postnatal day (P) 0 and 10 and compared it with that of *Notch1*, a known regulator of MG specification (Fig. 1) (Jadhav et al., 2006a; Jadhav et al., 2006b; Jadhav et al., 2009; Nelson et al., 2011). At P0, SOX2 and *Notch1* are expressed in retinal progenitors within the neuroblast layer (NBL) of the retina (Fig. 1A,G). SOX2 expression is also maintained in a subset of amacrine (starburst) cells occupying the future inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 1A, arrowhead) (Taranova et al., 2006). By P4, SOX2 and *Notch1* expression in the outer nuclear layer (ONL) is significantly reduced, concomitant with a decrease in the number of progenitor cells (Fig. 1B,H) (Young, 1985a; Young, 1985b). By P7, SOX2 and *Notch1* are no longer detected in the ONL, and their expression is restricted predominantly to the INL (Fig. 1C,I). This corresponding pattern of SOX2 and *Notch1* expression is observed at P10 and is maintained into adulthood (Fig. 1D-F,J-L).

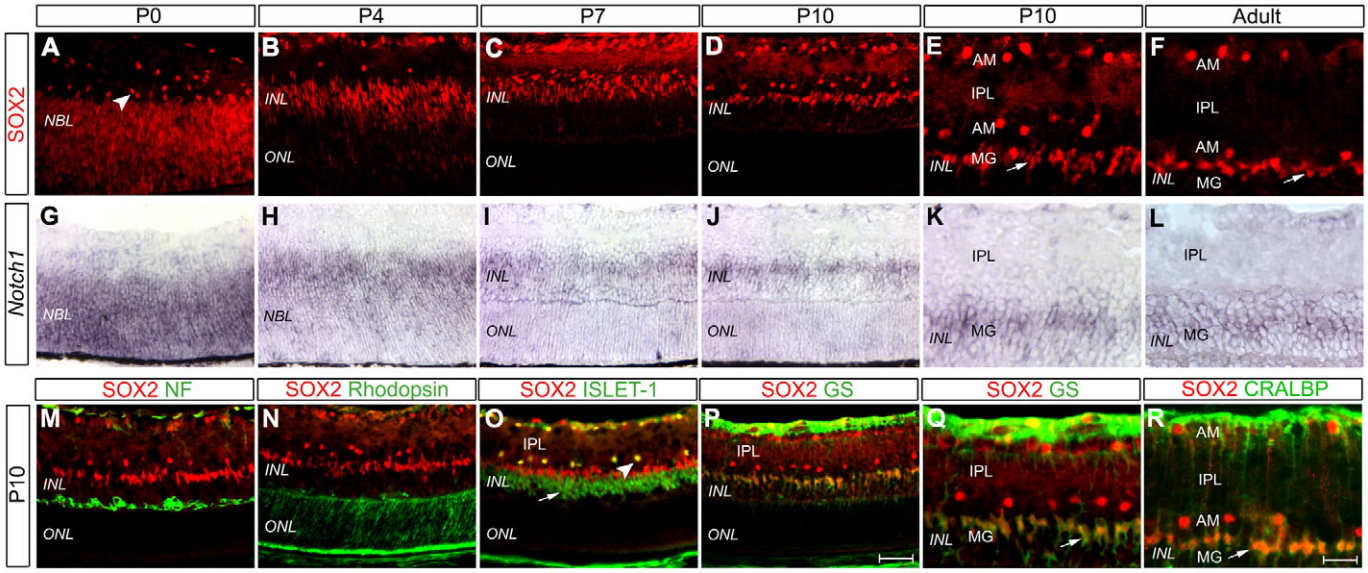

Fig. 1. SOX2 and NOTCH1 are expressed in postnatal retinal progenitor cells and in Müller glia. (**A-F**) Expression of SOX2 in the mouse retina was assessed by immunohistochemistry at the indicated ages. Arrowhead (A), amacrine cell; arrows (E,F), Müller glia (MG). (**G-L**) *Notch1* expression was examined by *in situ* hybridization. (**M-R**) SOX2 does not colocalize with NF (M) or rhodopsin (N). SOX2 is co-expressed with ISLET1 in amacrine cells (O, arrowhead) but not in bipolar cells (O, arrow). SOX2 colocalizes with GS (P, higher magnification in Q) and CRALBP (R) in MG at P10 (P,Q) and in the adult retina (R). AM, amacrine cells; GS, glutamine synthetase; IPL, inner plexiform layer; INL, inner nuclear layer; NBL, neuroblast layer; NF, neurofilament; ONL, outer nuclear layer. Scale bars: 65 μm in P for A-D,G-J,M-P; 30 μm in R for E,F,K,L,Q,R.

To determine the identity of SOX2-expressing cells in the INL, we compared the expression of SOX2 at P10 with the following markers of postmitotic neurons and glia: neurofilament (NF; horizontal cells), rhodopsin (rod photoreceptors), ISLET1 (subsets of amacrine, ganglion and bipolar cells), glutamine synthetase (GS; MG) and CRALBP (RLBP1 – Mouse Genome Informatics) (MG) (Fig. 1M-R). We find that SOX2 is mutually exclusive of NF (Fig. 1M) and rhodopsin (Fig. 1N). SOX2-positive amacrine cells express ISLET1 (Fig. 1O, arrowhead), whereas ISLET1-expressing bipolar cells in the INL do not co-express SOX2 (Fig. 1O, arrow) (Elshatory et al., 2007). Cells that maintain SOX2 in the INL are MG, as defined by the elongated cell body morphology and expression of both GS and CRALBP (Fig. 1P-R). In summary, SOX2 and *Notch1* display overlapping patterns of expression in the postnatal mouse retina and define RPCs and differentiating MG.

Temporally regulated ablation of *Sox2* and genetic induction of Notch signaling in the early postnatal retina

The Notch signaling pathway plays an important role in regulating NPC fate decisions; sustained activity of NOTCH1 is essential for the establishment and maintenance of MG cell identity (Furukawa et al., 2000; Jadhav et al., 2009). The role of SOX2 in MG cells is less well understood. We therefore sought to examine and compare the respective roles of SOX2 and NOTCH1 in the postnatal retina during MG development. To establish an experimental system allowing for analysis of SOX2 in differentiating MG, we first generated $Sox2^{COND/COND}$;*CAGGCre-ER*^{TM} (referred to as $Sox2^{MUTANT}$) animals, in which Cre expression is regulated by the ubiquitously active chicken β-actin promoter, and Cre activation is mediated by 4-hydroxytamoxifen (TM) (supplementary material Fig. S1A) (Hayashi and McMahon, 2002). To ablate *Sox2* during the period of MG cell development, P0-P1 $Sox2^{MUTANT}$ retinas were treated with TM *in vitro* and cultured for 5-7 days (supplementary material Fig. S1B). We find that expression of SOX2 in $Sox2^{MUTANT}$ retinas is reduced within 2 days, and is no longer detected in the INL at 4-5 days of culture (Fig. 2C; supplementary material Fig. S1S,T), whereas in $Sox2^{+/+}$;*CAGGCre-ER*^{TM} (referred to as $Sox2^{CONTROL}$) retinas cultured for 5 days, SOX2 is maintained in nascent MG in the INL and in a subset of amacrine cells (Fig. 2A; supplementary material Fig. S1S,T). Both retinal structural organization and expression of MG and rod photoreceptor markers are preserved in $Sox2^{CONTROL}$ retinas treated with TM at P0-P1 and cultured for 5 days (supplementary material Fig. S1C-R). When the fates of mosaically labeled RPCs are examined in TM-treated $Sox2^{CONTROL}$ and $Sox2^{MUTANT}$ retinas using electroporation of the Cre reporter construct pCALNL-EGFP at P1, no significant changes in the ratios of late-born MG, rod photoreceptors and bipolar cells are detected (supplementary material Fig. S2). These results suggest that TM-induced loss of *Sox2* in the postnatal retina is gradual and does not dramatically affect cell fate choices of RPCs at P1, thus making newly specified MG amenable to further analysis of SOX2 function in these cells.

