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p53 is Required for the Developmental Restriction in Müller Glial Proliferation in Mouse Retina

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ABSTRACT
Müller glia are normally mitotically quiescent cells, but in certain pathological states they can re-enter the mitotic cell cycle. While several cell cycle regulators have been shown to be important in this process, a role for the tumor suppressor, p53, has not been demonstrated. Here, we investigated a role for p53 in limiting the ability of Müller glia to proliferate in the mature mouse retina. Our data demonstrate that Müller glia undergo a developmental restriction in their potential to proliferate. Retinal explants or dissociated cultures treated with EGF become mitotically quiescent by the end of the second postnatal week. In contrast, Müller glia from adult *trp53*−/+ or *trp53*−/− mice displayed a greater ability to proliferate in response to EGF stimulation *in vitro.* The enhanced proliferative ability of *trp53* deficient mice correlates with a decreased expression of the mitotic inhibitor *Cdkn1a/p21*^{cip} and an increase in *c-myc*, a transcription factor that promotes cell cycle progression. These data show that p53 plays an essential role in limiting the potential of Müller glia to re-enter the mitotic cycle as the retina matures during postnatal development.

INTRODUCTION

Mammalian retinal progenitors undergo many rounds of cell division during development to produce the millions of neurons in the mature retina. After the first postnatal week, the progenitors withdraw from the mitotic cell cycle (Close et al., 2005; Rapaport et al., 2004; Young, 1985). However, in certain pathological conditions, Müller glia in the mature retina can re-enter the mitotic cell cycle (see for review Karl and Reh, 2010). Although even in these cases only a few Müller glia re-enter the mitotic cycle in the mature mouse retina, those that do may contribute to a regenerative response (like that present in nonmammalian vertebrates; see review by Karl and Reh, 2010) or a pathological process (i.e., proliferative vitreo-retinopathy; see review by Bringmann et al., 2006, 2009). Therefore, a better understanding of the mechanisms that limit the proliferation of the Müller glia may lead to the development of strategies to stimulate retinal regeneration and prevent proliferative pathologies.

While much is known about the cell cycle proteins that regulate proliferation of the retinal progenitors (Chong et al., 2009), the factors that regulate the transition from the high level of proliferation present in the developing retina to the mitotic quiescence of the Müller glia have received less attention. The cyclin dependent kinase inhibitor *Cdkn1b/p27*^{kip}, has been shown to be important in limiting the proliferation at the end of neurogenesis, and targeted deletion of this gene leads to excess proliferation of Müller glia (Dyer and Cepko, 2000; Levine et al., 2000). In the rat retina, there are at least two extracellular signals that contribute to a reduction in proliferation after the first postnatal week: an increase in TGF-beta signaling and a decline in signaling through the EGFR pathway (Close et al., 2005, 2006). Together these signals lead to an increase in the level of p27^{kip}, which as mentioned above, normally acts to restrict Müller glial proliferation. Although these studies all implicate p27^{kip} as an important regulator of Müller glial proliferation, there is evidence that additional regulators are involved; deletion of *Cdkn1b/p27*^{kip} leads to only a small increase in Müller glial proliferation (Vazquez-Chona et al., 2011) and most Müller glia do not enter the mitotic cycle in the knock-out animals.

Recently, a genome-wide analysis of gene expression in developing Müller glia demonstrated that these cells are very similar to astrocytes (Nelson et al., 2011); therefore, analysis of regulators of astrocyte proliferation could lead to identification of factors that control proliferation in Müller glia. A key regulator of cell proliferation in astrocytes and adult neural stem cells is the tumor suppressor p53 (Bogler et al., 1999; Gil-Perotin et al., 2006; Meletis et al., 2006; Yahanda et al., 1995;

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Zheng et al., 2008). While not thought to directly interact with the cell cycle proteins, p53 promotes cell cycle arrest by repressing *c-myc* transcription and by activating the *Cdkn1a p21^{cip}*, particularly after cell damage or stress (Cox and Lane, 1995; Ho et al., 2005; Kippin et al., 2005; Meletis et al., 2006; Zheng et al., 2008).

We therefore investigated whether p53 might be involved in limiting the proliferation of Müller glia in the retina. We first assessed the ability of glia from wild-type mice to grow in explant and dissociated culture with epidermal growth factor (EGF) stimulation, and found that their ability to grow *in vitro* declines over the second postnatal week. To functionally test for a role of p53 in inhibiting Müller glial proliferation, we tested whether Müller cells from *trp53*−/− mice displayed the same developmental restriction in growth ability. We found that Müller glia from *trp53*−/− and *trp53*+/− mice have a much greater potential for mitotic proliferation *in vitro*, even when taken from adult mice. These results show that p53 is an important limiting factor for proliferation of Müller glia.

MATERIALS AND METHODS

Mice

All animal procedures were approved by the IACUC at the University of Washington and housed in the Department of Comparative Medicine. Mice were C57BL/6, unless otherwise stated. Hes5-GFP transgenic mice (Basak and Taylor, 2007) were used to mark Müller glia in the retina (Nelson et al., 2011). All *trp53*−/−, *trp53*+/− (Donehower et al., 1992) and wild-type mice used for experiments were ~ 4- to 6-weeks-old, and were littermates.

Retinal Explants

Retinas from postnatal mice at various ages were cultured as explants. Retinas were isolated in cold HBSS and placed onto a 0.4 μm pore tissue culture insert (Millipore). Each insert was placed in a 6-well plate, and explants were cultured for various periods. Half the media (DMEM/F12 supplemented with 1% dialyzed fetal bovine serum (FBS), 0.6% D+ glucose, 0.2% $NaHCO_3$, 5 m*M* HEPES, L-glutamine (1 m*M*), 1 × B27, and 1 × N2) was changed daily, and recombinant mouse EGF (100 ng/mL; R&D systems) or vehicle (PBS) was also supplemented during each media change. BrdU (10 μg/mL; Sigma) or EdU (10 μg/mL; Invitrogen) was added to the media throughout the culture period.

NMDA Damage *In Vivo*

NMDA injury model was performed as described in Karl et al. (2008). Briefly, mice were anesthetized deeply with ketamine (130 mg/kg) and xylazine (8.8 mg/kg), and a single intravitreal injection of NMDA (Sigma) was performed to induce retinal damage. Intravitreal injections of EGF and EdU were performed daily starting 2 days after NMDA injection for 4 days, and retinas were collected for immunohistochemical analyses 7 days after NMDA injection.

