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Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina

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SUMMARY

The mechanisms of cell fate diversification in the retina are not fully understood. The seven principal cell types of the neural retina derive from a population of multipotent progenitors during development. These progenitors give rise to multiple cell types concurrently, suggesting that progenitors are a heterogeneous population. It is thought that differences in progenitor gene expression are responsible for differences in progenitor competence (i.e. potential) and, subsequently, fate diversification. To elucidate further the mechanisms of fate diversification, we assayed the expression of three transcription factors made by retinal progenitors: Ascl1 (Mash1), Ngn2 (Neurog2) and Olig2. We observed that progenitors were heterogeneous, expressing every possible combination of these transcription factors. To determine whether this progenitor heterogeneity correlated with different cell fate outcomes, we conducted Ascl1- and Ngn2-inducible expression fate mapping using the *CreER*^{TM}*/LoxP* system. We found that these two factors gave rise to markedly different distributions of cells. The Ngn2 lineage comprised all cell types, but retinal ganglion cells (RGCs) were exceedingly rare in the Ascl1 lineage. We next determined whether Ascl1 prevented RGC development. *Ascl1*-null mice had normal numbers of RGCs and, interestingly, we observed that a subset of Ascl1+ cells could give rise to cells expressing Math5 (Atoh7), a transcription factor required for RGC competence. Our results link progenitor heterogeneity to different fate outcomes. We show that *Ascl1* expression defines a competence-restricted progenitor lineage in the retina, providing a new mechanism to explain fate diversification.

KEY WORDS: Development, Neurogenesis, Retina, Mouse

INTRODUCTION

The retina comprises seven principal cell types: rod and cone photoreceptors, amacrine, bipolar and horizontal interneurons, Müller glia and retinal ganglion cells (RGCs). Retroviral lineage-tracing studies in rodents have shown that all of these cell types derive from a common multipotent progenitor population (Turner and Cepko, 1987; Turner et al., 1990), though fate-restricted progenitors were not observed. Nonetheless, the time of permanent cell cycle exit ('birthdate') for each cell type follows a stereotypical pattern (Carter-Dawson and LaVail, 1979; La Vail et al., 1991; Rapaport et al., 2004; Sidman, 1961; Young, 1985). This change in production of the different cell types over the period of retinogenesis has led several investigators to propose that the competence (potential) of progenitors changes over time (Reh and Kljavin, 1989; Watanabe and Raff, 1990), which probably depends on the combination of genes expressed by progenitors (Livesey and Cepko, 2001). Attempts to link directly heterogeneity in progenitors to differences in competence have failed to identify transcription factors that define restricted retinal progenitor populations.

One family of transcription factors that has been shown to be important in the regulation of cell fate is the basic helix-loop-helix (bHLH) family. Three of these transcription factors, *Olig2*, *Ngn2* (*Neurog2*) and *Ascl1* (*Mash1*), are expressed in subpopulations of progenitor cells throughout the nervous system, including the retina (Guillemot and Joyner, 1993; Jasoni and Reh, 1996; Jasoni et al., 1994; Lu et al., 2000; Nakamura et al., 2006; Shibasaki et al., 2007; Sommer et al., 1996; Takebayashi et al., 2000). Previous studies concluded that these factors are made by retinal progenitors; however, no study has yet analyzed whether these factors are expressed in overlapping or distinct progenitor populations, i.e. whether they indeed define progenitor heterogeneity.

To address this, we first examined Olig2, Ascl1 and Ngn2 expression in progenitors. We found cells that expressed every single, double and triple combination of these factors in the developing retina, consistent with the hypothesis that these factors define progenitor heterogeneity. To determine whether this transcription factor heterogeneity corresponded to different fate outcomes in the progenitors, we conducted Ascl1- and Ngn2-inducible expression fate mapping using the *CreER*^{TM}*/LoxP* system (Metzger and Chambon, 2001; Metzger et al., 1995). We found that Ascl1 and Ngn2 gave rise to very different distributions of retinal cells. Ascl1-expressing cells can give rise to all major types of retinal cells, though RGCs were not significantly represented. Interestingly, we found that Ascl1+ cells could express the RGC competence factor Math5 (J. A. Brzezinski, 4th, PhD thesis, University of Michigan, 2005) (Mu et al., 2005; Yang et al., 2003), but this subpopulation of cells did not adopt RGC fate. Together, our data show that gene expression heterogeneity in retinal progenitors leads to different fate choice outcomes: *Ascl1* expression defines a competence-restricted lineage in the retina.

MATERIALS AND METHODS

Animals and tamoxifen administration

All animals were used in accordance with University of Washington and UT Southwestern IACUC approved protocols. To trace the lineage of Ascl1+ progenitors, we used the *Ascl1*^{CreERT2} knock-in mouse strain in

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which CreER^{T2} replaces endogenous *Ascl1* (Kim et al., 2011a; Kim et al., 2011b). To track the expression of *Ascl1*, we used a previously characterized mouse knock-in line in which the coding region of *Ascl1* was replaced by a nuclear localized GFP element (Leung et al., 2007). Both *Ascl1*$^{GFP/+}$ and *Ascl1*^{CreERT2} have all of the 5′ and 3′ regulatory elements intact and GFP (or Cre) expression matches *Ascl1* expression (Leung et al., 2007). Heterozygous *Ascl1*$^{GFP/+}$ mice were intercrossed to generate wild-type, heterozygous and *Ascl1*$^{GFP/GFP}$-null mice, which did not survive past birth. To track Math5 expression we used *Math5*$^{Cre/+}$ knock-in mice (Yang et al., 2003). For the Ngn2 lineage, the coding region of Ngn2 was replaced by *CreER*^{TM} (*Ngn2*$^{CreERTM/+}$) (Ma and Wang, 2006; Zirlinger et al., 2002). To monitor Cre activity, we used a dual reporter mouse line (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$, Jackson Stock 007576, i.e. *mTmG*) that ubiquitously expressed membrane-localized tomato until recombination, at which point it expressed membrane-localized GFP (Muzumdar et al., 2007) from a *CMV*-β-*actin* enhancer/promoter element inserted into the *ROSA26* locus. To induce recombination, pregnant mice were given 2 mg tamoxifen (T5648, Sigma, St Louis, MO, USA; 10 mg/ml in corn oil) intraperitoneally at both embryonic day (E) 12.5 and E13.5, at both E14.5 and E15.5, or at E17.5. The eyes from these mice were collected for analysis at postnatal day (P) 0 or at P19-22. For short-term analysis, E14.0 mice were given 2 mg of tamoxifen and collected 12 or 23 hours later.

Immunohistochemistry and microscopy

Embryos, explants and P0 eyes were fixed for 1 hour in 2% paraformaldehyde (PFA). Adult mice were transcardially perfused with 10 ml of 2% PFA. The eyes were then processed for cryosectioning. Immunostaining was carried out using primary antibodies (below) and appropriate fluorescent secondary antibodies and streptavidin conjugates as previously described (Brzezinski et al., 2010).

The following primary antibodies were used: mouse anti-Ascl1 (1:100; 556604, BD Biosciences, San Jose, CA, USA), goat anti-Brn3a/b/c (pan-specific) (1:50; sc6026, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Brn3a (1:500; Eric Turner, Seattle Children's Research Institute and The University of Washington, Seattle, WA), rabbit anti-Brn3b (1:500; Eric Turner) rabbit anti-calbindin D-28K (1:500; ab1778, Millipore, Billerica, MA, USA), sheep anti-Chx10 (1:200; X1179P, Exalpha, Shirley, MA, USA), mouse anti-Cre recombinase (1:250; mab3120, Millipore), chicken anti-GFP (1:750; ab13970, Abcam, Cambridge, MA, USA), rabbit anti-neurofilament-M (1:500; ab1987, Millipore), goat anti-Ngn2 (1:50; sc19223, Santa Cruz), rabbit anti-Olig2 (1:250; ab33427, Abcam), goat anti-Otx2-biotin (1:150; BAF1979, R&D Systems, Minneapolis, MN, USA), rabbit anti-Pax6 (1:500; PRB-278P, Covance, Princeton, NJ, USA), mouse anti-PKC (1:250; P5704, Sigma), goat anti-Sox2 (1:100; sc17320, Santa Cruz) and rabbit anti-Sox9 (1:500; ab5535, Millipore).

Retrograde RGC labeling

RGC cell bodies were labeled by retrograde transport of biotinylated dextrans (Farah and Easter, 2005). Crystals of 3000 weight lysine fixable biotinylated dextran (Invitrogen) were liberally dissolved on Surgifoam (Ethicon, Somerville, NJ, USA) soaked in 3% L-α-lysophosphatidylcholine (Sigma). Eyes from recently euthanized lineage traced mice were immediately placed on the dextran-coated Surgifoam, optic nerve remnant down, and encased in 2% low-melt agarose/PBS. Eyes were placed in HBSS+ (HBSS with 0.05 M HEPES and 6 mg/ml glucose) for 2 hours under constant aeration. The Surgifoam and agarose were removed and the eyes placed in fresh HBSS+ overnight under constant aeration. A hole was made in the cornea and the eyes fixed for 3 hours in 2% PFA and processed for histology (as above).

Retinal explant cultures

E17.5 *Ascl1*$^{GFP/+}$ heterozygous and null retinas were collected in HBSS+ and placed RGC-layer up on 0.4 μm filter inserts (PICM03050, Milipore). Explants were grown for 9 days at the gas/media interface at 37°C and 5% CO_2. Half of the media (TR media: Neuralbasal supplemented with 1× penicillin/streptomycin, 1× N2 supplement, 1× L-glutamine and 1% dialyzed FBS) (Invitrogen) was changed every other day. To measure proliferation, E18.5 retinas from *Ascl1*$^{GFP/+}$ heterozygous and null embryos were cultured for 48 hours at 37°C and 5% CO_2 with constant nutation. At 47 hours of culture, 1 μg EdU (5′-ethynyl-2′-deoxyuridine) (Invitrogen) was added to the cultures and the explants rocked for an additional hour. Explants were quickly dissociated in trypsin, pelleted, resuspended in TR media, and plated on poly-D-lysine-coated glass coverslips. After 30 minutes, the media was removed and the cells fixed for 7 minutes with 2% PFA. Cells were stained for DAPI and EdU using a Click-iT 555 Kit according to the manufacturer's instructions (Invitrogen).