We previously showed that *Notch1* is a downstream transcriptional target of SOX2 in embryonic RPCs (Matsushima et al., 2011; Taranova et al., 2006). To determine whether ablation of *Sox2* in the postnatal retina leads to changes in the components of the Notch signaling pathway, we examined the expression of *Notch1* and *Hes5* in $Sox2^{MUTANT}$ compared with $Sox2^{CONTROL}$ retinas treated with TM and cultured for 5 days. We find that, in contrast to $Sox2^{CONTROL}$ retinas in which both *Notch1* and *Hes5* are maintained in the INL, expression of *Notch1* and *Hes5* is downregulated in $Sox2^{MUTANT}$ retinas (Fig. 2E,I versus 2G,K).

We next used a transgenic approach to genetically induce Notch signaling in $Sox2^{MUTANT}$ retinas via Cre-dependent activation of the *CALSL-NICD* transgene, which encodes the NOTCH1 intracellular domain (NICD) (Yang et al., 2004). We find that NICD activity restores the expression of *Notch1* and *Hes5* in $Sox2^{MUTANT}$;*CALSL-*

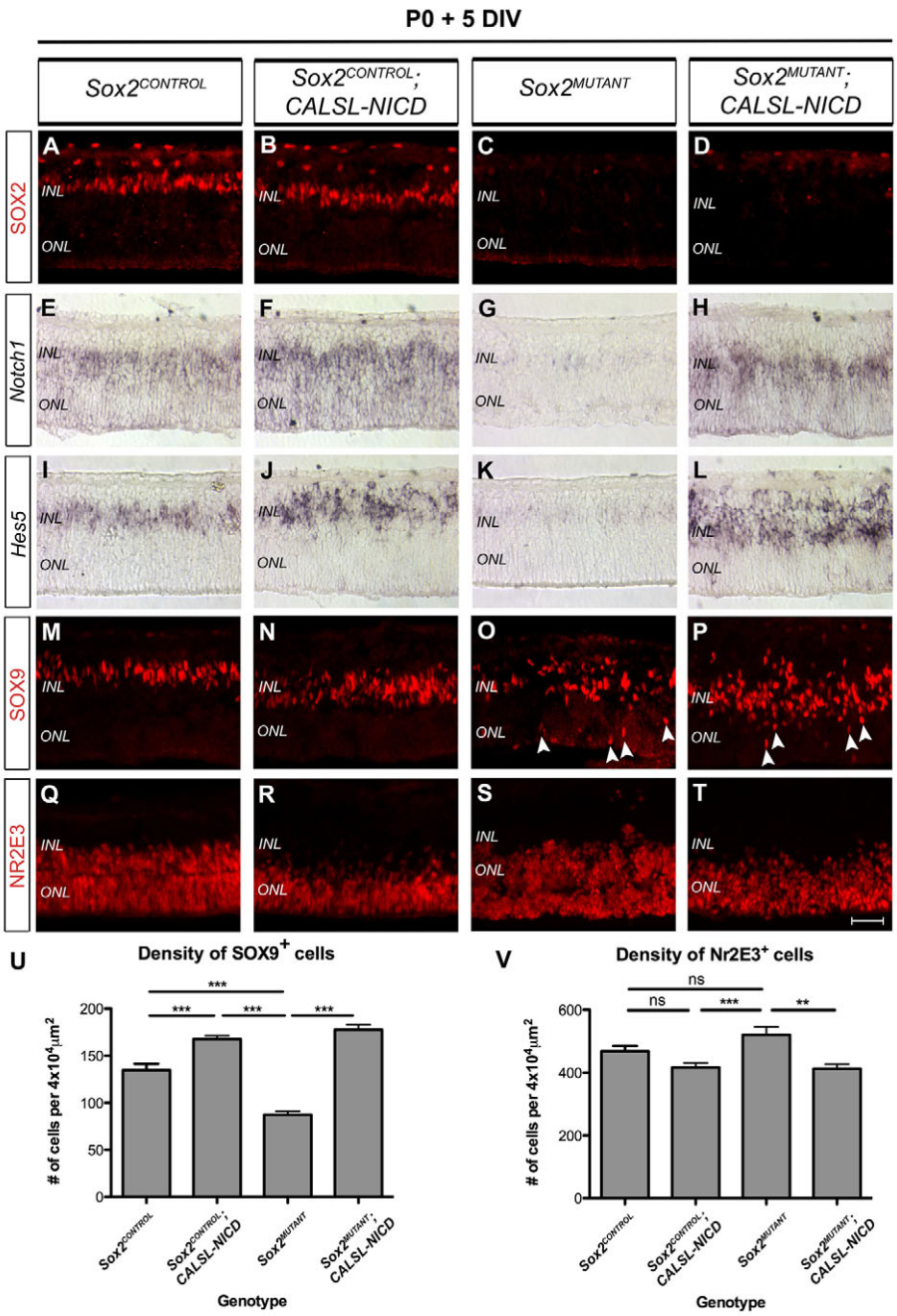

Fig. 2. Loss of NOTCH1 activity and disorganization of MG in *Sox2*^{MUTANT} retinas. (**A-D**) SOX2 expression is not detected in *Sox2*^{MUTANT} (C) and *Sox2*^{MUTANT}*;CALSL-NICD* (D) TM-treated retinas cultured for 5 days. (**E-L**) Expression (*in situ* hybridization) of *Notch1* (E-H) and *Hes5* (I-L) is lost in *Sox2*^{MUTANT} (G,K) retinas and is restored by NICD activity in *Sox2*^{MUTANT}*;CALSL-NICD* retinas (H,L). (**M-P**) SOX9-expressing MG are displaced to the ONL in both *Sox2*^{MUTANT} (O, arrowheads) and *Sox2*^{MUTANT}*;CALSL-NICD* (P, arrowheads) retinas. (**Q-T**) NR2E3-expressing rod photoreceptor precursors are reduced in number in *Sox2*^{CONTROL}*;CALSL-NICD* (R) and *Sox2*^{MUTANT}*;CALSL-NICD* (T) retinas, and are disorganized in *Sox2*^{MUTANT} retinas (S). (**U**,**V**) Total densities of SOX9-positive (U) and NR2E3-positive (V) cells were quantified on sections of TM-treated retinas of the indicated genotypes. ***P<0.0001; ns, not significant. Error bars indicate s.e.m. *Sox2*^{CONTROL} refers to *Sox2*$^{+/+}$*;CAGGCre-ER*^{TM}; *Sox2*^{MUTANT} refers to *Sox2*$^{COND/COND}$*;CAGGCre-ER*^{TM}. TM, 4-hydroxytamoxifen. Scale bar: 45 µm in A-T.

NICD retinas (Fig. 2H,L). These results demonstrate that expression of *Notch1* and its downstream effector *Hes5* in the postnatal retina is downregulated in the absence of SOX2, and can be ectopically maintained in SOX2-deficient retinas through activation of the *CALSL-NICD* transgene, thereby establishing an experimental premise to probe the functional relationship between SOX2 and NOTCH1 in the development of postnatal MG.

Reduced density and disorganization of MG in *Sox2*^{MUTANT} retinas

The generation of MG during the final divisions of postnatal RPCs is coupled with the genesis of rod photoreceptors (Gomes et al., 2011; Turner and Cepko, 1987). We examined the final densities of MG and rod photoreceptors in *Sox2*^{MUTANT} retinas cultured for 5 days using SOX9 and NR2E3 to mark MG and rod photoreceptor precursors, respectively. We find that, in contrast to SOX9-expressing MG in *Sox2*^{CONTROL} retinas, which are confined to the INL, cell bodies of MG in *Sox2*^{MUTANT} retinas treated with TM at P0-P1 and cultured for 5 days are found in both the INL and ONL and are significantly disorganized (Fig. 2O, arrowheads). Furthermore, compared with *Sox2*^{CONTROL} retinas, the total density of SOX9-positive nascent MG in *Sox2*^{MUTANT} retinas at day 5 of culture is reduced (87.0±3.7 versus 134.8±6.6, mean ± s.e.m.; P<0.0001) (Fig. 2M versus 2O,U). Conversely, the density of NR2E3-expresssing rod photoreceptor precursors in *Sox2*^{MUTANT} retinas is moderately increased compared with *Sox2*^{CONTROL} retinas (520.6±26.4 versus 468.5±17.1; P>0.05) (Fig. 2V), and the ONL is disorganized.