Dissociated Müller Glia Cell Culture

Postnatal day 12 (P12) Müller glia cultures were derived from pooled retinas of C57BL/6 littermates. Retinas were dissected after sacrifice and dissociated by placing into papain with 180 units/mL DNase (Worthington) and incubating at 37°C for 8–10 min. Cells were then briefly triturated, added to an equal volume of ovomucoid (Worthington), and spun at 300 g for 10 min at 4°C. Cells were plated in Neurobasal media, with 10% FBS (Clontech), 1 m*M* L-glutamine (Invitrogen), N2 (Invitrogen), 1% Penicilin–Streptomycin (Invitrogen), and EGF (100 ng/mL; R&D systems) at a density of two retinas per 10 cm^2 at 37°C in 5% CO_2. Half of the media was changed every 2–3 days. 4–7 days after dissociation, cells were passaged with TrypLE (Invitrogen). For the adult Müller glia, retinas were dissected from 4- to 6-week-old *trp53*−/− and *trp53*−/+ mice, dissociated, and cultured as described earlier. Adult cultures received 20 ng/mL EGF and were typically passaged every 4 days (plating 5,000 cells/cm^2). Seven days after initial dissociation, and every four days thereafter, cells were passaged and plated at a density of 5,000 cells/cm^2. Cell counts were obtained using a hemocytometer, and total cell number was determined at each passage. Cells were cultured in 1% FBS and B27 (Invitrogen) for four days prior to harvest.

RNA Extraction/Microarray/RT Qpcr

RNA was extracted from dissociated cells using Picopure RNA Isolation kit (Applied Biosystems). Affymetrix microarray analysis was carried out as described previously (Nelson et al., 2011). Expression levels were compared with previously reported expression data from Müller glia (Nelson et al., 2011; Roesch et al., 2008), astrocytes (Cahoy et al., 2008), and endothelial cells (Bell et al., 2001). For qPCR, RNA was isolated from retinas or explants using Trizol (Invitrogen). cDNA synthesis was carried out using iScript cDNA synthesis kit (BioRad), and real-time qPCR was performed using SsoFast EvaGreen Supermix (BioRad). *Gapdh* was used as the normalization control. Fold change in expression as compared with control samples was calculated and plotted for the target gene. Significance ($P < 0.05$) was determined by a paired *t*-test. Primer sequences were as follows: *p21cip/Cdkn1a* F 5′- CCTGGTGATGTCCGACCTG-3 ′, *p21cip/Cdkn1a* R 5′-CGGGACCGAAGAGACAACG-3′, *c-Myc* F 5′-ATGCCCCTCAACGT GAACTTC-3′, *c-Myc* R 5′-CGGAGTCGTAGTCGAGGTCATA-3′, *Gfap* F 5′-CCACCAAACTGGCTGATGTCTAC-3′, *Gfap* R 5′-TTCTCTCCAAATCCACACGAGC-3′, *Atf3* F 5′-AAATTGCTGCTGCCAAGTG-3′, *Atf3* R 5′-CCTTCAGCT

CAGCATTCACA-3′, *Btg2* F 5′-GGACGCACTGACCGATCATTA-3′, *Btg2* R 5′-ACAGCGATAGCCAGAACCTTT-3′, *Gapdh* F 5′-GGCATTGCTCTCAATGACAA-3′, and *Gapdh* R 5′-CTTGCTCAGTGTCCTTGCTG-3′.

Immunohistochemistry

Cells or retinas were fixed with 2% paraformaldehyde. Fixed explants or retinas were cryoprotected in 30% sucrose/PBS at 4°C overnight, embedded in OCT compound (Sakura Finetek), and sectioned at 12 μm using a cryostat. Immunohistochemistry (IHC) was carried out using standard protocols. EdU staining was performed following IHC using Click-iT EdU Alexa Fluor 555 or 647 imaging kit (Invitrogen). Imaging was performed using a confocal scanning microscope (Olympus). Primary antibodies used were: rat anti-BrdU (1:100, Accurate), chicken anti-GFP (1:500, Abcam), rabbit anti-Id1 (1:200, BioCheck), rabbit anti-Pax6 (1:600, Covance), mouse anti-s100β (1:1000, Sigma), rabbit anti-Sox2 (1:250, Abcam), rabbit anti-Sox9 (1:400, Millipore), and rabbit anti-GFAP (1:1000, Dako) antibodies. Goat or donkey antirabbit Alexa Fluor 568 (1:500, Invitrogen), goat antimouse 488 (1:400, Invitrogen), donkey antirabbit 488, a donkey antichicken 488 (Jackson Immunol, 1:500) and donkey antigoat 568 (1:400, Invitrogen) were used for secondary antibodies.

Western Blot

Each explant was homogenized in RIPA buffer. Prior to SDS–PAGE using 4–15% Tris-Glycine gradient gel (BioRad), each lysate was mixed with 5× sample buffer and boiled for 5 min. Proteins were transferred to a PVDF membrane, and Western blots were performed using a standard protocol. Primary antibodies used were: mouse anti-p27 (1:10,000, BD Transduction Labs) and mouse antibeta actin (1:20,000, Abcam). Anti-mouse HRP (1:10,000, BioRad) was used as a secondary antibody, and blots were exposed to X-ray films using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Signals were quantified using ImageJ.

RESULTS

Müller Glia in Retina from Young Mice Proliferate in Explant Culture

The retinal progenitor cells that generate both the neurons and the Müller glia in the developing retina terminally differentiate in the first postnatal week in a central to peripheral gradient in mice and rats (Rapaport et al., 2004; Young, 1985). The transition from proliferating progenitor to postmitotic Müller glia is in part regulated by extrinsic factors (e.g., EGF and TGF-beta) and intrinsic factors (e.g., $p27^{kip}$). In the rat retina, a decline in EGF receptor on the Müller glia over the second and third postnatal weeks of development leads to the inability of EGF to stimulate their proliferation (Close et al., 2005, 2006). To determine whether this decline in the ability of EGF to stimulate proliferation of the Müller glia after the second postnatal week is true more generally in rodent retina, we carried out a series of explant culture experiments in developing mouse retina.

We assessed the degree of Müller glial proliferation in explant cultures from mouse retina isolated at different developmental times. When the mouse retina was isolated from P6 or P12 animals and cultured in the presence of EGF, there was a robust incorporation of the S-phase marker BrdU into the Müller glia after 7 days (Fig. 1A,B). To verify that the proliferating cells were Müller glia, retinas from Hes5-GFP (Nelson et al., 2011) P12 mice were cultured for 5 days with EGF. EdU labeling colocalized with Hes5-GFP in these cultures (Fig. 1D,E), indicating that proliferating cells were Müller glia. When the same experiment was carried out with retinas from P16 or older mice, there was little to no BrdU labeling (Fig. 1C). Culture of retinal explants from mice at two-day intervals more clearly shows the time course of this decline in EGF-stimulated Müller glial proliferation (Fig. 1F). There was a large decline between P8 and P10, and a further decline between P10 and P14, such that in retinas taken from animals older than P14, only a few Müller glia per section incorporated BrdU after 7 days *in vitro* in the presence of EGF. We also used the M-phase marker, PH3, and found it showed a similar decline as a function of the age of the mouse retina that was explanted (Fig. 1G). The proliferation of Müller glia in the explant cultures required EGF, since without it there was little BrdU incorporation, even after 7 days (data not shown).