EdU birthdating

A single intraperitoneal injection of 200 μg EdU was given to an E13.5 timed pregnant BL/6 female. Retinas were harvested from P22 animals, fixed, cryopreserved and sectioned at 10 μm. Sections were stained for calbindin, pan-Brn3 and DAPI as described above. Sections were stained for EdU using a Click-iT 647 Kit (Invitrogen).

Cell counting and statistics

For the analysis of the overlap in expression of the progenitor transcription factors, we manually counted Ascl1-GFP+, Ngn2+, Sox2+ and Olig2+ nuclei from sections of E14.5 and E18.5 *Ascl1*$^{GFP/+}$ heterozygous and null retinas. We counted from at least seven 600× fields from two to four eyes for each time point, representing ~4300 cells at E14.5 and ~6500 cells at E18.5. Pan-Brn3+ RGCs were counted from at least five 400× fields from four eyes from E14.5 and E18.5 *Ascl1* heterozygous and null retinas. This represented ~1450 Brn3+ cells at E14.5 and ~1600 Brn3+ cells at E18.5. Differences in cell populations were evaluated by two-tailed unpaired *t*-tests. We counted Ascl1-GFP+, Math5-Cre+ and Brn3+ nuclei from at least five 600× fields from four eyes at E13.5 and E18.5, representing ~2100 and ~1250 cells, respectively. To determine the effects of *Ascl1* loss on proliferation in E18.5 explants, we counted DAPI+ and EdU+ nuclei from 17 or 18 200× fields representing three heterozygous (~15,600 DAPI+ nuclei) and three *Ascl1*-null (~14,000 DAPI+ nuclei) mice, respectively. Differences in the EdU+ fraction were evaluated using a two-tailed unpaired *t*-test.

For the Ascl1 and Ngn2 lineage traces, cell types were characterized by laminar position and unique morphology. The assignment of RGC versus displaced amacrine cell was made by the presence or absence, respectively, of pan-Brn3, Brn3a/b (in tandem), Neurofilament-M (NFM) or retrograde biotinylated dextran uptake. When counting GFP+ cells, we grouped them into 'clumps' based on their arrangement into radial units. Early born neurons near a radial unit were included because some neurons tangentially migrate a modest distance from their radial unit (Fekete et al., 1994; Reese et al., 1999). We counted 1591 isolated cells and clumps (3115 cells) from 20 eyes for the Ascl1 lineage traced at E12.5/E13.5 and 2528 isolated cells and clumps (4749 cells) from ten eyes for the Ascl1 lineage traced at E17.5. We counted 1075 isolated cells and clumps (1254 cells) from ten eyes for the Ngn2 lineage traced at E12.5/E13.5 and 827 isolated cells and clumps (1088 cells) from four eyes for the E17.5 Ngn2 lineage trace (see Table S1 in the supplementary material). Differences in fate distribution were evaluated by χ^2 tests. Retroviral lineage data and adult cell distributions were taken from Turner et al. (Turner et al., 1990) and Jeon et al. (Jeon et al., 1998), respectively. Differences in clump sizes were analyzed with the Mann-Whitney test. The binomial distribution was used to determine the probability of observing a certain number of RGCs given the number of cells sampled.

To determine the progenitor fraction in the E12.5/E13.5 Ascl1 lineage at P0, all of the GFP+ cells observed in 30 sections from four eyes were scored for the presence or absence of Sox9. The progenitor fraction from E14.0 Ascl1 lineage assayed 23 hours after tamoxifen was calculated in the same fashion (six sections, 212 GFP+ cells).

RESULTS

Ascl1 expression defines a subset of retinal progenitor cells

To determine the relationships between the three bHLH transcription factors known to be expressed in retinal progenitors, we used antibodies to both Olig2 and Ngn2 as well as an

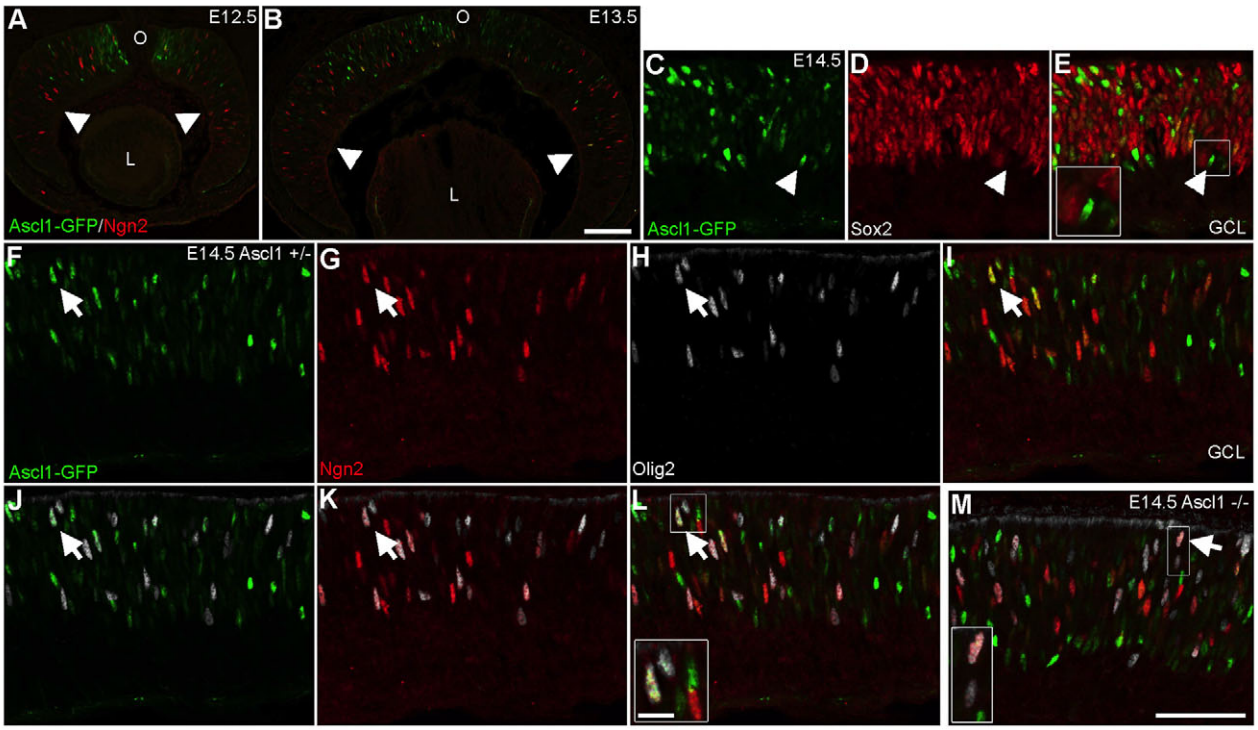

Fig. 1. Retinal progenitor transcription factor expression heterogeneity. (**A,B**) E12.5 (A) and E13.5 (B) *Ascl1*$^{GFP/+}$ mouse retinas stained for Ascl1-GFP (nuclear; green) and Ngn2 (red). Ascl1-GFP expression starts centrally and spreads towards the periphery with age (leading edge marked with arrowheads). (**C-E**) E14.5 retinas stained for Sox2 (red) and Ascl1-GFP (green). A small number of Ascl1– GFP+ cells do not express Sox2 (arrowhead; enlarged in inset). (**F-L**) Ascl1-GFP (green), Olig2 (gray) and Ngn2 (red) staining of the E14.5 retina. Cells that express Ascl1-GFP only; Olig2 only; Ngn2 only; Ascl1-GFP and Olig2; Ascl1-GFP and Ngn2; Olig2 and Ngn2; and all three transcription factors (arrows; enlarged in inset) are observed. (**M**) Ascl1-GFP, Olig2 and Ngn2 expression in E14.5 *Ascl1*$^{GFP/GFP}$-null retinas is similar to control (L). A co-expressing cell is enlarged in inset. GCL, ganglion cell layer; L, lens; O, optic nerve head. Scale bars: 100 μm for A,B; 50 μm for C-M; 10 μm for higher magnification insets.

Ascl1$^{GFP/+}$ line of mice (Leung et al., 2007). Ascl1-GFP expression in heterozygous knock-in mice was consistent with earlier reports on *Ascl1* expression in the mouse retina (Brown et al., 1998; Guillemot and Joyner, 1993; Hufnagel et al., 2010). We observed Ascl1-GFP expression in nuclei in the progenitor zone of the central retina at E12.5 (Fig. 1A). At E13.5, Ascl1-GFP expression had spread towards, but not yet reached, the far periphery (Fig. 1B). At E14.5, Ascl1-GFP extended to the far peripheral retina and GFP+ nuclei were absent from the ganglion cell layer (GCL) (Fig. 1C-E). The majority of Ascl1-GFP cells co-expressed Sox2, a pan-progenitor marker (71.9±4.12% s.d.) (Fig. 1C-E, Fig. 2D-E), but only a small fraction of the Sox2 progenitor population made Ascl1-GFP (25.6±3.99% s.d.) (Fig. 1C-E, Fig. 2E). The fraction (~28%) of the Ascl1-GFP+ cells that did not express Sox2 probably represents transient persistence of GFP in postmitotic cells derived from Ascl1+ progenitors; however, we cannot exclude the possibility that some postmitotic cells transiently express Ascl1. Similarly, only a subset of Sox2+ and Sox9+ progenitors expressed Olig2 (14.1±3.57% s.d.) and Ngn2 (17.2±3.10% s.d.), respectively (Fig. 2E).