Consistent with the role of NOTCH1 in regulating MG and rod photoreceptor cell fates in the developing retina, genetic activation of NICD in *Sox2*^{CONTROL}*;CALSL-NICD* TM-treated retinas promotes MG and attenuates photoreceptor cell genesis (Fig. 2M

versus 2N, 2Q versus 2R) (Bao and Cepko, 1997; Jadhav et al., 2006a; Jadhav et al., 2006b; Jadhav et al., 2009; Nelson et al., 2011; Yaron et al., 2006). Specifically, the density of SOX9-positive MG in *Sox2*^{CONTROL}*;CALSL-NICD* retinas is increased compared with *Sox2*^{CONTROL} retinas (167.7±3.7 versus 134.8±6.6; $P<0.001$) (Fig. 2M versus 2N,U), whereas the density of NR2E3-positive rod photoreceptor precursors is decreased (416.5±15.2 versus 468.5±17.1; $P>0.05$) (Fig. 2Q versus 2R,V).

Activation of the Notch signaling pathway in *Sox2*^{MUTANT}*;CALSL-NICD* retinas leads to an increase in the density of SOX9-positive MG, compared with *Sox2*^{MUTANT} retinas (177.8±5.4 versus 87.0±3.7; $P<0.0001$), to the extent observed in *Sox2*^{CONTROL}*;CALSL-NICD* retinas (167.7±3.7 versus 177.8±5.4; $P>0.05$) (Fig. 2O versus 2P,N,U). However, the vast majority of MG in *Sox2*^{MUTANT}*;CALSL-NICD* retinas remain displaced throughout the INL and ONL (Fig. 2P, arrowheads). Changes in density and disorganization of nascent MG in *Sox2*^{MUTANT} and *Sox2*^{MUTANT}*;CALSL-NICD* retinas are also observed by immunostaining against PAX6, vimentin and nestin (supplementary material Figs S3, S4). These data demonstrate that MG in *Sox2*^{MUTANT} retinas are formed but are reduced in number and are significantly disorganized. Furthermore, activation of the Notch signaling pathway in a *Sox2*-deficient background restores the density of *Sox2* mutant MG. However, NICD activity does not re-establish the correct laminar position of MG in *Sox2*^{MUTANT}*;CALSL-NICD* retinas, suggesting a role for SOX2 specifically in postnatal MG.

Aberrant MG morphology and laminar disorganization in *Sox2*^{MUTANT} retinas

MG are essential for the establishment and maintenance of the laminar organization in the postnatal retina (Bringmann et al., 2006; Willbold et al., 1997). Mutations leading to a reduced density of MG or that alter their ability to function as a structural scaffold are associated with the disruption of the inner and outer retinal membranes and of neuronal stratification, eventually resulting in retinal degeneration (Dubois-Dauphin et al., 1999; van Rossum et al., 2006). The reduced density and aberrant localization of MG throughout the INL and ONL in SOX2-deficient retinas, combined with the disorganization of the ONL, led us to examine the laminar architecture in *Sox2*^{MUTANT} retinas. Gross morphological analyses (Fig. 3A-D) coupled with β-catenin (adherens junctions; Fig. 3E-

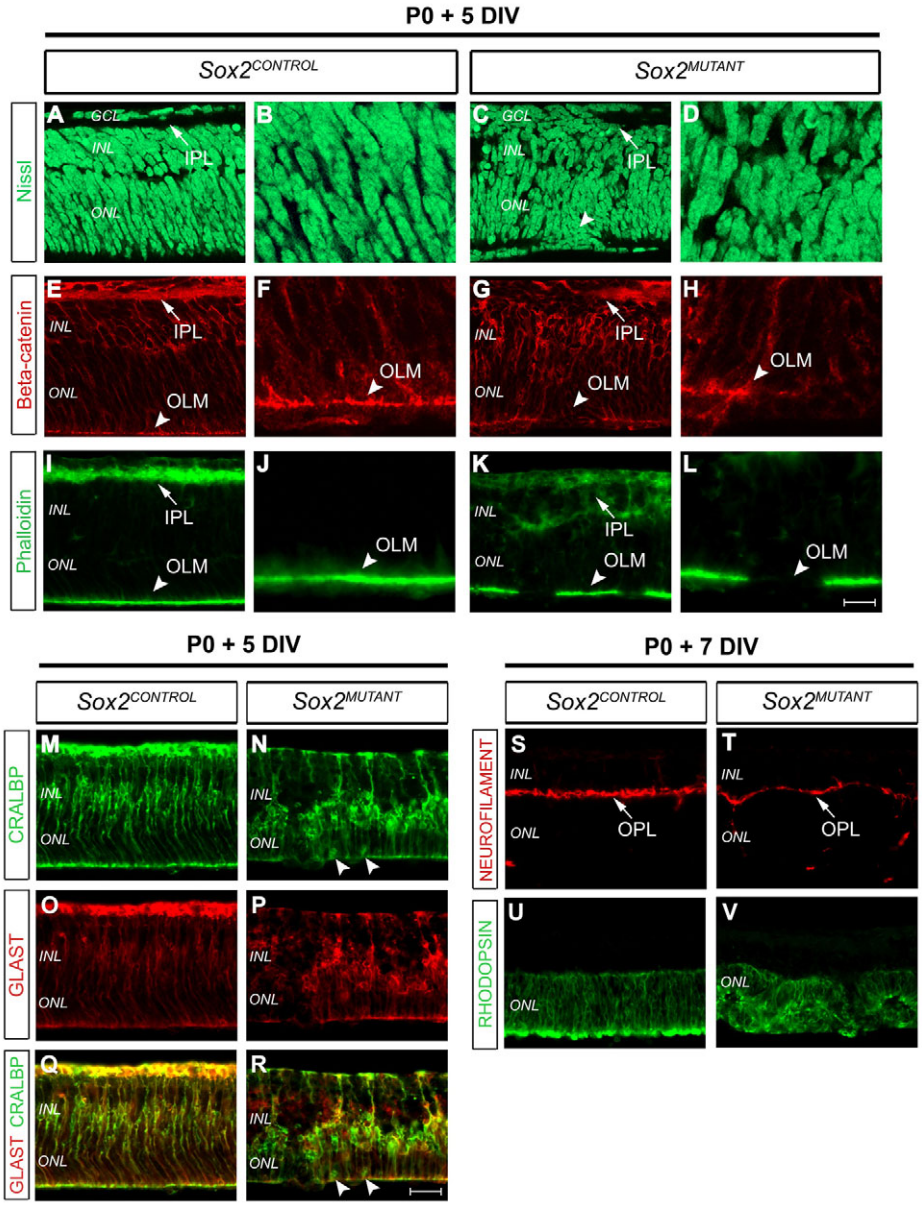

Fig. 3. Defects in retinal lamination and MG morphology in *Sox2*^{MUTANT} retinas. (**A-L**) Immunohistochemical analysis of sectioned retinas reveals disorganization of lamination in *Sox2*^{MUTANT} retinas treated with TM and cultured for 5 days. B,D,F,H,J,L are high magnification images of A,C,E,G,I,K. (A-D) In *Sox2*^{MUTANT} retinas, the ONL, INL and GCL are disorganized and cells are displaced beyond the OLM (C, arrowhead) and IPL (C, arrow), as shown by Nissl staining (A,B versus C,D). (E-H) Whereas β-catenin is enriched in adherens junctions of the IPL (E, arrow) and OLM (E,F, arrowheads) of *Sox2*^{CONTROL} retinas, β-catenin staining in the IPL (G, arrow) and OLM (G,H, arrowheads) of *Sox2*^{MUTANT} retinas is discontinuous. (I-L) Breaks in the IPL (arrows) and OLM (arrowheads) of *Sox2*^{MUTANT} retinas are visualized by the uneven distribution of F-actin (phalloidin), which is normally enriched in the IPL (I,K, arrows) and OLM (I-L, arrowheads). (**M-R**) Immunostaining against CRALBP (M,N) and GLAST (O,P) reveals the radial morphology of MG in *Sox2*^{CONTROL} retinas (M,O,Q), whereas MG in *Sox2*^{MUTANT} retinas are disorganized and displaced to the ONL (N,P,R, arrowheads). (**S**,**T**) The OPL, marked by NF, is disorganized in *Sox2*^{MUTANT} (T) compared with *Sox2*^{CONTROL} (S) retinas cultured for 7 days. (**U**,**V**) Rod photoreceptors form rosette structures in *Sox2*^{MUTANT} (V) compared with *Sox2*^{CONTROL} (U) retinas cultured for 7 days. GCL, ganglion cell layer; OLM, outer limiting membrane. Scale bars: 40 μm in R for M-R; 15 μm in L for B,D,F,H,J,L.