In most explant experiments, we assessed the number of Müller cells in the mitotic cycle at the end of 7 days of EGF treatment *in vitro*. However, to get a better idea of how soon the Müller glia re-enter the mitotic cell cycle after treatment with EGF in explants, we also assessed the number of BrdU+ and PH3+ cells in P10 explants at each day of culture (Fig. 1H,I). The first BrdU+ Müller glia were present after only 2 days *in vitro*, but their numbers steadily increase over the culture period (Fig. 1H). M-phase (PH3+) Müller glia were also observed after only 2 days *in vitro*, and their numbers appeared to reach a peak after 4 days; by 8 days there were few PH3+ cells (Fig. 1I). These data suggest that the Müller glia respond to the mitogen within a few days *in vitro* and continue to multiply for almost a week in the explants. However, even with the continued treatment with EGF, there appears to be a decline in Müller glial proliferation in explants cultured for more than a week. This may reflect some intrinsic limit to the number of cell cycles they can undergo, or may reflect technical limitations with the explant culture system.

Müller Glia from P12 Mice Grow Well in Dissociated Cultures

Although it is difficult to grow Müller glia from the mature mouse retina, and this has led investigators to

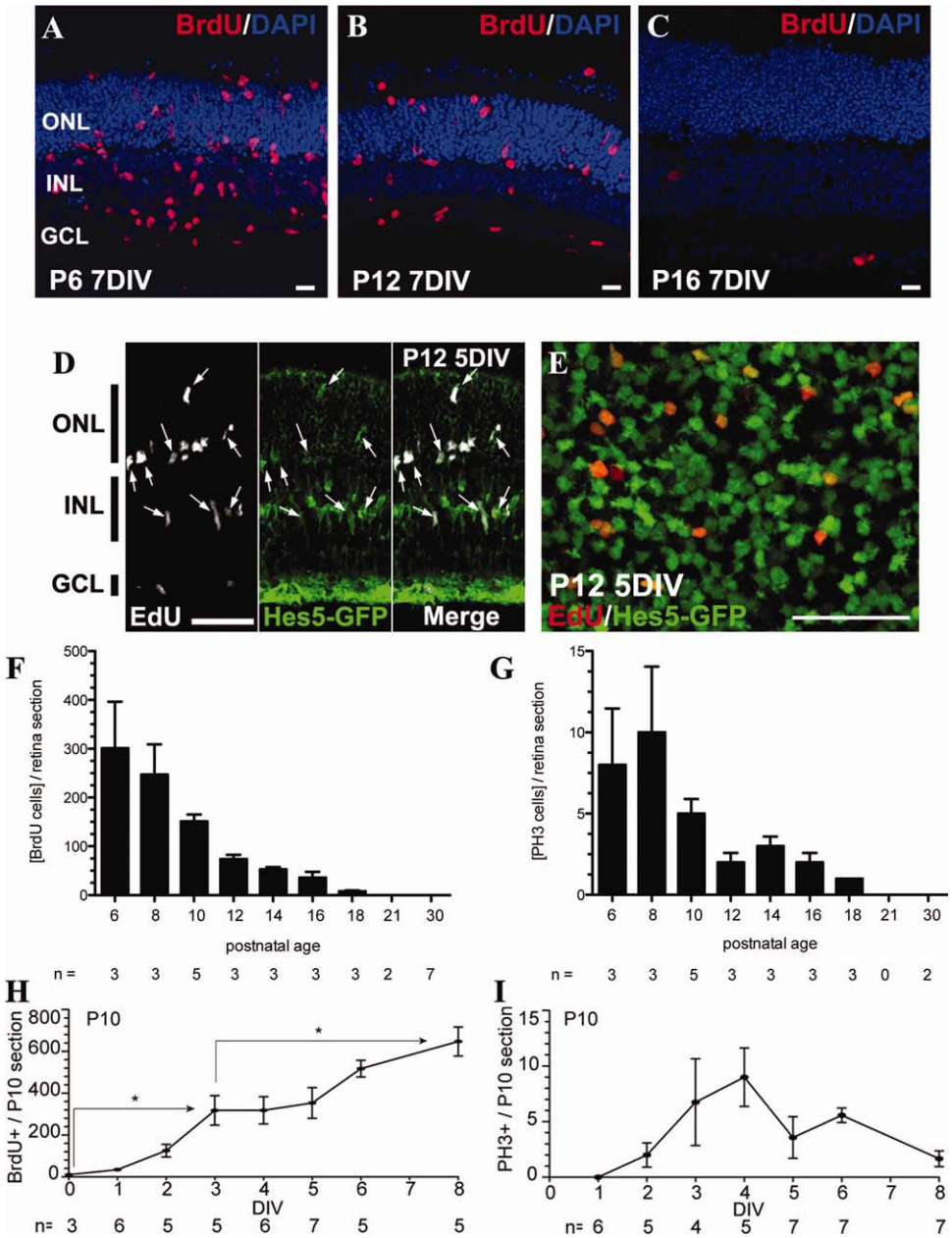

Fig. 1. Stimulation of Müller glial proliferation in retinal explants *ex vivo* is age-dependent. Retinal explants were analyzed by immunostaining of flatmounts (**E**) or after tissue sectioning (**A-D**, **F-I**). (A) P6 retina cultured for 7 days (7DIV) showed BrdU-labeled cells (S-phase cells) across the entire retina. (B) P12, 7 DIV; BrdU- labeled cells were still present. (C) P16, 7DIV; almost no BrdU+ cells were found after 7DIV. (D) P12, 5DIV; the majority of EdU+ cells (white) expressed Hes5-GFP (green) (arrows), a marker for Müller glia. (E) P12, 5 DIV; a 3 μm single optical section of confocal image taken in the inner nuclear layer (INL) of a retinal explant showing EdU+/Hes5-GFP+ double positive Müller cell nuclei. (F) Retinal explants cultured at increasing postnatal ages showed a decline in Müller glial proliferation. (G) Retinal explants at increasing postnatal ages decline in phosphohistone-3 positive (PH3+) M-phase cells. (H) At P10, one day after explantation few BrdU-labeled cells were present; however, after 4 DIV, BrdU-labeled proliferating cells were found across the entire retina, and continued to increase to 8 DIV. (I) Time course of the PH3+ dividing Müller glia in P10 retina explants. The number of PH3+ cells peaked at 4DIV, along with BrdU+ cells. Scale bars: A–C 10 μm and D,E 50 μm. *$P < 0.01$ with *t*-test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

use oncogenes to immortalize them for *in vitro* studies (e.g., Otteson and Phillips, 2010), our results from the explant culture experiments suggested that Müller glia from retinas of mice less than 14 days postnatal might be better able to expand *in vitro* as dissociated cells than Müller glia isolated from adult animals. To test for this possibility, we dissociated retinas from P12 mice and plated the cells in medium containing serum and EGF. Most of the neurons did not survive when dissociated at this age, and the dead neurons were removed after the first day of culture; however, the Müller glia attached to the plate within the first 24 h and over