To determine whether these transcription factors were expressed in the same retinal progenitor cells, we compared the expression of Ascl1-GFP with that of Ngn2 and Olig2. At E12.5 and E13.5, the Ngn2 expression domain extended further peripherally than Ascl1-GFP (Fig. 1A,B) and three populations of cells were present: Ascl1-GFP+, Ngn2+ and cells that expressed both markers (Fig. 1A,B). At E14.5, we observed similar numbers of cells expressing Olig2 or Ngn2, whereas about 50% more expressed Ascl1-GFP (Fig. 1F-L, Fig. 2A). Interestingly, we found that all seven molecularly distinct populations of cells existed in the retina at E14.5 (Fig. 1F-L, Fig. 2C). The most abundant population expressed solely Ascl1-GFP (19.3±4.49 s.d. cells per 100 μm) (Fig. 2C). Some of these Ascl1-GFP-only cells are likely to be postmitotic; however, this single labeled population was larger than the Ascl1-GFP+, Sox2-negative population identified in parallel experiments (7.75±1.58 s.d. cells per 100 μm) (Fig. 2D), indicating that most of the Ascl1-GFP-only cells are progenitors. The six other population combinations were less common (about three to nine cells per 100 μm), but when pooled (31.2 cells per 100 μm) accounted for 50% more cells than Ascl1-GFP-only (Fig. 2C). Of the cells that made Ascl1-GFP, it was equally likely that they co-expressed Ngn2 or Olig2 (Fig. 2B). A slightly smaller fraction of the Ascl1-GFP+ population expressed all three markers (20.1±4.75% s.d.) (Fig. 2B). Examining three transcription factors, we found considerable progenitor heterogeneity in our snapshots of retinal development at E14.5 and E18.5 (see Fig. S1 in the supplementary material).

Ascl1+ progenitors form a fate-restricted lineage

The above results indicate that there is considerable heterogeneity in the progenitor population with respect to bHLH gene expression. One way to assess whether these progenitor populations are equivalent is to determine whether they generate different lineages. The lineages of Ngn2+ progenitor cells have already been analyzed

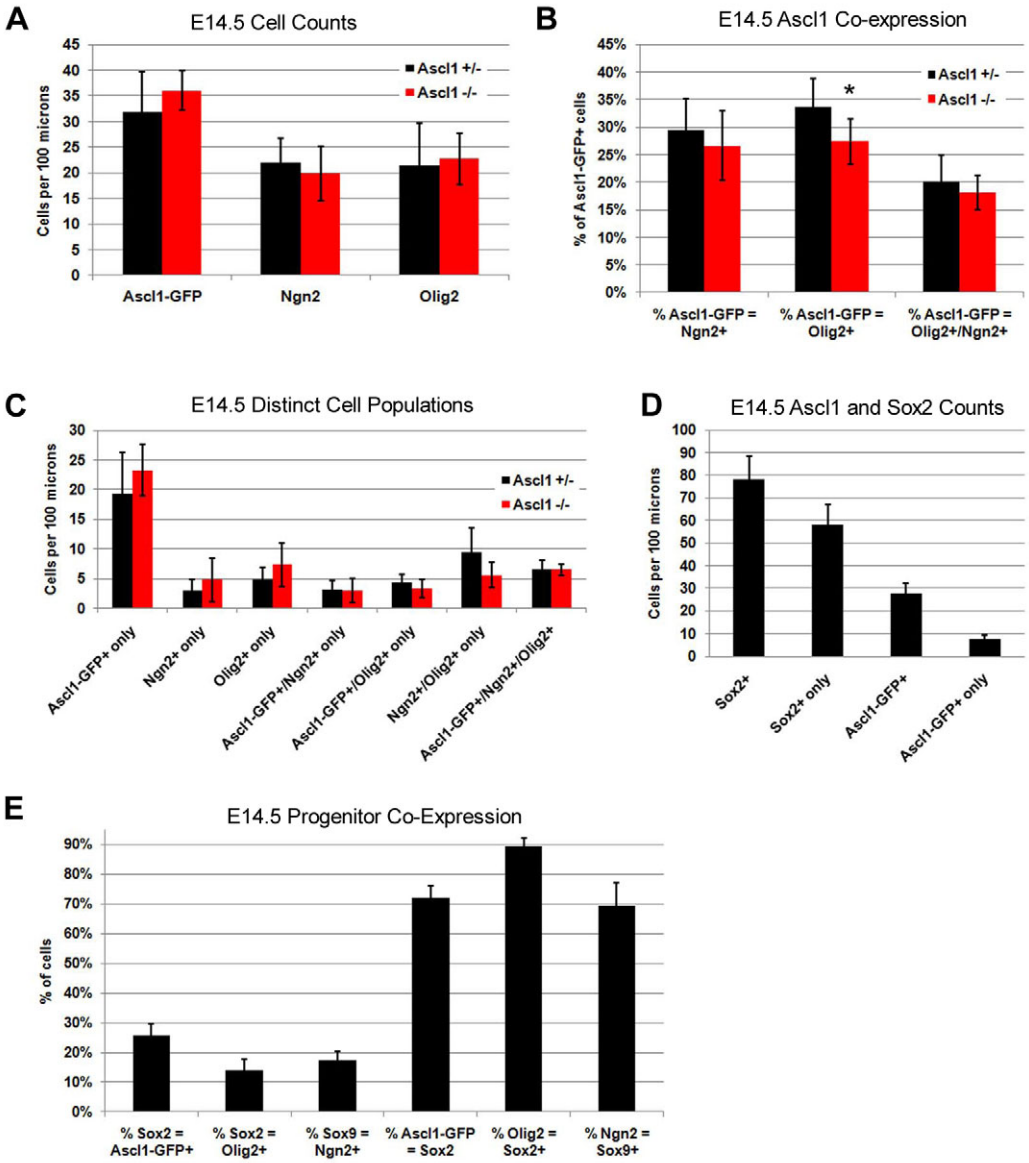

Fig. 2. Transcription factor expression in E14.5 *Ascl1*$^{GFP/+}$ heterozygous and null mouse retinas. (**A**) Plot showing the number of cells that express Ascl1-GFP, Ngn2 and Olig2 at E14.5. The number of cells that express each factor is similar in *Ascl1* mutants (red) and heterozygotes (black). (**B**) Plot showing the percent of Ascl1-GFP+ cells that co-express Ngn2, Olig2 or both. *Ascl1* mutants (red) have fewer Ascl1-GFP+ cells that make Olig2 than controls (black) (unpaired *t*-test, *$P \leq 0.05$). (**C**) Plot showing the number of cells that express each of seven combinations of transcription factors. *Ascl1*$^{GFP/GFP}$-null mice have a similar distribution of cells. (**D**) Plot of Sox2+ and Ascl1-GFP+ cell numbers at E14.5. (**E**) Plot showing the percentage of progenitors that co-express Sox2 or Sox9. Error bars for all panels represent ±s.d.

(Ma and Wang, 2006), but this type of analysis has not been reported for Ascl1+ progenitors. To determine the potential of Ascl1+ cells we conducted a conditional expression fate-mapping experiment utilizing *Ascl1*$^{CreERT2/+}$ knock-in mice (Kim et al., 2011a; Kim et al., 2011b), crossed to reporter mice that express membrane localized GFP only after Cre-based recombination. Tamoxifen was used to induce CreER^{T2} localization to the nucleus and cause recombination. We injected tamoxifen at E12.5 and E13.5 to ensure that the earliest cohort of Ascl1+ cells would be represented in the lineage and to increase the total number of cells that underwent recombination. We immunostained adult retinas from reporter mice lacking *Ascl1-Cre* (*Ascl1*$^{+/+}$) as a control and did not detect any GFP+ cells. No GFP+ cells in *Ascl1*$^{CreERT2/+}$ mice were detected if tamoxifen was not administered (data not shown). We observed GFP+ cells distributed in all layers of the retina in E12.5/E13.5 *Ascl1*$^{CreERT2/+}$ lineage-traced mice (Fig. 3B-M, see Fig. S2 in the supplementary material). GFP+ cells were often found in small groups (clumps) or in isolation (Fig. 3B-M). We observed GFP+ cells in peripheral retina, which strongly suggests that recombination events occur at the leading edge of the *Ascl1* expression domain. However, we did not observe GFP+ cells in the far peripheral retina, consistent with the lack of *Ascl1* expression in that domain at E12.5 and E13.5, indicating that CreER activity was neither protracted nor delayed.