H), phalloidin (F-actin; Fig. 3I-L) and NF (horizontal cells; Fig. 3S,T) staining reveal the disruption of the outer limiting membrane (OLM) and the disorganization of the inner and outer plexiform layers (IPL and OPL) in *Sox2*^{MUTANT} retinas compared with *Sox2*^{CONTROL} retinas.

We next assessed the morphology of postnatal MG in *Sox2*^{CONTROL} and *Sox2*^{MUTANT} retinas by immunostaining against CRALBP and GLAST (SLC1A3 – Mouse Genome Informatics) (Fig. 3M-R). In *Sox2*^{CONTROL} retinas, MG display a characteristic radial morphology, with cell bodies located within the INL and processes that extend towards the apical and basal retinal boundaries (Fig. 3M,O,Q). By contrast, the radial morphology of *Sox2*^{MUTANT} MG is disrupted, and the cell bodies are irregularly shaped and mislocalized to the ONL (Fig. 3N,P,R; supplementary material Fig. S1T). The reduced density and aberrant morphology of MG in *Sox2*^{MUTANT} retinas and the disruption of the OLM are accompanied by the disorganization of the ONL, such that rhodopsin-positive photoreceptors are no longer contained within a defined cellular layer but protrude through the breaks in the OLM and form rosette-like structures (Fig. 3U versus 3V). The severity of retinal disorganization in *Sox2*^{MUTANT} retinas correlates with the kinetics of TM-induced *Sox2* ablation (supplementary material Fig. S1T) and the duration of culture period, with a subset of retinas displaying severe degeneration at day 7 of culture (supplementary material Fig. S1U, Table S3). Together, these data reveal the aberrant morphology of postnatal *Sox2*^{MUTANT} MG and the defects in retinal laminar architecture caused by ablation of *Sox2* in the postnatal retina.

Disruption of cell cycle quiescence in nascent *Sox2*^{MUTANT} MG

Cell cycle quiescence, along with the confinement of RPC marker expression strictly to the INL, serve to distinguish newly specified MG from proliferating RPCs (supplementary material Fig. S3). However, postnatal MG that are forced to re-enter the cell cycle, either by the administration of mitogenic factors or in response to retinal injury, are capable of undergoing interkinetic nuclear migration (INM) and cell division (Close et al., 2006; Joly et al., 2011; Karl et al., 2008; Ueki et al., 2012). SOX2 is crucial to cell cycle dynamics in the retina, cortical subventricular zone and in glial cells in the hippocampus (Pevny and Nicolis, 2010; Ferri et al., 2004). We asked whether displacement of MG cell bodies to the ONL in *Sox2*^{MUTANT} retinas is associated with dysregulation of their cell cycle state. We first assessed the incorporation of BrdU (2-hour pulse) and presence of phospho-histone H3 (PH3), marking cells in the S and M phases of the cell cycle, respectively, in *Sox2*^{CONTROL} and *Sox2*^{MUTANT} retinas (Fig. 4A-D). We find that by day 5 in culture, cells that incorporate BrdU and exhibit PH3 are no longer present in the central regions of *Sox2*^{CONTROL} retinas (Fig. 4A,C), whereas they persist in the peripheral retinal regions (Fig. 4A, inset; supplementary material Fig. S1M, inset). By contrast, BrdU-positive and PH3-positive cells are observed in both the central and peripheral regions in *Sox2*^{MUTANT} retinas (Fig. 4B,D). The total number of PH3-positive cells is significantly increased in *Sox2*^{MUTANT} relative to *Sox2*^{CONTROL} retinas (32.70±3.88 versus 16.20±4.05; P=0.0055) (Fig. 4O). Consistent with both the decrease in MG cell density and the

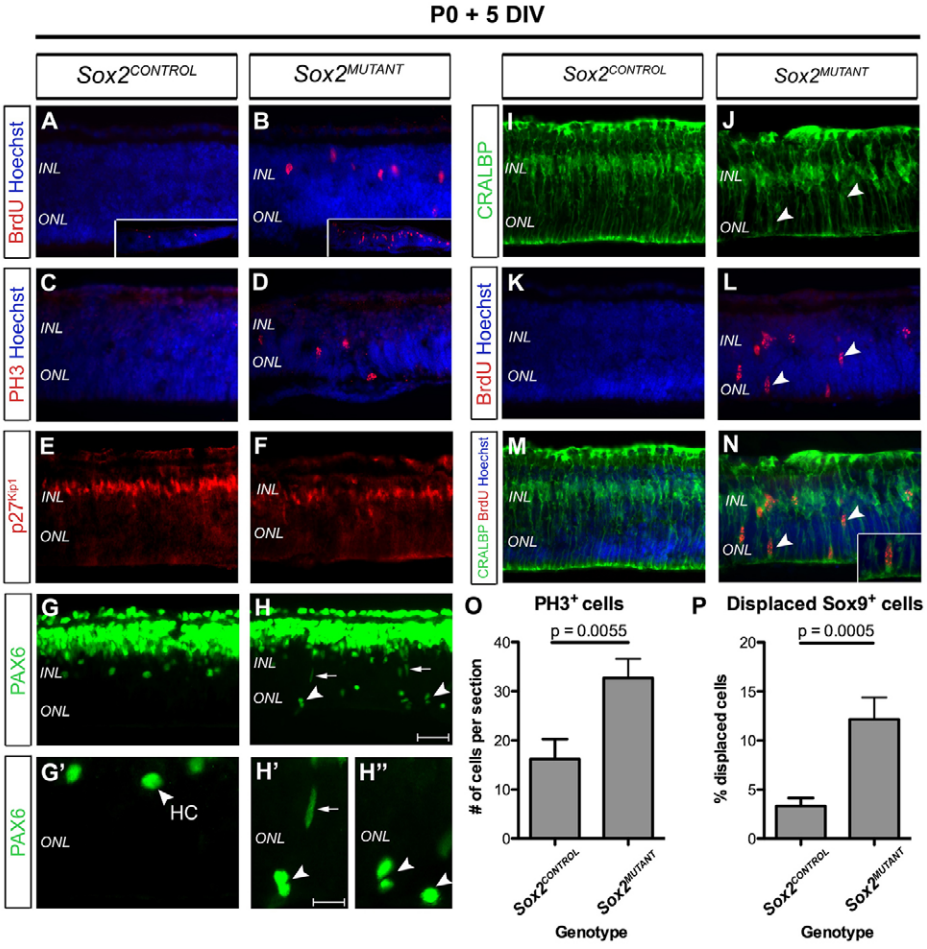

Fig. 4. MG in *Sox2*^{MUTANT} retinas express mitotic cell cycle markers. (**A-D**) The number of cells incorporating BrdU (A,B) and exhibiting PH3 (C,D) in the central and peripheral regions of *Sox2*^{MUTANT} retinas is increased (insets in A,B). (**E**,**F**) Expression of p27^{Kip1} in the INL of *Sox2*^{MUTANT} retinas (F) is decreased compared with *Sox2*^{CONTROL} retinas. (**G-H″**) PAX6 is not detected in the ONL, but marks amacrine cells, MG and horizontal cells (G′, arrowhead) in the INL (G,G′) in *Sox2*^{CONTROL} retinas. In *Sox2*^{MUTANT} retinas, PAX6 is found in elongated cells (H,H′, arrows) and in cells with a round morphology in the ONL (H,H′,H″, arrowheads). (**I-N**) CRALBP-positive MG do not incorporate BrdU in *Sox2*^{CONTROL} retinas (I,K,M), but are BrdU positive in *Sox2*^{MUTANT} retinas (J,L,N, arrowheads, inset). (**O**) The number of PH3-positive cells is higher in *Sox2*^{MUTANT} than *Sox2*^{CONTROL} retinas. (**P**) The percentage of SOX9-expressing MG displaced to the ONL is increased in *Sox2*^{MUTANT} (12.16±2.23) compared with *Sox2*^{CONTROL} (3.31±0.84) retinas (two-tailed *t*-test; P=0.0005). Data are mean ± s.e.m. HC, horizontal cell. Scale bars: 45 μm in H for A-H,I-N; 25 μm in H′ for G′-H″.

increase in proliferating cells, expression of the cell cycle inhibitor $p27^{Kip1}$ (CDKN1B – Mouse Genome Informatics) is reduced in $Sox2^{MUTANT}$ retinas (Fig. 4E versus 4F).