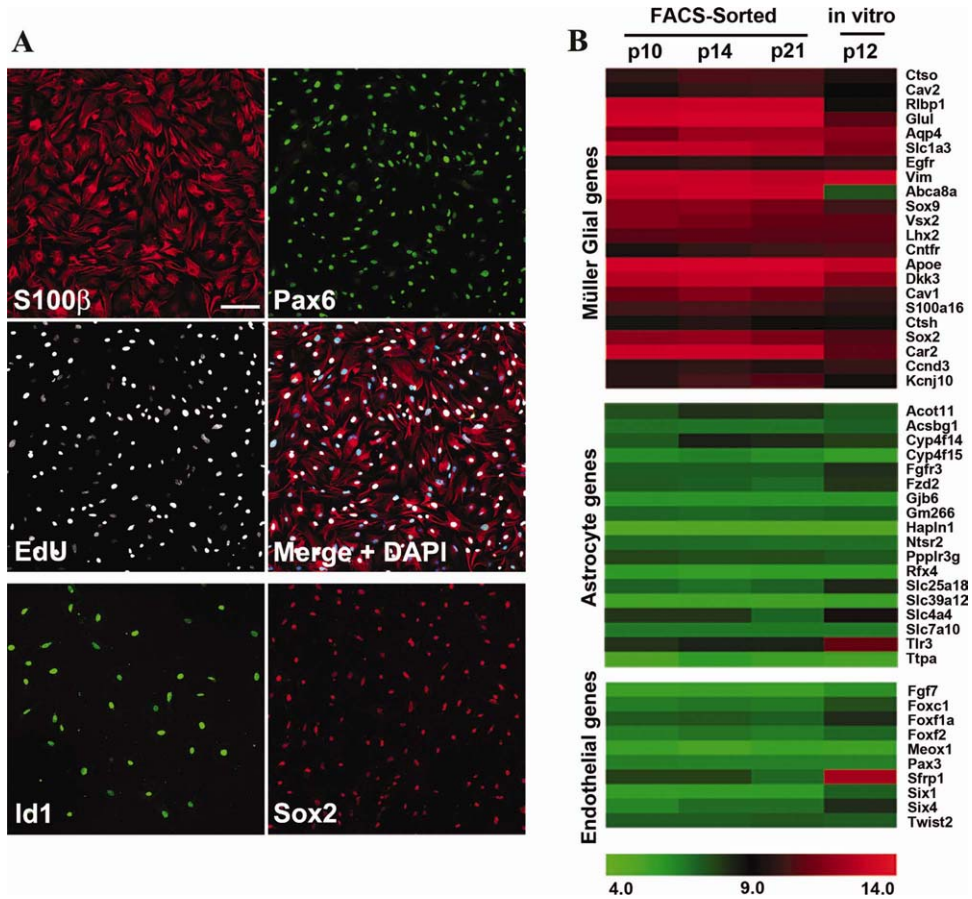

Fig. 2. Müller glia from P12 mice can be maintained in dissociated cell culture. (**A**) Müller glia from P12 retinas 5 DIV expressed S100β (red) and Pax6 (green) and incorporated EdU (white). Müller glia expressed Id1 (green, lower panel) and Sox2 (red, lower panel). Scale bar: 100 μm. (**B**) Microarray analysis of FACS-sorted Hes5-GFP+ Müller glia from P10, P14, and P21 retinas compared with P12 Müller glia cultures. Müller glial genes were highly enriched in cultured Müller glia. Astrocyte and endothelial cell-specific genes were not highly expressed in P12 Müller glia cultures. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the next 5 days expanded to form confluent monolayers (Fig. 2A). The cells were passaged after 4 days *in vitro*, and this further reduced the numbers of surviving neurons.

We analyzed the cultures using several different antibody markers of Müller glia, and by comparing their gene expression with freshly dissociated, FACS-sorted Müller glia from various ages of developing retina (data from Nelson et al., 2011). After 5 days of culture, the vast majority of the cells were labeled for the Müller glial markers S100β, Id1, Pax6, and Sox2 (Figs. 2A and 5A). In addition, EdU labeling throughout the culture period showed that most of the Müller glia re-entered the cell cycle (92.4%+/− 3.9), consistent with the proliferation ability of Müller glia in explants of this age (Fig. 1). Gene expression studies of Müller glia freshly isolated from retina have identified additional markers, including *Glul, Vim, Sox9, Aqp4, Ctsh, Car2, Ccnd3, Dkk3,* and *Apoe*. The gene expression analysis shows that nearly all of these were expressed at similar levels in the dissociated cell cultures as they were in the FACS-sorted Müller glia (Fig. 2B). Since, the retina also contains other cell types—astrocytes and endothelial cells—that could potentially proliferate in dissociated cell cultures, we also used the gene expression data to determine whether these cells made up a significant contaminating population. Although astrocytes express many of the same genes as Müller glia, there is also a cohort of genes expressed specifically in astrocytes and not Müller glia. Expression of astrocyte-specific genes was low in dissociated Müller glial cultures, similar to the levels present in freshly isolated Müller glia (Fig. 2B). Lastly, genes enriched in endothelial cells were not highly expressed in the dissociated Müller glial cultures (Fig. 2B), and there were fewer than 1% SIB4+ microglia/endothelial cells, so there appears to be only a low level of contamination from this source. Thus, the Müller glia in P12 mouse retina express most Müller glial markers at close to mature levels, but can still be expanded through at least one passage *in vitro*. In contrast, repeated attempts to establish Müller glia from mature mouse retina met with relatively little