When we assayed the cell types present in E12.5/E13.5 *Ascl1* lineage-traced animals at 19-21 days of age, we found that all of the principal cell types in the retina were well represented except the ganglion cells (Fig. 3H-P, see Table S1 in the supplementary material). Co-labeling for calbindin to mark horizontal and amacrine cells (Fig. 3H,I), Pax6 to label amacrine (among other) cells (Fig. 3J), Otx2 to mark photoreceptors and bipolar cells (see Fig. S2 in the supplementary material), or Sox9 to label Müller glia (data not shown) confirmed that all these cells types were present in the Ascl1 lineage; however, the vast majority of the cells in the Ascl1 lineage in the ganglion cell layer were displaced amacrine cells as shown by: (1) the lack of labeling by ganglion cell-specific markers pan-Brn3 (Brn3a, b, c), Brn3a/b (in tandem) or Neurofilament-M (NFM) (Fig. 3B-D) (Nixon et al., 1989; Xiang et al., 1995); (2) the inability to label the cells with retrograde uptake of biotinylated dextran (Fig. 3E) (Farah and Easter, 2005); or (3)

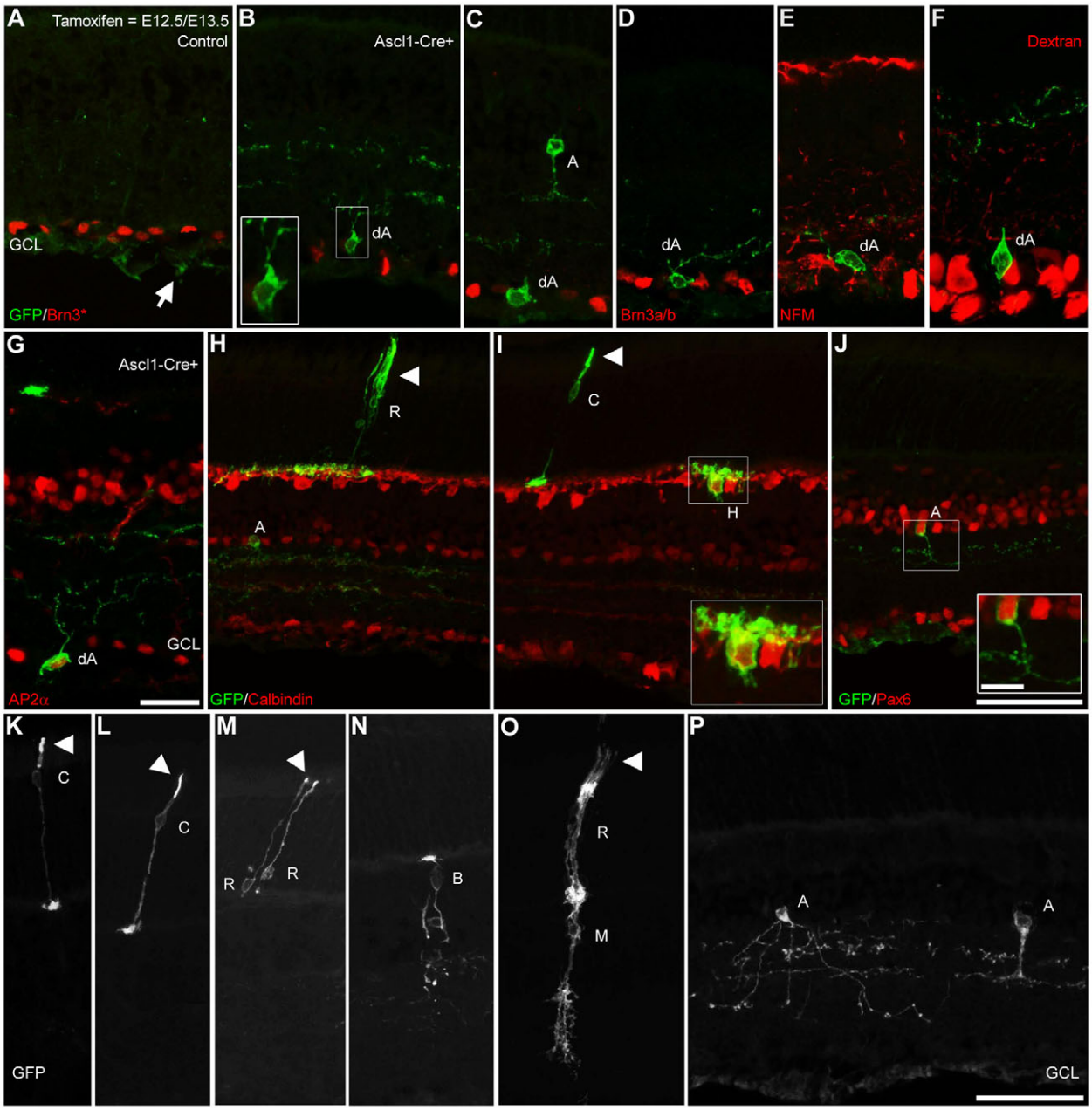

Fig. 3. The E12.5/E13.5 Ascl1 lineage. Tamoxifen was administered at E12.5 and E13.5 and mice were examined as adults. (**A**) A control animal lacking the *Ascl1-Cre* (*Ascl1*$^{+/+}$) transgene. There is a fibrous GFP antibody-specific background in the ganglion cell layer (GCL; arrow) and inner plexiform layer. (IPL). (**B-P**) Examples of *Ascl1*$^{CreERT2/+}$ lineage-traced cells (isolated or in clumps) (green or gray) with cell-type specific labeling in A-J (red). (B-J) Displaced amacrines (dA), inner nuclear layer (INL) amacrines (A), rods (R) and horizontal cells (H) are seen. (K-P) Examples of cones (C), rods, bipolar cells (B), amacrine cells and Müller glia (M) seen in the Ascl1 lineage. (B-G) Cells in the GCL are labeled by pan-Brn3 (Brn3*), Brn3a/b, Neurofilament-M (NFM) or with retrograde uptake of biotinylated dextran to label retinal ganglion cells (RGCs), and with AP2α to label displaced amacrines (all in red). Nearly all GCL cells are label-negative displaced amacrine cells. (H-J) Horizontal and amacrine cells are co-labeled with calbindin (red) or Pax6 (red). Arrowheads denote outer segments of photoreceptors. Scale bars: 50 μm for A-C,H-P; 25 μm for D-G; 10 μm for insets.

the presence of AP2α staining, which specifically labels the bulk of displaced amacrine cells (Fig. 3G) (Bassett et al., 2007). In our analysis of the Ascl1 lineage, we identified only seven RGCs in the 3115 GFP+ cells sampled (see Table S1 in the supplementary material). The paucity of ganglion cells in the Ascl1 lineage was not due to lack of production at this age; we conducted a birthdating analysis and found that 14% of cells that exited the cell cycle at E13.5 adopted RGC fate (see Fig. S3, Table S2 in the supplementary material). If we conservatively assume that RGCs should be generated at their adult frequency (0.6%) (Jeon et al., 1998) at the time of tamoxifen injection, then the probability of observing at least seven RGCs in our sample size is 0.00181 (binomial distribution, see Table S3 in the supplementary material), demonstrating that RGCs are significantly under-represented in the

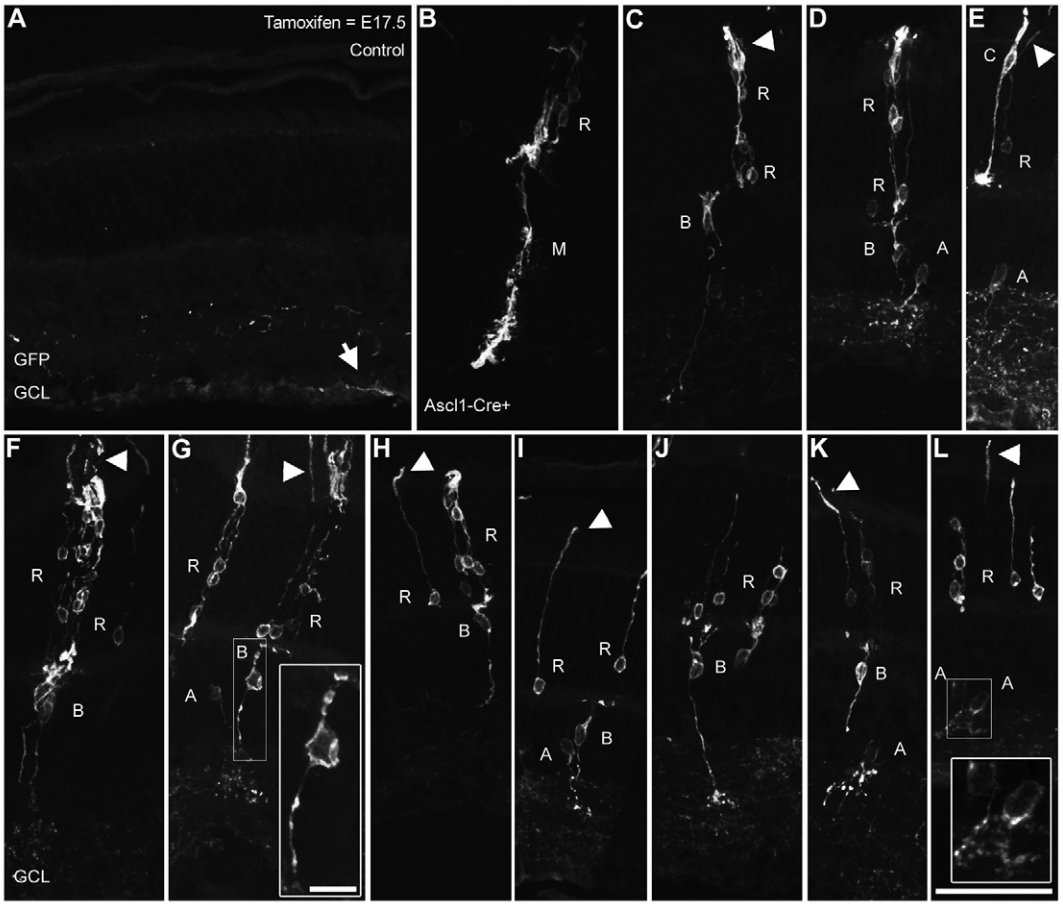

Fig. 4. The E17.5 Ascl1 lineage. Tamoxifen was given at E17.5 and mice were examined as adults. (**A**) A control animal lacking *Ascl1-Cre* (*Ascl1*$^{+/+}$) has only background GFP staining (gray, arrow). (**B-L**) Examples of *Ascl1*$^{CreERT2/+}$ lineage-traced cells (gray). Shown are examples of rods (R), amacrines (A), bipolars (B), cones (C) and Müller glia (M). Arrowheads denote photoreceptor outer segments. Scale bars: 50 μm for A-L: 10 μm for insets.

Ascl1 lineage. Only five of the ten animals we examined had GFP+ RGCs. Indeed, the RGC frequency (0.322±0.488% s.d.) was not statistically different from zero (*t*-test, $P>0.05$) (see Fig. S4 in the supplementary material). Thus, although an occasional ganglion cell was seen in some Ascl1 lineage-traced mice, we can conclude that the Ascl1 lineage effectively lacks RGCs.