We next examined the expression of PAX6, which marks MG cells, as well as amacrine and horizontal cells (HCs) in the INL (Fig. 4G-H″). In accordance with the depletion of dividing RPCs in the central retinal regions at day 5 of culture, PAX6-positive cells are rarely detected in the ONL of $Sox2^{CONTROL}$ retinas, whereas postnatal MG in the INL maintain PAX6 expression (Fig. 4G,G′; supplementary material Fig. S4A,A′, arrowheads). By contrast, PAX6-positive cells that undergo INM and co-express PCNA (Fig. 4H,H′, arrows; supplementary material Fig. S5B,D,F,F′, arrows), and PAX6-positive PCNA-negative cells with a round morphology that have exited the cell cycle (Fig. 4H,H′,H″, arrowheads; supplementary material Fig. S5B,D,F,F′, arrowheads), are found in the ONL of $Sox2^{MUTANT}$ retinas. Consistent with the INM, the number of SOX9-expressing MG that are displaced to the ONL is increased in $Sox2^{MUTANT}$ relative to $Sox2^{CONTROL}$ retinas (Fig. 4P). Importantly, cells that incorporate BrdU in $Sox2^{MUTANT}$ retinas are located in both the INL and ONL and co-express CRALBP (Fig. 4I-N, arrowheads; inset in 4N), confirming their identity as MG. Thus, ablation of *Sox2* in the postnatal retina leads to the re-entry of MG into the cell cycle.

Sox2 mutant postnatal MG undergo cell division

To determine whether postnatal MG that undergo INM and upregulate mitotic markers in $Sox2^{MUTANT}$ retinas undergo cell division, we electroporated P0-P1 retinas with GLASTp-dsRED2 plasmid and monitored the behavior of labeled cells in TM-treated retinal slices at days 3-4 of culture using time-lapse microscopy (Fig. 5; supplementary material Fig. S6) (Mizutani et al., 2007). GLASTp-dsRED2 marks a small subset of electroporated cells, the vast majority of which exhibit the characteristic radial morphology of MG at day 4 in culture (supplementary material Fig. S6E,F).

Consistent with their identity as MG, GLASTp-dsRED2-positive cells in $Sox2^{CONTROL}$ retinas display long processes that extend towards the apical and basal retinal boundaries, do not undergo INM, and maintain a cell body position within the INL (Fig. 5A,A′; supplementary material Fig. S6B, Movie 1). In contrast to MG in $Sox2^{CONTROL}$ retinas and in accordance with the expression of proliferative markers, a subset of $Sox2^{MUTANT}$ MG captured during a 24-hour imaging period at days 3-4 of culture migrate towards the apical retinal surface and undergo cell division (Fig. 5B,B′; supplementary material Movies 2-4). Importantly, daughter cells resulting from $Sox2^{MUTANT}$ MG cell divisions do not re-establish radial morphology, losing both RPC and MG identity (supplementary material Movies 2-4). Together, these results demonstrate that, in the absence of SOX2, postnatal MG cells divide at the apical retinal boundary, suggesting a role for SOX2 in the maintenance of nascent MG by preventing their depletion through cell division.

Mosaic ablation of *Sox2* in the postnatal retina leads to cell division of nascent MG

The ability of postnatal and adult MG cells to re-enter the cell cycle is tightly associated with their response to retinal injury (Dyer and Cepko, 2000; Jadhav et al., 2009; Joly et al., 2011; Ooto et al., 2004). To confirm that cell division of postnatal MG is caused by the loss of SOX2 in these cells specifically, as opposed to being triggered by non-cell-autonomous factors, we ablated *Sox2* in a mosaic manner. The pCRALBP-CreEGFP-Nuc construct, which encodes a CreEGFP fusion protein driven by the *Cralbp* regulatory element, was used to electroporate retinas at P1 (Fig. 6A) (Matsuda and Cepko, 2004; Matsuda and Cepko, 2007). Fate-mapping analysis of pCRALBP-CreEGFP-Nuc with the Cre reporter pCALNL-dsRED in retinas cultured for 5 days confirms its restricted activity predominantly in postnatal MG (supplementary material Fig. S7A-D). At day 5 of culture, CreEGFP colocalizes with SOX2 and CRALBP strictly in MG in the INL (Fig. 6C; supplementary material Fig. S7E-G). In contrast to MG in $Sox2^{+/+}$ retinas, which maintain their quiescent state, a subset of CreEGFP-expressing MG in $Sox2^{COND/COND}$ retinas exhibit PH3 (Fig. 6E, arrowhead), migrate to the apical retinal boundary (Fig. 6F, arrowheads) and undergo cell division (Fig. 6G; supplementary material Movie 5). These results, combined with the observed MG cell division in TM-treated $Sox2^{MUTANT}$ retinas, strongly suggest that SOX2 is required in nascent MG specifically to prevent cell cycle entry and aberrant cell division.

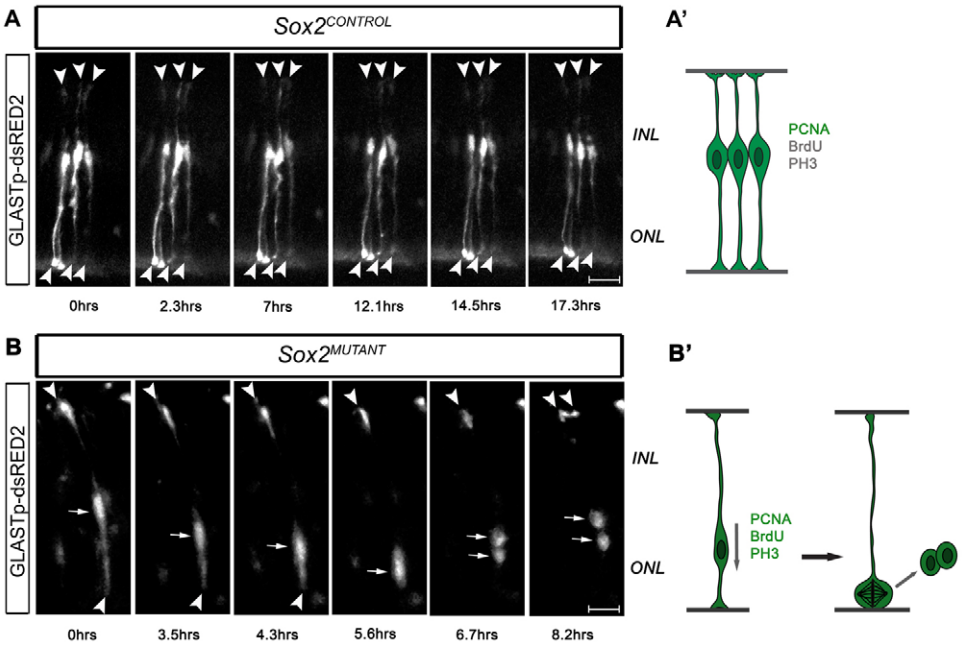

Fig. 5. Cell division of MG in $Sox2^{MUTANT}$ retinas. (**A**,**A′**) Time-lapse imaging of GLASTp-dsRED2-expressing cells in slices of TM-treated $Sox2^{CONTROL}$ retinas at day 3-4 of culture illustrates MG radial morphology, maintenance of apical and basal cellular processes (arrowheads) and limited cell body movement (illustrated in A′). (**B**,**B′**) A GLASTp-dsRED2-labeled MG imaged in the $Sox2^{MUTANT}$ retina migrates to the ONL and undergoes cell division, followed by separation of the two daughter cells (arrows) and splitting of the basal cellular process (arrowheads) (illustrated in B′). Image series were collected on 200 µm retinal sections every 40 minutes over 8-18 hours. Scale bars: 30 µm in A; 20 µm in B.