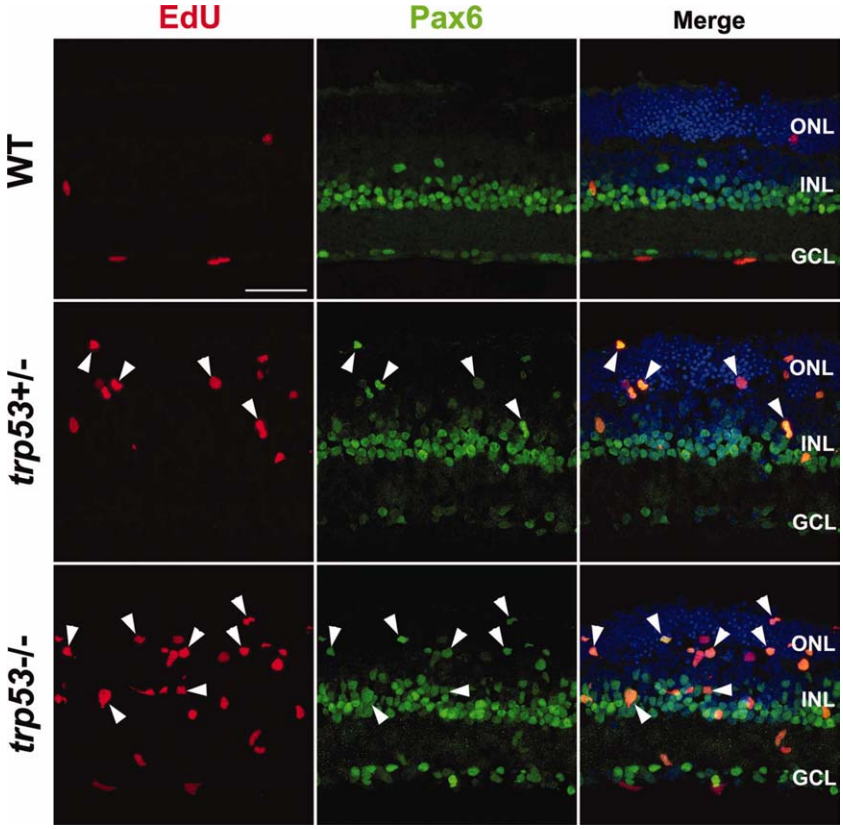

Fig. 3. Loss of *trp53* stimulates cell proliferation in adult retinal explants with EGF treatment for 5 days. *trp53*−/− adult retinal explants showed cell proliferation (EdU+ cells; red) when treated with EGF (lower panels), whereas wild-type explants did not (upper panels). *trp53*+/− explants showed intermediate level of cell proliferation (middle panels). EdU+ cells were primarily in the INL and ONL and were Pax6+ (arrowheads). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

success. Thus, for both dissociated Müller cells and explant cultured Müller cells, there appears to be a developmental restriction in their ability to proliferate *in vitro*.

Müller Glia from *trp53*−/− or *trp53*+/− Mice Proliferate in Mature Mouse Retina *In Vitro*

The tumor suppressor p53 is a key regulator of cell proliferation in astrocytes. Since, Müller glial cells have an overall gene expression profile that is similar to astrocytes (Nelson et al., 2011); we postulated that their proliferation might also be regulated by p53. To test this hypothesis, we assessed Müller glial proliferation in adult wild-type, *trp53*+/− and *trp53*−/− mouse retinas, a stage when there is no Müller glial proliferation in wild-type mice (Fig. 1). Previous studies have shown that while some of the *trp53*−/− mice die during embryogenesis, most survive and develop normally. No developmental phenotype has been identified in the *trp53*+/− mice. We analyzed the retinas of *trp53*−/− mature animals, and did not detect any gross developmental abnormalities (data not shown).

To assess a role for p53 in Müller glial proliferation, we set up explant cultures of adult (>6 week) mouse retinas from wild-type, *trp53*+/− and *trp53*−/− animals. The retinas were cultured for 5 days, with EGF to stimulate Müller glial proliferation and EdU to label the S-phase cells. When we analyzed these cultures, there was a large increase in the number of EdU+ Müller glia (Pax6+/Sox9+) in the *trp53*−/− and *trp53*+/− mouse retinas (Figs. 3 and 4). There were a greater number of EdU+ Müller cells in the retinas from the *trp53*−/− when compared with the *trp53*+/− mice, and loss of a single allele of *trp53* was sufficient to promote re-entry of the adult Müller glia into the mitotic cell cycle (Fig. 3). The Pax6+/EdU+ cells in the *trp53*−/− and *trp53*+/− retinas were present in both the INL, and the ONL (Fig. 3), similar to the P12 explants (Fig. 1B,D). There were also many cells in the ganglion cell layer that took up EdU that were not labeled with Pax6 or Sox9 (Fig. 3); we presume these are microglia or endothelial cells and they were not included in our analysis.

The above data show that the Müller glia in adult *trp53*−/− or *trp53*−/+ mouse retina proliferate when placed in explant culture and treated with EGF. Figure 4 demonstrates that this effect requires EGF, since when this growth factor was not included in the medium, there was only a low level of proliferation (Fig. 4A,B). Quantitation of the effect of EGF on proliferation of *trp53*−/− Müller glia revealed that over 10% of the Sox9+ Müller glia re-enter the cell cycle upon EGF