In addition to the absence of ganglion cells in the Ascl1 lineage, the relative numbers of cells in the E12.5/E13.5 Ascl1 lineage was different from the normal adult mouse retina (Jeon et al., 1998). Horizontals, cones and amacrines were over-represented whereas rods were under-represented (see Fig. S4 in the supplementary material). This early fate bias suggests that many Ascl1+ progenitors exited the cell cycle near the time of tamoxifen administration. Many of the later-born cell types (rods, bipolars, glia) were found in multicellular clumps, suggesting that some Ascl1+ progenitors labeled at E12.5/E13.5 divided multiple times before adopting a cell fate. To examine this further, we gave mice tamoxifen at E12.5 and E13.5 or at E14.5 and E15.5 and examined retinas at birth (P0). We observed photoreceptors, amacrines, horizontal cells and progenitors in the lineages traced from both time points but, again, no RGCs were identified (see Fig. S5 in the supplementary material). About one third (37.5±8.4% s.e.m.) of GFP+ cells in the E12.5/E13.5 trace were Sox9+ progenitors at P0. We also gave tamoxifen at E14.0 and examined the Ascl1 lineage 12 and 23 hours later. We observed few GFP+ cells at 12 hours but numerous GFP+ cells at 23 hours (see Fig. S6 in the supplementary material). Of these, half were Sox9+ progenitors (49.4±8.4% s.e.m.) whereas none co-expressed the RGC marker pan-Brn3 (see Fig. S6 in the supplementary material). Taken together, these data show that recombination and subsequent GFP expression takes place quickly following tamoxifen treatment, that Cre activity is not protracted, that recombination takes place in proliferative Ascl1+ cells and that Ascl1+ cells can remain as progenitors for several days.

We next examined the Ascl1 lineage after inducing Cre recombination at E17.5. There were more GFP+ cells seen in lineage-traced animals, correlating with a greater number of Ascl1+cells at E17.5 compared with E13.5. GFP+ cells were seen in isolation and in small clumps, both in the central and far peripheral parts of the retina (Fig. 4B-L). Very few cells were observed in the GCL and none of them co-expressed pan-Brn3 (see Table S1 in the supplementary material). We observed few early-born cell types (cones, horizontals and displaced amacrines). These occurrences were almost always observed in the peripheral retina, where a small number of these cells are still being born on E17.5. Most of the cells in the E17.5 Ascl1 lineage were rods and amacrine cells (Fig. 4B-L). However, multicellular clumps that contained postnatally generated cell types, such as Müller cells and bipolar cells, were observed, indicating that some of the Ascl1+

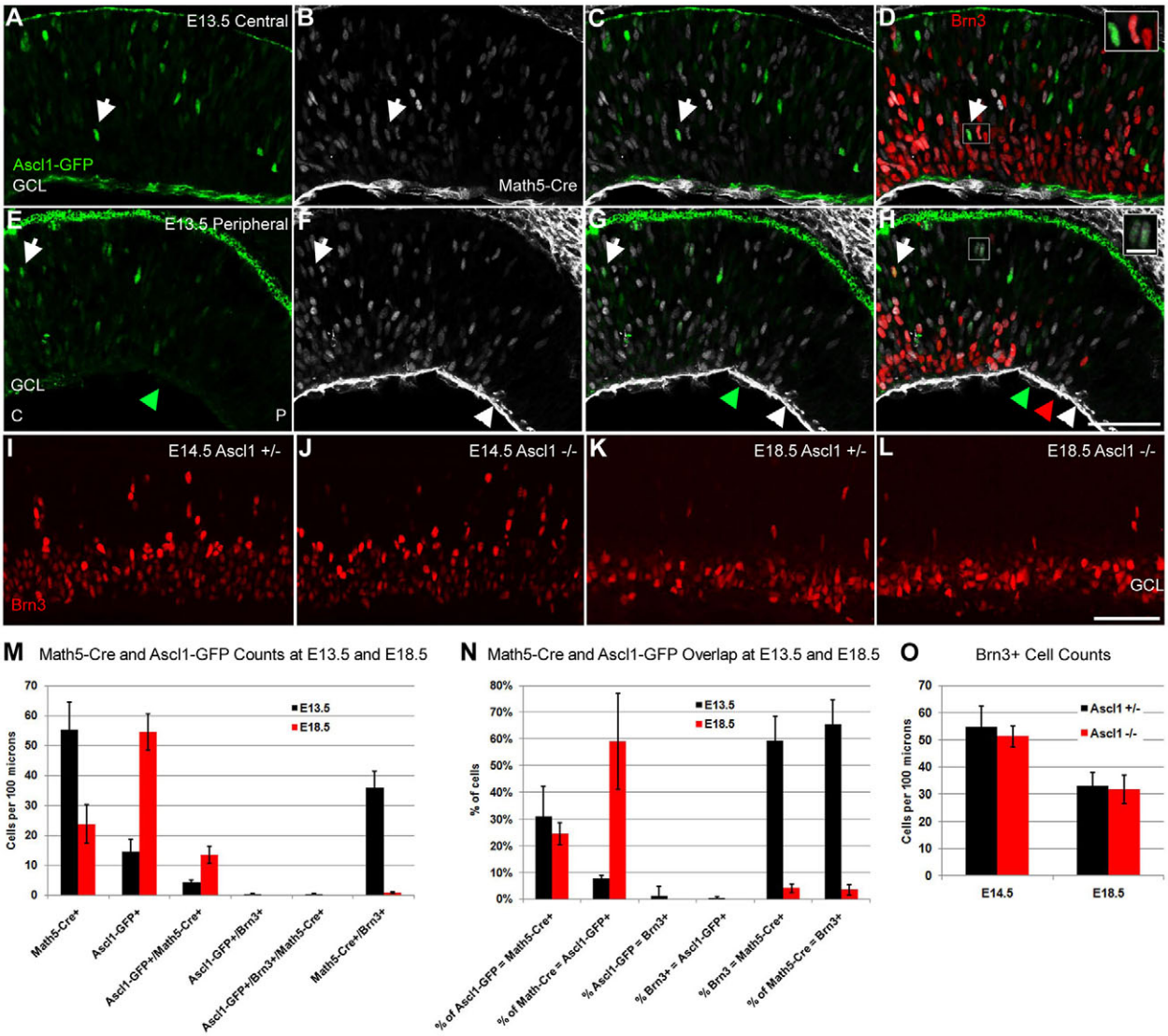

Fig. 5. Loss of *Ascl1* does not affect retinal ganglion cell (RGC) numbers. (**A-H**) E13.5 *Ascl1*$^{GFP/+}$*::Math5*$^{Cre/+}$ transheterozygous mice stained for Ascl1-GFP (green), Math5-Cre (gray) and pan-Brn3 (red). (A-D) The central retinas of E13.5 animals have many cells that co-express Math5-Cre and Brn3. Numerous Ascl1-GFP+ cells co-express Math5-Cre (arrows), but none co-expresses Ascl1-GFP and Brn3. (E-H) E13.5 peripheral retina showing the leading edge of Math5-Cre (white arrowhead), Brn3 (red arrowhead) and Ascl1-GFP (green arrowhead) expression. Only one cell made Ascl1-GFP, Math5-Cre and Brn3 (arrows). (**I-L**) Pan-Brn3 stains of E14.5 (I,J) and E18.5 (K,L) *Ascl1*$^{GFP/+}$ heterozygous (I,K) and null (J,L) retinas. C, central; GCL, ganglion cell layer; P, peripheral. Scale bars: 50 μm for A-L; 10 μm for insets. (**M**) Plot of Ascl1-GFP, Math5-Cre and Brn3 cell counts at E13.5 (black) and E18.5 (red). (**N**) Plot of overlap between Ascl1-GFP and Math5-Cre at E13.5 (black) and E18.5 (red). (**O**) Plot showing the number of Brn3+ RGCs at E14.5 and E18.5 for *Ascl1*$^{GFP/+}$ heterozygotes (black) and null (red) retinas. Error bars represent ±s.d. for all panels.

progenitors continue to generate progeny for several days (Fig. 4B-L). Taken together, our data show that *Ascl1* expression defines a population of competence-restricted progenitors in the retina.

Ascl1 itself does not inhibit RGC competence

Our lineage studies show that Ascl1+ progenitors do not become RGCs. RGC development requires the transcription factor *Math5* (Brown et al., 2001; Wang et al., 2001). *Math5* is expressed in postmitotic cells and establishes RGC competence, but only a subset of Math5+ cells adopts RGC fate (J. A. Brzezinski, 4th, PhD thesis, University of Michigan, 2005) (Mu et al., 2005; Yang et al., 2003). In principle, Ascl1 could act by preventing the expression of Math5 in the Ascl1 lineage, thereby inhibiting RGC fate. Alternatively, Ascl1+ progenitors could give rise to Math5+ postmitotic cells and this subpopulation, owing to its previous expression of Ascl1, would be inhibited from RGC fate. To test these hypotheses, we crossed *Ascl1*$^{GFP/+}$ mice to *Math5*$^{Cre/+}$ mice and looked for overlap of GFP and Cre proteins, which both persist transiently after *Ascl1* and *Math5* expression terminates. We examined mice at E13.5 (the peak of RGC formation) and at E18.5 (the tail of RGC formation) for pan-Brn3 co-expression. At E13.5, 36% of the cells in the retina are pan-Brn3+ RGCs (see Fig. S3 in the supplementary material) and most of the Math5-Cre+ cells co-expressed pan-Brn3 (65.7±8.99% s.d.) (Fig. 5A-D,M,N). By contrast, we observed only one instance of an Ascl1-GFP+ cell that co-expressed pan-Brn3 (Fig. 5E-H,M,N, see Table S4 in the supplementary material). Nonetheless, we observed that many Ascl1-GFP+ cells co-expressed Math5-Cre at this age (31.0±11.1% s.d.) (Fig. 5A-D,M,N). The same was true at E18.5; by