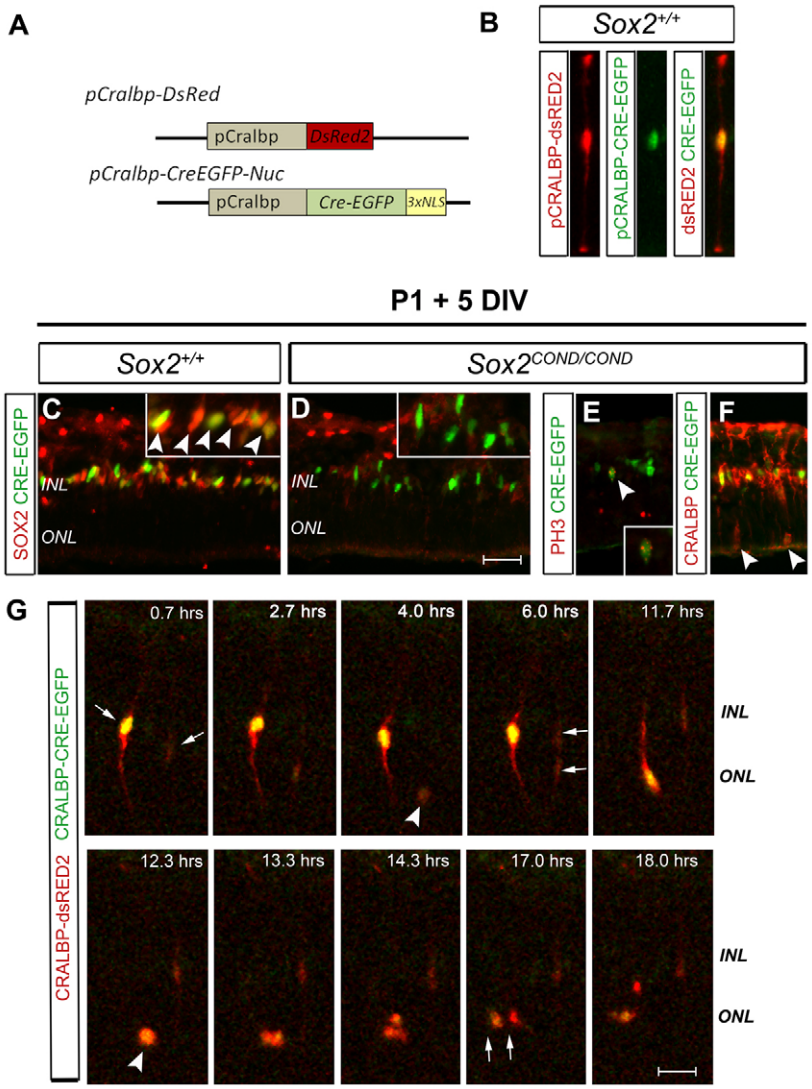

Fig. 6. Mosaic ablation of *Sox2* leads to ectopic cell division of nascent MG. (**A**) pCRALBP-dsRED2 and pCRALBP-CreEGFP-Nuc DNA constructs. (**B**) CRALBP-dsRED2 colocalizes with CRALBP-CreEGFP in MG in retinas co-electroporated at P1 and cultured for 5 days. (**C**) In $Sox2^{+/+}$ retinas, CRALBP-CreEGFP is co-expressed with SOX2 in the INL (C, arrowheads). (**D**) SOX2 is not detected in EGFP-positive cells in $Sox2^{COND/COND}$ retinas. (**E**,**F**) A subset of CRALBP-CreEGFP-expressing MG in $Sox2^{COND/COND}$ retinas exhibit PH3 (E, arrowhead, inset) and are displaced to the ONL (F, arrowheads). (**G**) CRALBP-CreEGFP/CRALBP-dsRED2 double-labeled MG (arrows) in $Sox2^{COND/COND}$ retinas undergo interkinetic nuclear migration and cell division (see supplementary material Movie 5). Arrowheads indicate mitotic MG at the apical retinal boundary. Confocal images (G) were collected from 200 μm retinal sections every 40 minutes over an 18-hour period. Scale bars: 45 μm in C-F; 35 μm in G.

Induction of ectopic NOTCH1 activity restores MG cell identity to $Sox2^{MUTANT}$ cells but does not secure their quiescent state

Radial morphology and cell cycle quiescence are distinguishing characteristics of postnatal MG cells, and are disrupted due to loss of SOX2 in the postnatal retina. To examine whether genetic activation of the Notch signaling pathway restores these characteristic features of MG, in addition to their cell density, we examined MG morphology and retinal laminar architecture in $Sox2^{MUTANT}$;*CALSL-NICD* retinas (Fig. 7). In contrast to the aberrant morphology of MG in $Sox2^{MUTANT}$ retinas, the morphology of MG in $Sox2^{MUTANT}$;*CALSL-NICD* retinas is restored by the activity of NICD. Despite the displacement of MG cell bodies to the ONL in $Sox2^{MUTANT}$;*CALSL-NICD* retinas (Fig. 2P), their processes are maintained at the apical and basal retinal boundaries (Fig. 7C versus 7D). Furthermore, restoration of MG cell density and morphology in $Sox2^{MUTANT}$;*CALSL-NICD* retinas is accompanied by restoration of the OLM, as marked by phalloidin staining (Fig. 7G versus 7H). These data demonstrate that the Notch signaling pathway functions downstream of SOX2 to maintain MG cell molecular identity and radial morphology.

We next assessed whether the quiescent state of nascent *Sox2* mutant MG is restored by the NICD activity in $Sox2^{MUTANT}$;*CALSL-NICD* retinas. We find that the expression pattern of PCNA, marking predominantly MG in the INL, is indistinguishable in $Sox2^{CONTROL}$ and $Sox2^{CONTROL}$;*CALSL-NICD* retinas (Fig. 8A,B; supplementary material Fig. S3Q-T), whereas the mitotic marker PH3 is undetectable (Fig. 8E,F). In sharp contrast, expression of PCNA and presence of PH3 are observed throughout the INL and ONL of $Sox2^{MUTANT}$;*CALSL-NICD* retinas (Fig. 8D,H).

To further examine the effect of ectopic NICD activity on the mitotic behavior of postnatal MG, we followed the fates of GLASTp-dsRED2-labeled cells in slices of $Sox2^{CONTROL}$;*CALSL-NICD* and $Sox2^{MUTANT}$;*CALSL-NICD* retinas using time-lapse imaging at days 3-4 of culture (Fig. 8I,J; supplementary material Movies 6-9). In accordance with the lack of PH3, GLASTp-dsRED2-labeled MG cells in $Sox2^{CONTROL}$;*CALSL-NICD* retinas do not undergo INM or cell division (Fig. 8J; supplementary material Movie 6). By contrast, GLASTp-dsRED2-expressing MG in $Sox2^{MUTANT}$;*CALSL-NICD* retinas undergo INM and divide at the apical retinal boundary (Fig. 8I; supplementary material Movies 7-9). The proportions of GLASTp-dsRED2-labeled cells undergoing cell division during the 12-hour imaging periods in both $Sox2^{MUTANT}$ (16.14±1.86; n=19) and $Sox2^{MUTANT}$;*CALSL-NICD* (16.68±1.90; n=11) retinas are significantly higher than in $Sox2^{CONTROL}$ (1.73±0.58; n=24) and $Sox2^{CONTROL}$;*CALSL-NICD* (0.37±0.37;