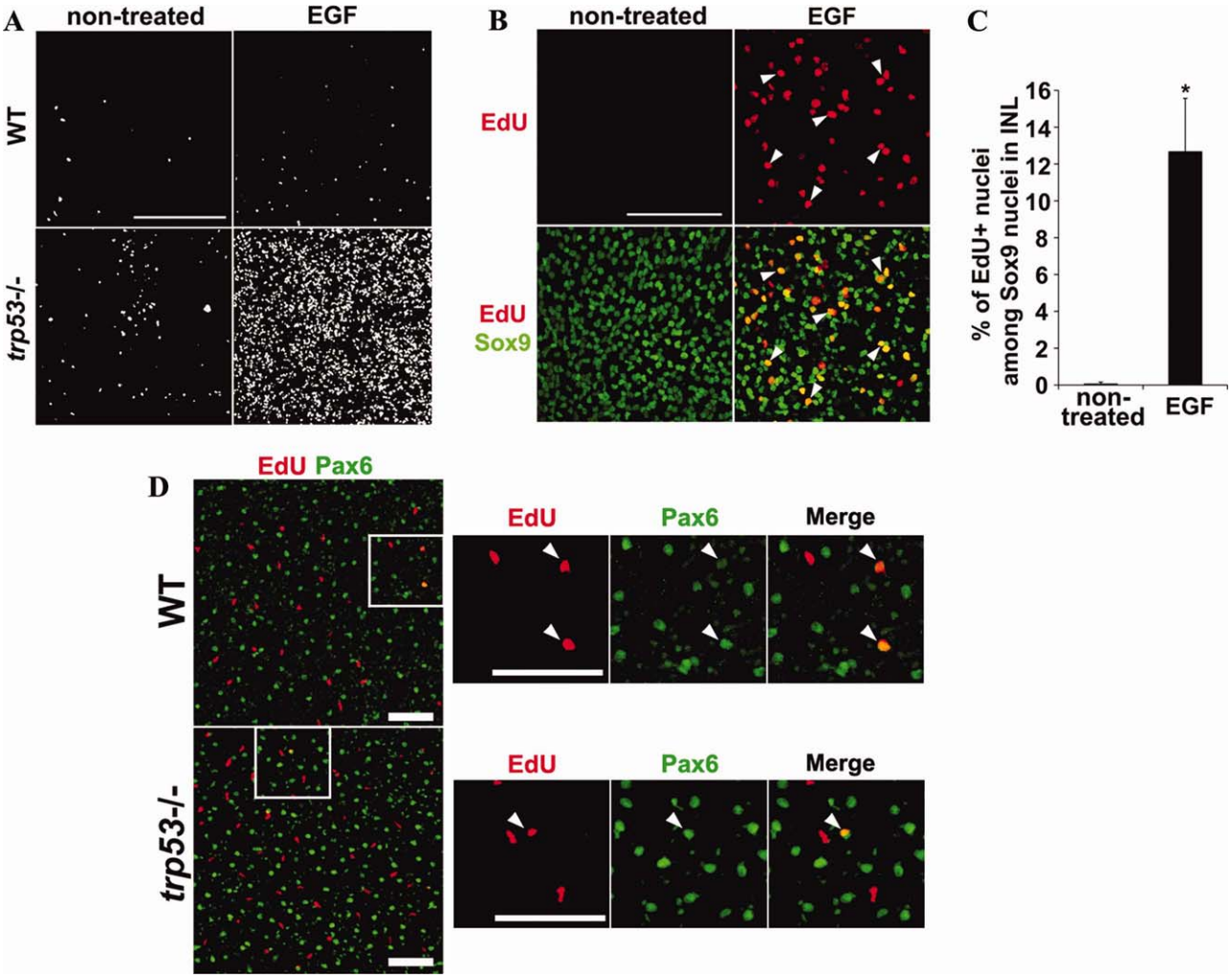

Fig. 4. Proliferating cells in *trp53*−/− adult retinal explants are Müller glia. Adult retinas from indicated genotype were cultured as explants for 5 days with or without EGF. (**A**) EdU^+ positive cells in ONL and INL in retinal flatmounts. Many more cells proliferated in *trp53*−/− retina with EGF treatment compared with untreated *trp53*−/− retinas. (**B**) Three micrometer single slice images of *trp53*−/− INL on retinal flatmounts. EdU (red) colocalized with Sox9 (green), a marker for Müller cell nuclei (arrowheads). (**C**) Percentage of EdU+/Sox9+ (proliferating Müller glia) among total Sox9+ nuclei (total Müller glia) in *trp53*−/− INL was calculated from a random field imaged for each retinal flatmount 12.7 ± 2.87% of total Sox9+ nuclei were also EdU+ when explants were treated with EGF. $^*P < 0.01$ (*t*-test). Error bars are in SEM ($n = 5$). (**D**) WT or *trp53*−/− mice were injected with NMDA intravitreally to induce retinal damage. Intravitreal injection of EGF and EdU were performed to stimulate cell proliferation and to detect proliferating cells, respectively. Representative 1 mm single slice images of WT and *trp53*−/− INL. The arrows point out Pax6+/EdU+ cells in the INL in both WT and *trp53*−/−. Scale bars: 100 mm. Scale bars in A, B, and D: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stimulation (Fig. 4C). These results further suggest that neuronal damage, other than that which occurs from explant culture, is not required for the adult *trp53*−/− Müller glia to proliferate. Despite the robust proliferation observed in *trp53*−/− retinas *in vitro*, *in vivo* damage by NMDA and intraocular EGF injection failed to stimulate Muller glial proliferation over that observed in wild-type retinas (Fig. 4D).

Glial cultures isolated from wild-type adult mice show very little, to no, ability to proliferate in dissociated cell cultures. To determine whether the effects of p53 loss that we observed on Müller glia in explant culture extended to dissociated cell culture, we enzymatically dissociated adult (>28 day) retinas from wild-type, *trp53*+/− and *trp53*−/− mice, and cultured the cells under similar conditions used for the P12 Müller glia. We saw no evidence of Müller glial cell proliferation in the wild-type cultures, though some cells survived for over one week (Fig. 5A). However, in the *trp53*−/− or *trp53*+/− cultures, many of the Müller glia re-entered the mitotic cell cycle and within a week established confluent monolayers of cells (Fig. 5A). The cells could be passaged for at least 4 weeks in *trp53*+/− mice, and for more than 8 weeks in *trp53*−/− mice. We calculated the doubling time for the *trp53*−/− to be ∼ 31–36 h and that of the *trp53*+/− to be greater than 60 h. We verified that the majority of cells in the cultures were Müller glia by using the same markers described above (Fig. 5B).

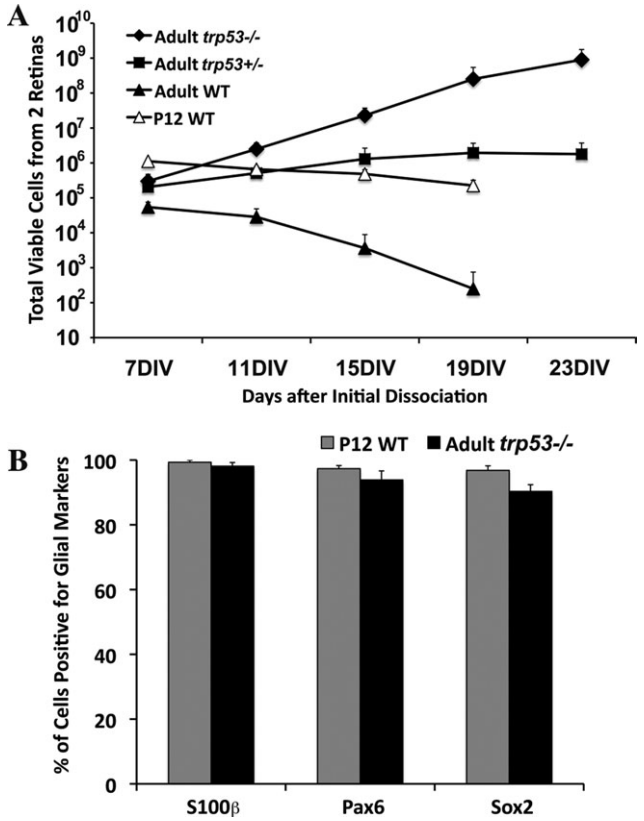

Fig. 5. *trp53*−/− dissociated adult Müller glia show an increased rate of proliferation over *trp53*+/− or wild-type. (**A**) Cells were passaged every 4 days and replated at 50,000 cells/10 cm^2. Total number of cells at each passage are shown. WT adult and P12 cultures did not expand after 11 days. The doubling time of *trp53*+/− was ∼ 63 h in the first two weeks, but after 19 days *trp53*+/− no longer expanded. The doubling time of *trp53*−/− remained relatively constant. (**B**) Percentage of cells positive for glial markers: S100β, Pax6, and Sox2, in dissociated Müller glial cultures from P12 wild-type and adult *trp53*−/− retinas. Error bars indicate SD (n = 3).