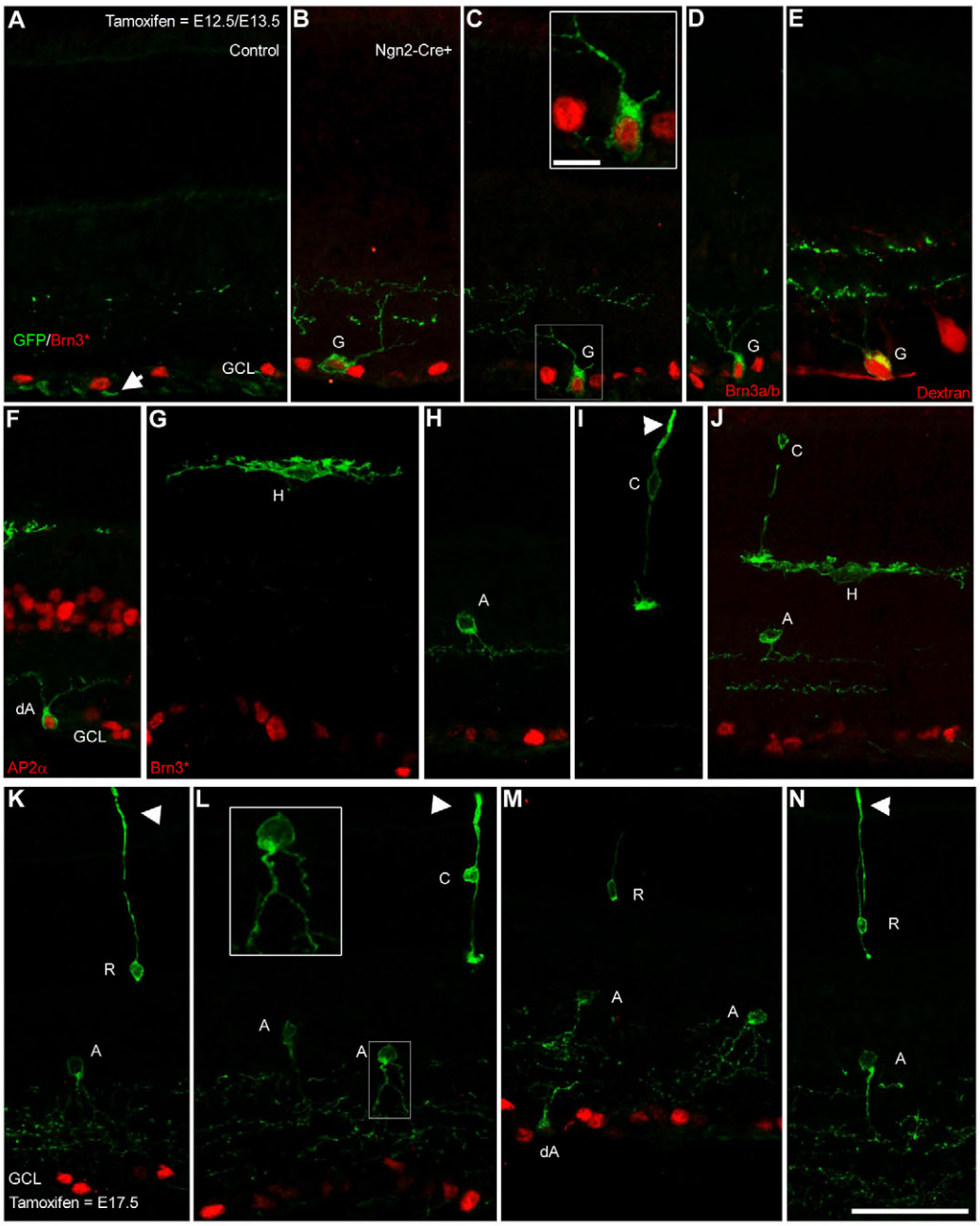

Fig. 6. Ngn2 lineage traces. Tamoxifen was given at E12.5 and E13.5 or at E17.5 and mice were examined as adults. (**A-J**) E12.5/E13.5 *Ngn2*$^{CreERTM/+}$ lineage traces (green) co-stained with pan-Brn3 (Brn3*), Brn3a/b, or by retrograde uptake of biotinylated dextran to mark retinal ganglion cells (RGCs; red). (A) Control mice lacking *Ngn2-Cre* (*Ngn2*$^{+/+}$) have only fibrous GFP background (arrow). (B-J) Ngn2 lineage-traced RGCs (G), horizontals (H), cones (C) and amacrines (A) are shown. (F) Displaced amacrines (dA) are marked with AP2α staining (red) in the ganglion cell layer (GCL). (**K-N**) E17.5 Ngn2 lineage trace showing rods (R), amacrines (A), displaced amacrines (dA) and cones (C). Arrowheads mark photoreceptor outer segments. Scale bars: 50 μm for AN; 10 μm for insets.

this age, most Math5-Cre+ cells co-expressed Ascl1-GFP (59.11±17.98% s.d.) (Fig. 5M,N, see Fig. S7 in the supplementary material), but we did not detect Ascl1-GFP+/Brn3+ cells.

The fact that Ascl1+ progenitor cells can give rise to Math5-expressing cells, but not Brn3+ ganglion cells suggests that prior (or concurrent) Ascl1 expression prevents Math5+ cells from adopting RGC fate. If Ascl1 prevents Brn3 expression in the progeny of these progenitors, then the loss of *Ascl1* should cause an increase in RGC number. To test this hypothesis, we examined the number of pan-Brn3+ RGCs at both E14.5 and E18.5 in *Ascl1*$^{GFP/+}$ heterozygous and null retinas and found no differences at either age (Fig. 5I-L,O). The absence of an effect on ganglion cell fate in the *Ascl1*$^{GFP/GFP}$ retinas might be due to compensation by Ngn2 or Olig2. However, when we analyzed *Ascl1*$^{GFP/GFP}$ retinas for the expression of Ngn2 and Olig2, we found that the number of Ngn2+ or Olig2+ cells in *Ascl1*-null mice was not significantly different compared with heterozygous mice at E14.5 (Fig. 1M, Fig. 2A,C). The only significant difference we observed was that a slightly smaller fraction of Ascl1-GFP+ cells co-expressed Olig2 in the absence of *Ascl1* (Fig. 2B). We also examined *Ascl1*$^{GFP/GFP}$ retinas at E18.5. At this age, all populations of progenitors (Sox2+, Ascl1-GFP+, Ngn2+ and Olig2+) were modestly decreased (~15-30%) in *Ascl1* mutants (see Fig. S1 in the supplementary material). Thus, *Ascl1* is not required for the repression of the RGC fate in this lineage and neither compensation nor cross-regulation by Ngn2 or Olig2 can explain this result.

Ascl1+ and Ngn2+ progenitors give rise to distinct lineages

Previous conditional expression fate-mapping experiments demonstrated that Ngn2+ progenitors can give rise to all retinal cell types, including RGCs (Ma and Wang, 2006). As Ascl1 and