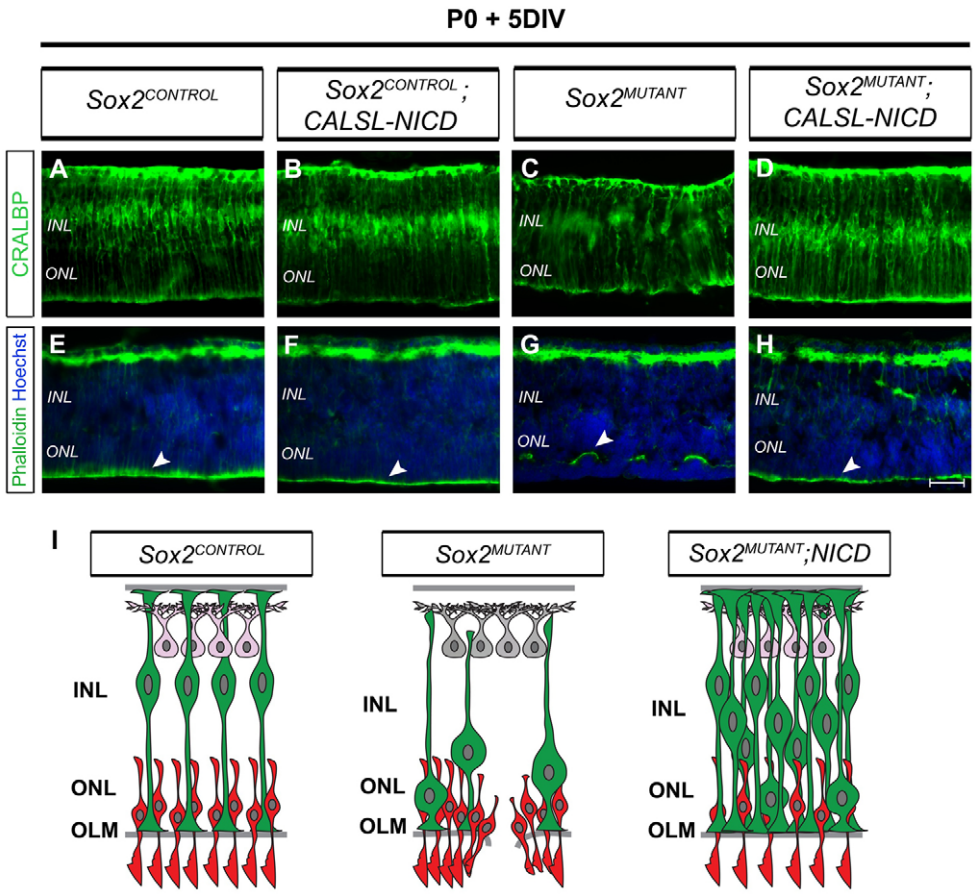

Fig. 7. Activation of Notch signaling in *Sox2*^{MUTANT}*;CALSL-NICD* retinas restores MG cell morphology and retinal architecture. (**A-D**) CRALBP reveals restoration of MG morphology and retinal architecture in *Sox2*^{MUTANT}*;CALSL-NICD* retinas, as compared with *Sox2*^{MUTANT} retinas (C versus D). (**E-H**) The integrity of the OLM is restored in *Sox2*^{MUTANT}*;CALSL-NICD* retinas (H, arrowhead) compared with *Sox2*^{MUTANT} retinas (G, arrowhead). (**I**) Comparison of MG morphology and retinal organization between *Sox2*^{CONTROL}, *Sox2*^{MUTANT} and *Sox2*^{MUTANT}*;CALSL-NICD* retinas. MG in *Sox2*^{MUTANT} retinas are reduced in number and are displaced to the ONL. NICD activity restores the number of MG, but not their INL position. MG, green; rod photoreceptors, red; amacrine cells, gray, pink. Scale bar: 45 μm in A-H.

n=8) retinas (*P*<0.0001) (Fig. 8J). These findings, coupled with the restoration of MG cell density and morphology in *Sox2*^{MUTANT}*;CALSL-NICD* retinas, provide evidence that SOX2 maintains the identity of postnatal MG via the Notch signaling pathway, but functions in a Notch-independent manner to prevent the aberrant cell division of nascent MG.

DISCUSSION

In this study, we show that SOX2 plays an essential role in maintaining the structural organization of the postnatal retina and the quiescence of nascent MG. Utilizing real-time imaging we find that loss of SOX2 forces MG to aberrantly divide into a pair of postmitotic daughter cells, leading to MG cell depletion and retinal degeneration. We further establish that activation of Notch signaling restores MG characteristics to *Sox2* mutant cells, but is insufficient to secure their progenitor state quiescence. We propose a model by which SOX2 maintains a quiescent progenitor cell state of nascent MG by preventing their progression through the cell cycle to terminal division.

MG represent a dormant progenitor cell population. They maintain characteristics of postnatal RPCs, including radial morphology and the expression of NPC markers. Although they retain a neurogenic capacity, MG proliferate and give rise to new neurons only when stimulated by signals that arise in response to retinal injury (Jadhav et al., 2009; Roesch et al., 2008). Here, we show that ablation of *Sox2* during the period of MG cell development leads to their gradual depletion, as evidenced by a decrease in the expression of the MG markers SOX9, PAX6, p27^{Kip1} and PCNA. Moreover, this reduction in MG cell number is accompanied by the disruption of their radial morphology.

Unlike the subsets of specialized adult glial stem cells in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, MG constitute the principal glial cell population in the retina, and thus their primary role lies in maintaining retinal homeostasis (Kriegstein and Alvarez-Buylla, 2009). Spanning the entire width of the retina, MG define the retinal boundaries through the formation of intercellular junctions, function as a neuronal scaffold, and play the decisive role in the establishment of retinal laminar pattern and polarity (Bringmann et al., 2006; Willbold et al., 1997). Consistent with this role, the loss of MG in the postnatal retina results in retinal degeneration (Dubois-Dauphin et al., 1999; Rich et al., 1995). In agreement with these observations, we find that the gradual loss of MG cells in *Sox2*^{MUTANT} retinas leads to severe retinal disorganization followed by retinal degeneration, suggesting an essential role for SOX2 in the maintenance of retinal homeostasis through its function in nascent MG.

In the developing and adult CNS, SOX2 serves as a marker of multipotent NPCs and controls the maintenance of NPC identity (Ellis et al., 2004; Favaro et al., 2009; Ferri et al., 2004; Pevny and Nicolis, 2010). Disruption of SOX2 function is associated with the loss of proliferative and differentiation capacity in embryonic and adult NPCs. However, to date, the precise mechanism underlying the requirement for SOX2 in the maintenance of NPCs has not been characterized. In this study, we demonstrate that SOX2 functions to maintain postnatal MG, the NPC-like cells of the retina, by safeguarding them from entering the cell cycle and undergoing aberrant cell division.