Mechanism of p53 Regulation of Müller Glial Proliferation

To determine the mechanism by which loss of p53 leads to increased Müller glial proliferation, we analyzed the expression of known p53 targets in an Affymetrix cDNA array dataset of wild-type glia isolated from Hes5-GFP+ mice (Nelson et al., 2011). Several known p53 targets change substantially in Müller glia as they mature; in particular, members of the cyclin dependent kinase inhibitor family are upregulated in Müller cells in the first two postnatal weeks. In addition, two other well-established p53 targets, *Btg2*, a mitotic inhibitor, and *Atf3*, a p53 regulated transcription factor, also showed increases in expression in the Müller cells as they mature.

To determine whether any of these genes were relevant to the increase in Müller glial proliferation, we carried out qPCR of *trp53*−/− and wild-type retinas that had been cultured as explants for 5 days (Fig. 6A–D). We found that the level of expression of *Cdkn1a*/$p21^{cip}$, was significantly higher in wild-type retinas than in *trp53*−/− retinas (Fig. 6A). In addition, the levels of *c-myc* increased to a greater degree in the *trp53*−/− retinas than in the wild-type retinas (Fig. 6B). These differences are enhanced by treatment with EGF. We also found that there was an increase in the p53 regulated transcription factor, *Atf3*, in the *trp53*−/− retinas (Fig. 6C). By contrast, we did not observe a significant change in another p53 target, *Btg2* (Fig. 6D). The effects of the loss of p53 on Müller glia were not due to a reduction in the cell cycle regulator $p27^{kip}$ (Fig. 6E,F), suggesting that the p53-regulated pathways relevant to Müller glial proliferation are independent of the previously characterized $p27^{kip}$ regulation. Together, our data support a model in which p53 normally leads to an inhibition of Müller glial proliferation through an increase in the mitotic inhibitor *Cdkn1a*/$p21^{cip}$ and a repression of the cell cycle progression factor, *c-myc*. In addition to these changes in gene expression, we also analyzed the *trp53*−/− retinas for signs of reactive gliosis. Targeted deletion of cell cycle regulators has been shown to lead to Müller glial reactivity (Levine et al., 2000; Vazquez-Chona et al., 2011), and loss of *trp53* could have similar effects. Indeed, when we analyzed the *trp53*−/− retinas for GFAP expression, we found that both with qPCR and immunofluorescence, there was an increase in expression of this marker of reactive gliosis (Fig. 7A,B).

DISCUSSION

We have found that Müller glia from mouse retina undergo a developmental restriction in their ability to re-enter the mitotic cell cycle in the second postnatal week, even in the presence of high levels of EGF. This phenomenon occurs in both explant cultures and dissociated cell cultures. However, in *trp53*−/− and *trp53*+/− mice this developmental restriction no longer occurs and Müller glia can be stimulated to divide in the presence of EGF even in mature retina, possibly through a reduced expression of the cyclin dependent kinase inhibitor $p21^{cip}$/*Cdkn1a*, or through an increase in expression of *c-myc*.

During retinal development, the progenitors undergo extensive cell divisions to generate the large number of retinal cells that comprise this tissue. At the end of the first postnatal week, however, most of the progenitor cells have withdrawn from the cell cycle in mice and rats, and the neurons remain postmitotic throughout life. In contrast, while the Müller glia does not actively proliferate in the mature retina, they retain competence to re-enter the mitotic cell cycle in response to mitogens for almost a week after they have been generated. Nevertheless, adding even high levels of EGF to either explant or dissociated cell cultures of mouse Müller glia after P12 does not stimulate their proliferation. A similar developmental decline has been previously reported in the rat retina, and this is mediated in part by an increase in the response of the cells to TGF-beta signaling and a decline in their responsiveness to EGF (Close et al., 2006). Our data suggest that this decline in