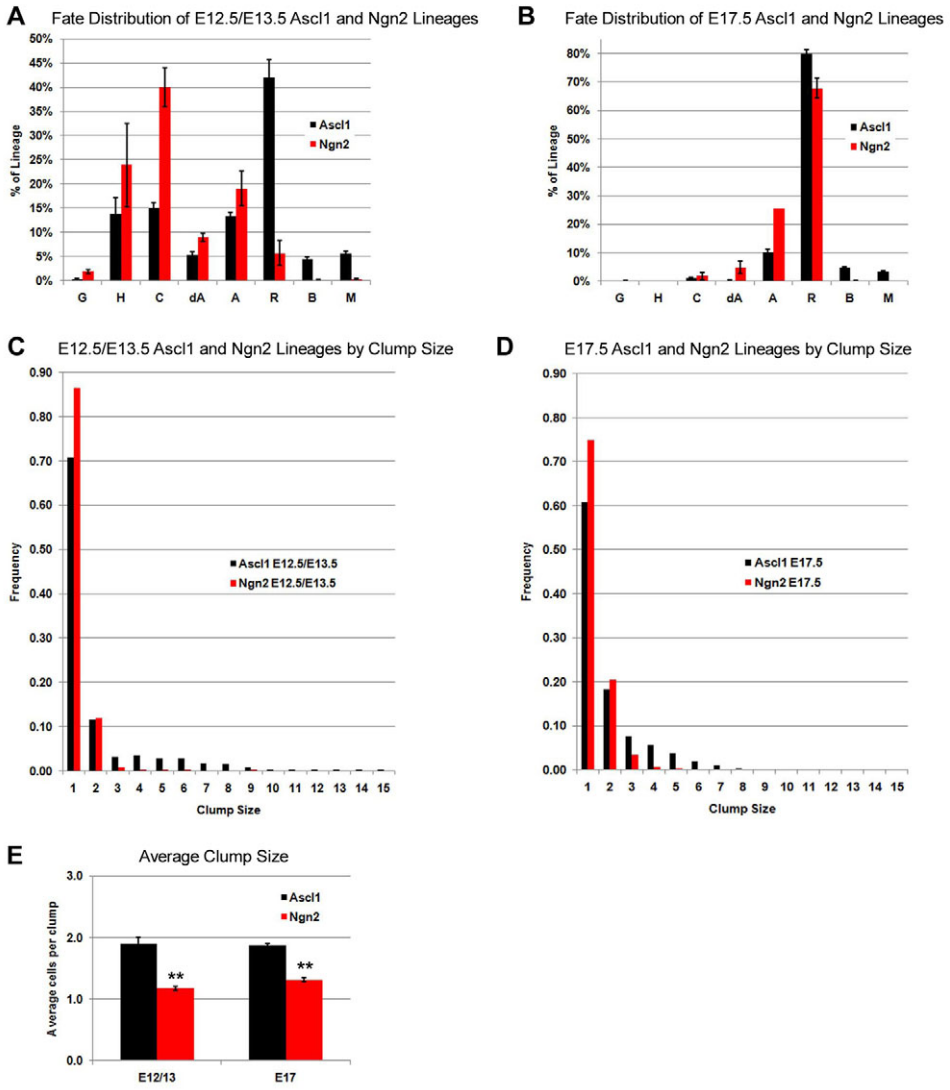

Fig. 7. Fate distribution and clump sizes in the Ascl1 and Ngn2 lineages. (**A**) Plot of the fate distributions of E12.5/E13.5 Ascl1 (black) and Ngn2 (red) lineages. The Ascl1 lineage and Ngn2 lineages have significantly different distributions (χ^2, $P<0.001$); the Ascl1 lineage nearly lacks retinal ganglion cells (RGCs) and contains more late-born cell fates. (**B**) Distribution of E17.5 Ascl1 (black) and Ngn2 (red) lineages. Compared with the Ngn2 lineage, the Ascl1 lineage has more late-born cell types (χ^2, $P<0.001$). G, RGC; H, horizontal; C, cone; dA, displaced amacrine; A, amacrine; R, rod; B, bipolar; M, Müller glia. Error bars represent ±s.e.m. (**C**,**D**) Histogram showing clump size frequencies in the E12.5/E13.5 (C) and E17.5 (D) Ascl1 (black) and Ngn2 (red) lineages. The maximum clump size is 15 cells for the Ascl1 lineage and nine cells for the Ngn2 lineage at E12.5/13.5 (12 and seven cells, respectively, at E17.5). (**E**) Plot showing the average size of clumps in the Ascl1 and Ngn2 lineages. Ascl1 clumps are twice as large as Ngn2 clumps (Mann-Whitney test, $**P<0.001$). Error bars represent ±s.d.

Ngn2 have partially overlapping domains at several time points (Figs 1, 2, see Fig. S1 in the supplementary material), we tested whether differences in expression of these factors in progenitors leads to distinct lineages. We carried out lineage analysis of *Ngn2*$^{CreERTM/+}$ mice after administering tamoxifen at E12.5/E13.5 and at E17.5. Adult control mice without *Ngn2-Cre* (*Ngn2*$^{+/+}$) and *Ngn2*$^{CreERTM/+}$ mice that did not receive tamoxifen lacked GFP+ cells (Fig. 6A) (data not shown). *Ngn2*$^{CreERTM/+}$ mice given tamoxifen at E12.5/E13.5 had GFP+ cells in all layers of the retina and were typically seen in isolation or in clumps of two to three cells spanning the central and far peripheral retina (Fig. 6B-J). Most abundant in the E12.5/E13.5 Ngn2 lineage trace were cones, horizontals and amacrine cells (Fig. 6F-J). There were few rods, bipolars or Müller glia from this trace, which contrasts significantly with the Ascl1 lineage trace (χ^2 test, $P<0.001$) (Fig. 7A, see Table S1 in the supplementary material). GFP+ RGCs were present in every Ngn2 lineage-traced animal we examined (Fig. 6B-E), and though not numerous, RGCs were more frequently observed in the Ngn2 lineage (1.95±0.92 s.d.) versus the Ascl1 lineage (0.322±0.488% s.d.) (Mann-Whitney, $P=0.001$, Fig. 7A, see Fig. S4 in the supplementary material). Although the Ngn2 lineage was strongly biased towards cell fates born around the time of tamoxifen administration, RGCs formed only a small, though significant (*t*-test, $P<0.01$), fraction of the Ngn2 lineage.

In the E17.5 Ngn2 lineage trace, most of the cells we observed were rods, but a few early-born cell types were seen (e.g. RGCs and cones), primarily in peripheral parts of the retina (Fig. 6K-N). In contrast to the Ascl1 lineage, the latest generated cell types, bipolars and Müller glia, were nearly absent from the E17.5 Ngn2 lineage (χ^2 test, $P<0.001$) (Fig. 7B, see Table S1 in the supplementary material). As before, the E17.5 Ngn2 lineage distribution is highly biased towards the cell types born at the time of tamoxifen administration. Together, our data show that Ascl1 and Ngn2 lineages are distinct. First, RGCs are significantly more abundant in the Ngn2 lineage. Second, the Ngn2 lineage is more heavily biased towards cell types that exit the cell cycle shortly after tamoxifen treatment.

The differences in fate distributions suggested differential proliferative ability of the Ascl1+ and Ngn2+ progenitor populations. We counted the number of cells in clumps traced from both time points as an indirect measure of the proliferative ability of these progenitors. We plotted the frequency of clump sizes and observed a Pareto-like (L-shaped) distribution for both lineages at

both time points (Fig. 7C,D). The average clump size was about twice as large in the Ascl1 lineage compared with the Ngn2 lineage at both time points (Mann-Whitney test, $P<0.001$) (Fig. 7E). The high frequency of one- and two-cell clumps in the Ngn2 lineage (98.5%) suggests that Ngn2+ progenitors are typically in their last cell cycle.

As noted above, all populations of E18.5 progenitors were modestly decreased (~15-30%) in *Ascl1*$^{GFP/GFP}$-null retinas (see Fig. S1 in the supplementary material), consistent with a previous study that suggested that *Ascl1* non-autonomously maintains the retinal progenitor pool (Nelson et al., 2009). We also found that E18.5 *Ascl1*$^{GFP/GFP}$-null explants cultured for 2 days in vitro (DIV) had 50% fewer EdU+ (S-phase) nuclei (progenitors) at the end of the culture period compared with heterozygous explants (see Fig. S8 in the supplementary material). Lastly, we examined E17.5 retinal explants cultured for 9 DIV and found that although *Ascl1*-null retinas were noticeably smaller, all seven principal retinal cell types were present (data not shown, see Figs S1, S9 in the supplementary material). Together these results suggest that Ascl1 is required for proliferation, which cannot be compensated for by Olig2 or Ngn2.

DISCUSSION

Here, we report that retinal progenitors are heterogeneous in their expression of the bHLH transcription factors Ascl1, Ngn2 and Olig2. This progenitor heterogeneity is reflected in differences in fate; Ascl1 and Ngn2 lineages are distinct from each other. The Ascl1+ progenitor cells do not significantly generate RGCs, but can give rise to the other six principal retinal cell fates. *Ascl1* expression, therefore, defines a subpopulation of competence-restricted progenitors during retinal development. Interestingly, *Ascl1* itself is not required to restrict RGC competence, suggesting a mechanism by which factors upstream of *Ascl1* limit competence in the retina.

Ascl1 defines a competence-restricted retinal lineage

Ascl1+ progenitors did not significantly generate RGCs at any time point. Although we cannot formally rule out that a biologically relevant, rare RGC subtype(s) derives from the Ascl1 lineage, our data strongly argue against this possibility. First, Ascl1-GFP and pan-Brn3 co-expression data suggests that this putative subtype would have to be exceedingly rare during development (one cell or fewer per retina) (binomial distribution, $P<0.00001$, see Table S5 in the supplementary material). Second, whereas Brn3a/b/c expression might not label all ganglion cell subtypes (Badea and Nathans, 2011), retrograde dextran uptake labels all RGCs; nonetheless, we did not observe a significant number of Brn3+ or dextran-labeled RGCs in the Ascl1 lineage.

Retroviral lineage-tracing studies have shown that all seven retinal cell types derive from a common progenitor population (Turner and Cepko, 1987; Turner et al., 1990). However, throughout most of retinal development, several cell types are being formed concurrently (La Vail et al., 1991; Rapaport et al., 2004; Sidman, 1961; Young, 1985). This implies that retinal progenitor cells form a heterogeneous population that expresses different intrinsic factors and responds differentially to extrinsic cues (Livesey and Cepko, 2001; Taylor and Reh, 1990). Previous studies have demonstrated that *Ascl1* is expressed in a subset of retinal progenitors, and proposed that *Ascl1* expression defines a particular stage in the progenitor cells (Jasoni and Reh, 1996; Jasoni et al., 1994). Our Ascl1 lineage-tracing experiment confirmed this proposal, as Ascl1+ progenitors significantly generated all retinal cell types except for RGCs. This is the first molecularly defined progenitor population that has competence to form all but one cell type in the retina. Analogously, Ascl1+ cells give rise to a competence-restricted lineage in the spinal cord (Battiste et al., 2007), forebrain (Parras et al., 2002) and other regions of the CNS (Kim et al., 2008).

Although lineage-restricted progenitors have not been previously shown to exist in the mouse retina in vivo, it has been shown that committed precursors exist in the fish retina. Raymond and colleagues first demonstrated that many of the rod photoreceptors in the teleost retina are generated through a committed rod precursor (Raymond and Rivlin, 1987). More recently, immature horizontal cells in the inner nuclear layer of the zebrafish retina have been shown to undergo a mitotic division to generate two horizontal cells (Godinho et al., 2007). In *Ath5-GFP* transgenic zebrafish, GFP is made by cells in their terminal division such that one daughter becomes a ganglion cell and the other adopts a different neural fate (Poggi et al., 2005). This cell is somewhat different from the committed horizontal and rod precursors, but nevertheless suggests that, in fish, there is something unique about progenitor cells in their last cell division. Our results suggest that Ngn2 expression might mark mouse progenitors in their final mitotic division since nearly all of the traced cells were found in one- to two-cell clumps and because their cell fates were strongly biased towards those born at the time of tamoxifen treatment. However, we did not find any clear pattern to the types of progeny generated in two-cell clumps in the Ngn2 or Ascl1 lineages (data not shown) (Ma and Wang, 2006). This appears to be true in vitro as well, where single isolated progenitors that undergo their final mitotic division in culture do not show a bias towards generating cells of the same identity (Gomes et al., 2011).