Cell cycle quiescence is an essential feature of MG that distinguishes them from postnatal RPCs and enables them to

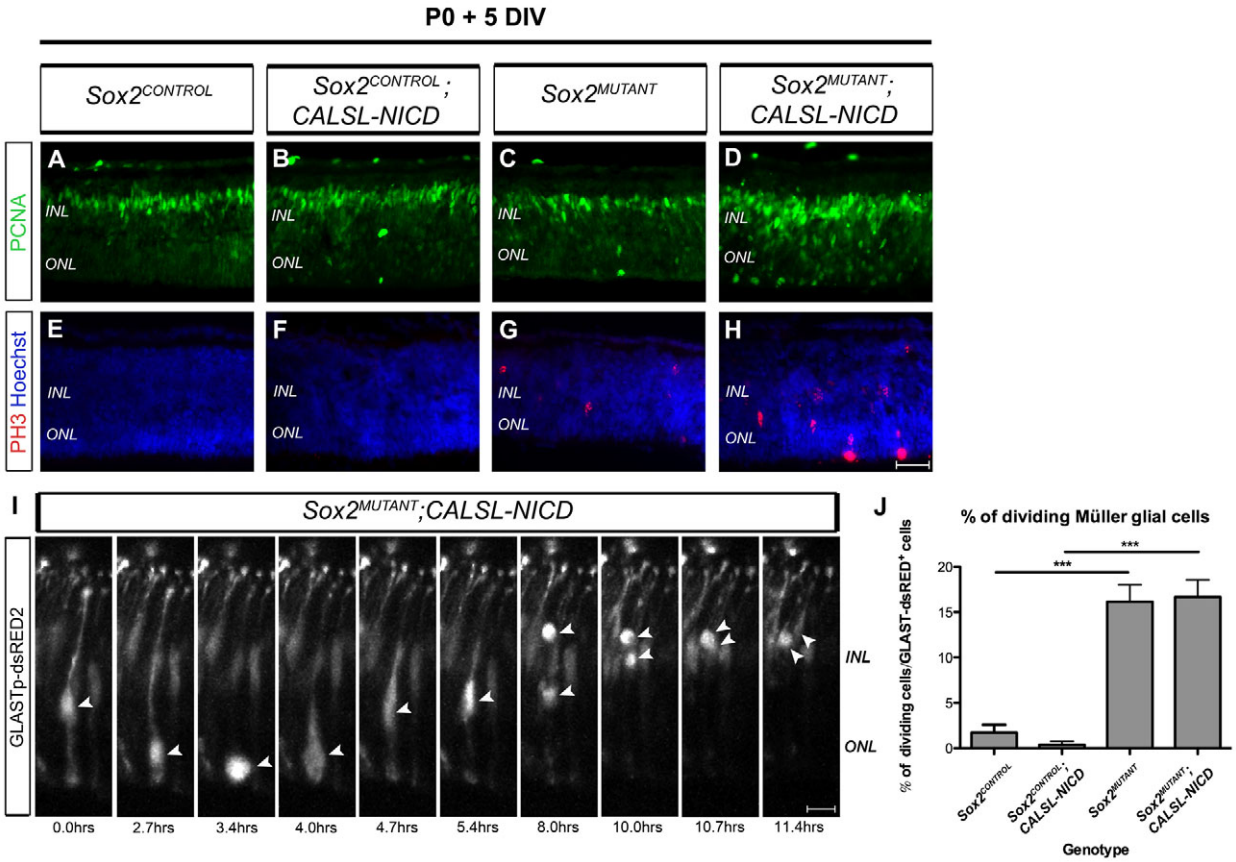

Fig. 8. NICD activity does not restore the quiescent state of MG in *Sox2*^{MUTANT}*;CALSL-NICD* retinas. (**A-D**) PCNA-expressing cells are present in both the INL and ONL of *Sox2*^{MUTANT}*;CALSL-NICD* retinas (D). (**E-H**) PH3-positive cells are found in *Sox2*^{MUTANT} (G) and *Sox2*^{MUTANT}*;CALSL-NICD* (H) retinas. (**I**) Daughter cell resulting from a dividing GLASTp-dsRED2-labeled MG (arrowheads) re-establishes radial morphology in *Sox2*^{MUTANT}*;CALSL-NICD* retina. The image series were collected on 200 μm sections every 40 minutes over a 12-hour period. (**J**) The percentage of dividing GLASTp-dsRED2-positive MG is significantly increased in *Sox2*^{MUTANT} and *Sox2*^{MUTANT}*;CALSL-NICD* retinas ($P<0.0001$) compared with *Sox2*^{CONTROL} and *Sox2*^{CONTROL}*;CALSL-NICD* retinas. ***$P<0.0001$. Error bars indicate s.e.m. Scale bars: 45 μm in A-H; 30 μm in I.

function in maintaining retinal homeostasis. Here we show that MG in *Sox2*^{MUTANT} retinas are formed, but are not maintained in the absence of *Sox2*. Using real-time imaging, we demonstrate that, unlike quiescent MG, *Sox2* mutant MG undergo INM and cell division at the apical retinal boundary. Divisions of MG in *Sox2*^{MUTANT} retinas produce daughter cells that do not display the morphological and molecular characteristics of MG, losing both MG and progenitor cell identity. The moderate increase in rod photoreceptor precursors in *Sox2*^{MUTANT} retinas suggests that a subset of daughter cells produced by dividing *Sox2* mutant MG might acquire rod photoreceptor cell fate. However, ectopic expression of PAX6 in a subset of these daughter cells is inconsistent with this idea. Rather, we conclude that daughter cells resulting from *Sox2*^{MUTANT} MG divisions exit the cell cycle and transiently acquire a somewhat complex identity. This conclusion is supported by studies demonstrating that NPCs are incapable of proper neuronal or glial differentiation when the function of SOX2 is perturbed (Bylund et al., 2003; Ehm et al., 2010; Favaro et al., 2009; Ferri et al., 2004; Graham et al., 2003; Holmberg et al., 2008; Matsushima et al., 2011).

Similar to SOX2, Notch signaling serves to maintain NPC identity in the developing and adult CNS (Austin et al., 1995; Ehm et al., 2010; Furukawa et al., 2000; Henrique et al., 1997; Holmberg et al., 2008; Takatsuka et al., 2004; Tomita et al., 1996; Yaron et al., 2006). Studies in multiple systems, including the developing mouse retina, provide evidence for a possible interaction between SOX2 and Notch (Ehm et al., 2010; Holmberg et al., 2008; Matsushima et al., 2011; Takatsuka et al., 2004; Taranova et al., 2006; Yaron et al., 2006). These studies establish that the ability of Notch signaling to maintain NPC identity depends on functional SOXB1 proteins.

Examination of the relationship between Notch signaling and SOX2 in nascent MG reveals that *Sox2*^{MUTANT} MG downregulate both *Notch1* and its downstream target, *Hes5*, confirming that the expression of components of the Notch signaling pathway in MG depends on SOX2. Importantly, genetic induction of Notch signaling prevents the depletion of MG in *Sox2*^{MUTANT}*;CALSL-NICD* retinas, as evidenced by the restoration of MG radial morphology, leading to an improvement in retinal architecture. However, Notch activity does not secure the quiescent state of *Sox2*^{MUTANT} MG, while NICD itself has little effect on cell proliferation in the postnatal retina (Bao and Cepko, 1997; Furukawa et al., 2000). Real-time visualization of MG in *Sox2*^{MUTANT}*;CALSL-NICD* retinas suggests that the daughter cells resulting from *Sox2*^{MUTANT} MG cell divisions are capable of re-establishing radial morphology and cell body position in the INL. The increase in MG cell density and restoration of their radial morphology indicate that NICD activity specifically rescues the fates of *Sox2*^{MUTANT} MG cells.

Collectively, these data strongly suggest that, in addition to maintaining MG cell identity via the Notch signaling pathway,

SOX2 functions in a Notch-independent manner to prevent the depletion of nascent MG through cell cycle progression and terminal cell division. Thus, we uncoupled the roles of SOX2 and Notch signaling in MG development. We established that whereas Notch signaling functions as a MG cell fate determinant, SOX2 is essential to prevent their terminal cell cycle exit. We further show that there is an obligatory requirement for at least one round of cell division in the absence of SOX2 as a prelude to terminal progenitor cell differentiation.

Together, our data highlight the essential roles of SOX2 and the Notch signaling pathway in the regulation of the quiescent state of MG in the postnatal mouse retina. The mechanisms by which quiescent cells preserve their ability to resume proliferation after weeks or even years of cell cycle arrest are not known. Our study provides a general paradigm of how SOX2 functions in NPCs and will have implications for our understanding of the pathways that balance stem cell quiescence and proliferation in the developing and adult CNS.

Acknowledgements
This work is dedicated to the memory of Dr Larysa H. Pevny, for whose insight, encouragement and guidance were are eternally grateful. We thank Drs Eva S. Anton, William D. Snider and Susana da Silva for critically evaluating the manuscript; the UNC Neuroscience Center Confocal and Multiphoton Imaging and *In Situ* Core Facilities, especially Dr Robert E. Peterson and Dr Megumi Aita, for their help.

Funding
This work was supported by grants to L.P. from the National Institutes of Health [EY018261, EY022341 and MH071822]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071878/-/DC1

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