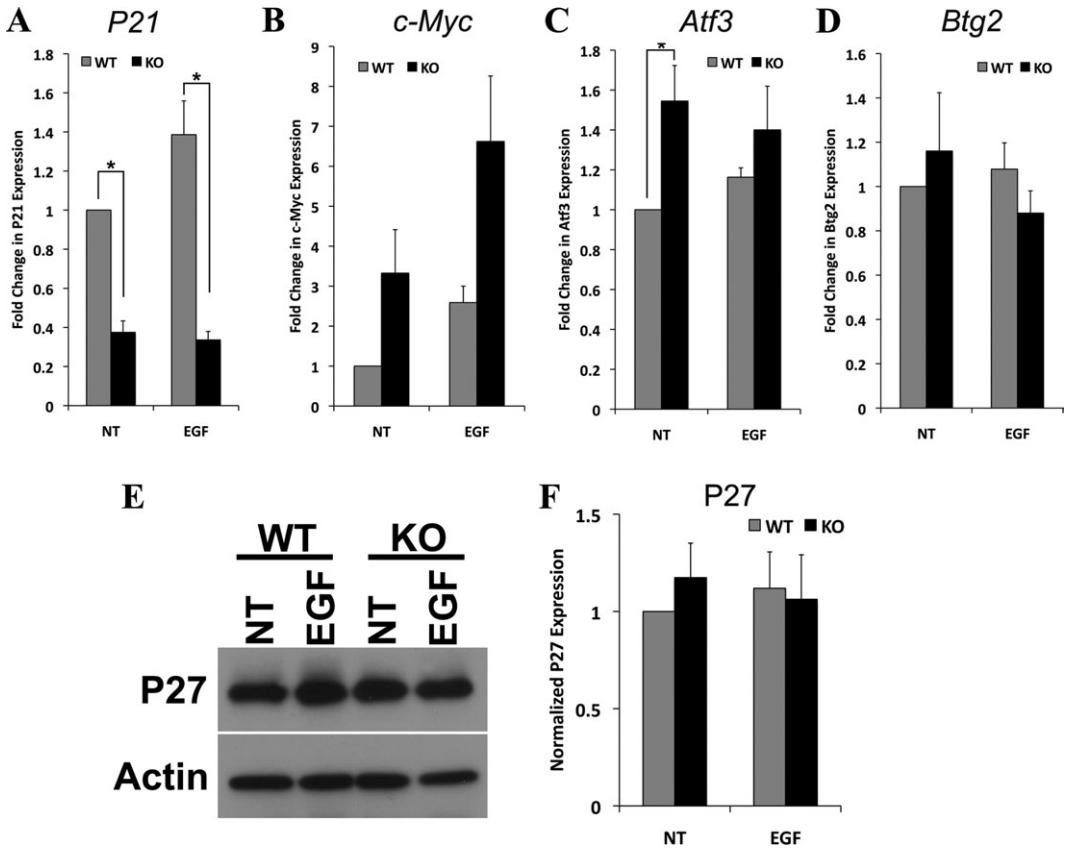

Fig. 6. Expression of *trp53* regulated genes in retinal explants (5 DIV). (**A**) *trp53*−/− (KO) adult explants had significantly decreased expression of $p21^{cip}$ compared with wild-type after 5 DIV with or without EGF by qPCR. (**B,C**). There was a trend towards increased *c-myc* and *Atf3* expression in *trp53*−/− explants compared with wild-type. (**D**) No difference in *Btg2* expression between *trp53*−/− and wild-type explants. (**E**) Representative Western blot for $p27^{kip}$. β-actin was used as a loading control. (**F**) Normalized $p27^{kip}$ protein expression in adult wild-type and *trp53*−/− explants with or without EGF treatment was quantified and plotted. Loss of p53 does not affect $p27^{kip}$ expression in adult retinal explants. $^*P < 0.05$ with paired *t*-test ($n = 3$). Error bars are in SEM.

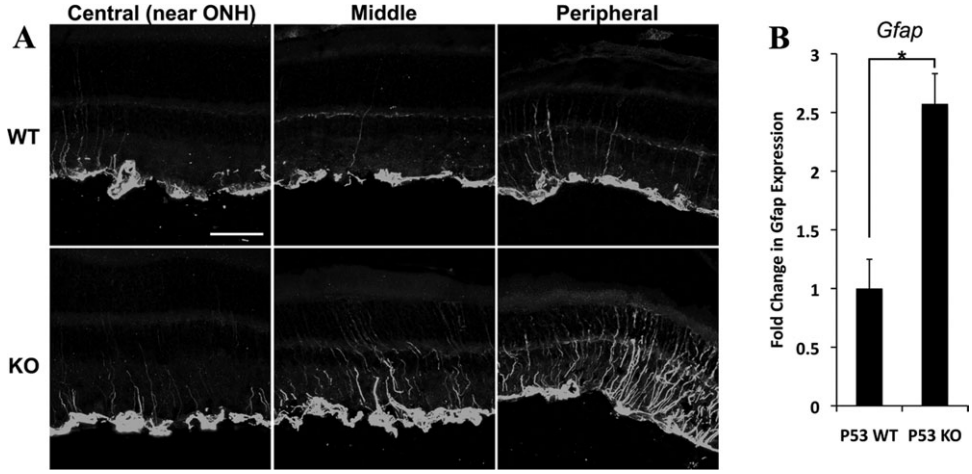

Fig. 7. Adult *trp53*−/− retina expresses increased level of GFAP compared with wild-type. Retinas from adult *trp53*−/− (KO) and wild-type (WT) littermates were collected for IHC (**A**) or qPCR (**B**) to analyze expression of GFAP, a marker for Müller glial activation. (A) Increased GFAP expression was observed in *trp53*−/− retina (bottom panels). GFAP expression in wild-type adult retina was limited to Müller cell endfeet (upper panels). Scale bar: 50 μm. ONH, optic nerve head. (B) qPCR shows significant increase in *Gfap* mRNA expression in *trp53*−/− retinas compared with wild-type littermates. *Gapdh* was the normalization control. $^*P < 0.01$. Error bars are in SEM ($n = 9$).

Müller glial response to EGF and other mitogens might be due in part to a p53 mediated increase in $p21^{cip}$ and a concomitant decrease in c-myc expression.

Previous studies have implicated Cdki's in the inhibition of cell proliferation in Müller glia. The cell cycle regulator $p27^{kip}$ has been shown to be critical for the

maintenance of Müller glia in a mitotically quiescent state (Levine et al., 2000). Loss of this gene, even in mature animals, leads to a re-entry of Müller glia into the cell cycle (Vázquez-Chona et al., 2011). In our analysis of *trp53−/−* mice, we did not observe changes in expression of $p27^{kip}$ (Fig. 6E,F), but rather we found that a related gene, *Cdkn1a/p21^{cip}*, was significantly down-regulated in the *trp53−/−* retina (Fig. 6A). These results suggest that these two Cdki's may both be necessary for regulating Müller glial proliferation, though p53 likely affects the expression of a large number of genes, and the effects on *Cdkn1a/p21^{cip}* may only partly be responsible for the *trp53−/−* phenotype in the Müller glia.

Previous studies have shown that p53 is a key regulator of cell proliferation in CNS glia and adult neural stem cells (Bogler et al., 1999; Gil-Perotin et al., 2006; Meletis et al., 2006; Yahanda et al., 1995; Zheng et al., 2008). The mechanisms by which p53 inhibits Müller glial proliferation may be similar to those operating in astrocytes or adult neural stem cells. In astrocytes, p53 promotes cell cycle arrest by repressing *c-myc* transcription and/or by activating the cyclin dependent kinase inhibitor $p21^{cip}$/Cdkn1a (Cox and Lane, 1995; Ho et al., 2005; Kippin et al., 2005; Meletis et al., 2006; Zheng et al., 2008). The activation of *Cdkn1a/p21^{cip}* by p53 is likely to be direct, while there is evidence that p53 represses *c-myc* expression via indirect mechanisms (Cannell et al., 2010; Sachdeva et al., 2009). In the *trp53−/−* Müller glia, we found an increase in *c-myc* and a reduction in *Cdkn1a/p21^{cip}*. In the brain of *trp53−/−* mice, similar changes in astrocytes are thought to lead to astrocytomas (Zheng et al., 2008). We have not found evidence of glial tumors in the retinas of the *trp53−/−* mice; however, we have observed an increase in GFAP expression, somewhat analogous to that observed in the *Cdkn1b/p27^{kip}* knockout mice (Levine et al., 2000; Vázquez-Chona et al., 2011), suggesting that some of the same downstream effects may occur with perturbations in these pathways. Thus, although Müller glia and astrocytes share a common role for p53 in the regulation of *c-myc*, *p21^{cip}/Cdkn1a* and possibly other cell cycle regulatory genes, Müller glia appear to have additional inhibitors of cell proliferation that are active *in vivo*.

Consistent with the possibility that there are additional regulators of proliferation in Müller glia are our own *in vivo* experiments in the *trp53−/−* mice. Although there is a robust proliferative response *in vitro*, we failed to see this same effect *in vivo*, even after multiple injections of mitogenic factors. These results suggest that the *in vitro* environment reduces the effectiveness of these additional mitotic inhibitors, perhaps by down-regulating known inhibitory factors, like $p27^{kip}$ or alternatively by up-regulating factors that promote re-entry into the mitotic cell cycle, such as cyclinD1. Further studies of the changes that occur upon explant culturing the retina may allow the identification of the additional regulatory factors.

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