Our study demonstrates that the E12.5/E13.5 Ascl1 and Ngn2 lineages are quite distinct from each other and from retroviral lineages traced at the same time points (see Figs S4, S10 in the supplementary material) (Turner et al., 1990). However, these lineage-tracing techniques are quite different from each other. First, only progenitors can be infected and labeled by retroviruses, but it is possible for Ascl1- and Ngn2-Cre to persist transiently and catalyze recombination in newly postmitotic cells. Thus, the average clump size in the Ascl1 and Ngn2 lineages should be lower than that in the retroviral lineages, but the maximum clump size should be similar. Second, although clumps seen in the E12.5/E13.5 Ascl1 and Ngn2 lineage traces were sparsely distributed, we cannot be certain they were clones. Thus, we might be overestimating the number of progeny that Ascl1+ and Ngn2+ cells generate.

A major difference between these three lineage traces was the clump/clone size distribution. Whereas some retroviral clones were larger than 50 cells (Turner et al., 1990), we did not observe any clumps in the Ascl1 lineage that contained more than 15 cells and no clumps in the Ngn2 lineage that contained more than nine cells (see Fig. S10 in the supplementary material). Most of the clumps in the Ascl1 and Ngn2 lineages contained one or two cells (82.4% and 98.5%, respectively), but only a small fraction of retroviral clones (~33%) contained one or two cells. These differences in the clump/clone size distributions strongly suggest the following model (Fig. 8). Retinal progenitors that do not express Ascl1 or Ngn2 can undergo a large number of mitotic divisions, whereas progenitors that express Ascl1 undergo few mitotic divisions and those that express Ngn2 are in their last cell cycle. This model is consistent with observations in other regions of the CNS, where it has been proposed that Ngn2-expressing progenitors in the spinal cord are in their last cell division (Helms et al., 2005) and that Ascl1

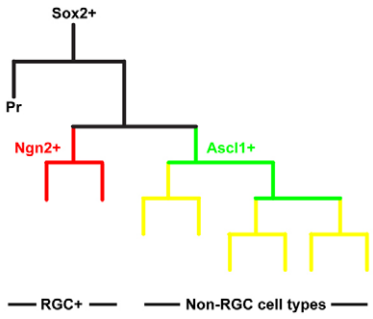

Fig. 8. Model based on lineage and expression data described in this report. Progenitors (Pr, Sox2+) that express neither Ascl1 nor Ngn2 have the potential to generate large clones (>16 cells) and adopt all cell fates. Ascl1+ progenitors (green) undergo fewer cell divisions (<16 cells) and generate all cell fates except retinal ganglion cells (RGCs), thus defining a competence-restricted lineage. Ngn2+ progenitors (red) can adopt RGC and all other cell fates, but appear to be in their last cell division. Many, or perhaps all, Ascl1+ progenitors in their last cell cycle co-express Ngn2 (yellow) and subsequently adopt non-RGC fates.

overexpression can inhibit proliferation in certain contexts (Alvarez-Rodriguez and Pons, 2009; Bertrand et al., 2002; Farah et al., 2000).

The Ascl1, Ngn2 and retroviral lineages have distinct distributions of other cell fates (see Fig. S4 in the supplementary material). These distinctions, with the exception of RGCs, can be attributed to differences in proliferation. Ascl1+ cells at E12.5/E13.5 primarily adopted early neural fates, but some of the recombined cells proliferate until at least P0 and generate later fates, such as rods, bipolars and Müller glia. By contrast, the Ngn2 lineage fate distribution is consistent with near total cell cycle exit at the time of tamoxifen administration. The retroviral lineages from these same ages contained large clones with, predominantly, cells born later in development, such as rods, bipolars and amacrine cells (Turner et al., 1990). Small retroviral clones (<16 cells) had more early-born fates, but did not match the fate distribution of the Ascl1 or Ngn2 lineages (see Table S1 in the supplementary material) (Turner et al., 1990). Although the Ngn2 lineage cannot be an obligate subset of the Ascl1 lineage, our data suggest that these two lineages overlap such that co-expressing cells are in their final cell division (Fig. 8).

We observed that Ngn2+ progenitors generated few RGCs. Because RGCs and horizontal cells are similar in number and their birthdates overlap extensively (Jeon et al., 1998; Rapaport et al., 2004) these cell types should be similarly represented in the E12.5/E13.5 Ngn2 lineage trace. However, RGC frequency was considerably lower (~12-fold) than horizontal cells, indicating that only a subset of RGCs come from Ngn2+ progenitors. This suggests that Ngn2 lineage is largely RGC-competence restricted, probably a result of considerable overlap with the Ascl1 lineage. It is unclear whether Olig2+ progenitors are also competence restricted. An initial examination of the E14.5 Olig2 lineage revealed RGCs, amacrines and horizontal cells (Shibasaki et al., 2007), suggesting that Olig2+ progenitors might be more similar to Ngn2+ than to Ascl1+ progenitors in the retina.

Progenitor heterogeneity correlates with different fate outcomes

Previous studies have demonstrated progenitor heterogeneity in the retina. For example, progenitor cells from late retina differentiate in response to increases in cAMP, whereas those from early embryonic stages do not (Taylor and Reh, 1990). Analogously, EGF and related EGFR ligands are mitogenic for late progenitors, but not early ones (Anchan et al., 1991; Lillien and Cepko, 1992). Progenitors are heterogeneous in their expression of the cyclin-dependent kinase inhibitors $p57^{kip2}$ and $p27^{kip1}$ (Dyer and Cepko, 2001). In our experiments, we found that all seven distinct single, double and triple bHLH transcription factor combinations were represented, along with Sox2+ progenitors that did not express Ascl1, Ngn2 or Olig2. In single-cell gene expression-profiling experiments, roughly similar fractions of progenitors expressed *Ascl1*, *Ngn2* or both factors (Trimarchi et al., 2008). Although we have not analyzed the Olig2 lineage, at least two populations of cells, Ascl1+ and Ngn2+, have different fate and proliferative potentials. Thus, progenitor heterogeneity correlates with different fate choice outcomes in the retina.

How do *Ascl1*, *Ngn2* and *Olig2* regulate cell diversity in the retina? One model proposes that different bHLH transcription factors bias retinal progenitor cells, or their postmitotic progeny, to specific cell fates (Ohsawa and Kageyama, 2008); each factor (or combination of factors) is instructive for a specific cell fate. This seems unlikely for *Ascl1* and *Ngn2*, as mice deficient in these transcription factors are able to generate all of the seven principal retinal cell types (Akagi et al., 2004; Hufnagel et al., 2010; Skowronska-Krawczyk et al., 2009; Tomita et al., 2000). Alternatively, these factors could bias progenitors towards neural fates. *Ascl1* and *Ngn2* mutants have increased glia at the expense of neurons, whereas overexpression of these factors drives neurons (Akagi et al., 2004; Cai et al., 2000; Nelson et al., 2009; Tomita et al., 2000; Tomita et al., 1996). A third possibility is that these transcription factors differentially regulate progenitor cell cycle dynamics; our lineage data show that Ascl1+ and Ngn2+ cells have different proliferative and fate potentials. Perhaps a combination of Ascl1 and Ngn2 reach a threshold and induce terminal differentiation. Alternatively, Ascl1, but not Ngn2, might autonomously promote proliferation of progenitors by activating cell-cycle genes (Castro et al., 2011). Lastly, these transcription factors might differentially activate cell non-autonomous signaling mechanisms. Previously, we have shown that *Ascl1*-null mice express lower levels of the Notch ligands *Dll1* and *Dll3* in progenitors, resulting in diminished Notch signaling within the retina (Nelson et al., 2009). One consequence of reduced Notch signaling might be a progressive depletion of progenitors, a phenotype that we observed in *Ascl1*-null mice. Notch ligand reduction is not observed in *Ngn2*-deficient retinas, demonstrating a difference between these transcription factors in sustaining Notch signaling and the progenitor pool (Nelson et al., 2009; Ohsawa and Kageyama, 2008).

Ascl1 is not required to restrict RGC competence

The bHLH transcription factor *Math5* is required for RGC formation (Brown et al., 2001; Wang et al., 2001). *Math5* is required for RGC competence, but only a small subset of Math5+ cells adopts RGC fate (J. A. Brzezinski, 4th, PhD thesis, University of Michigan, 2005) (Mu et al., 2005; Yang et al., 2003). How are Ascl1+ cells prevented from becoming RGCs? First, Ascl1 might repress Math5 and prevent RGC competence. This is unlikely as we saw numerous Ascl1+/Math5+ co-labeled cells and *Math5* expression is unchanged in *Ascl1*-null mice (Hufnagel et al., 2010; Nelson et al., 2009). Second, Ascl1 might restrict RGC competence independently of Math5. This also seems unlikely because *Ascl1* mutant mice had normal numbers of RGCs during development. Ngn2+ cells, which can give rise to Math5+ cells and RGCs,

engineered to co-express *Ascl1* were still able to make *Math5* and become RGCs (Hufnagel et al., 2010). Also, overexpression of *Ascl1* in chicken retina did not change the number of RGCs (Mao et al., 2009). Third, it is possible that Ascl1, Ngn2 and/or Olig2 function redundantly to repress RGC formation. This is consistent with the paucity of RGCs in our Ascl1 and Ngn2 lineages traces and with our Ascl1, Ngn2 and Olig2 expression data. In support of this possibility, there is a modest increase in the number of RGCs and amount of *Math5* expression in *Ascl1*/*Ngn2* double knockout mice (Akagi et al., 2004). Lastly, another factor might restrict RGC competence in Ascl1+ cells. *Dicer* conditional null retinas, which cannot make microRNAs, lack *Ascl1* expression (Georgi and Reh, 2010). However, unlike *Ascl1*-null mice, conditional *Dicer*-null retinas have extra RGCs. This raises the possibility that the factors that regulate *Ascl1* expression also restrict RGC competence. Together, our data suggest a developmental mechanism in which factors upstream of, or redundant with, *Ascl1* restrict RGC competence to promote cell fate diversification in the retina.

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Competing interests statement
The authors declare no competing financial interests.

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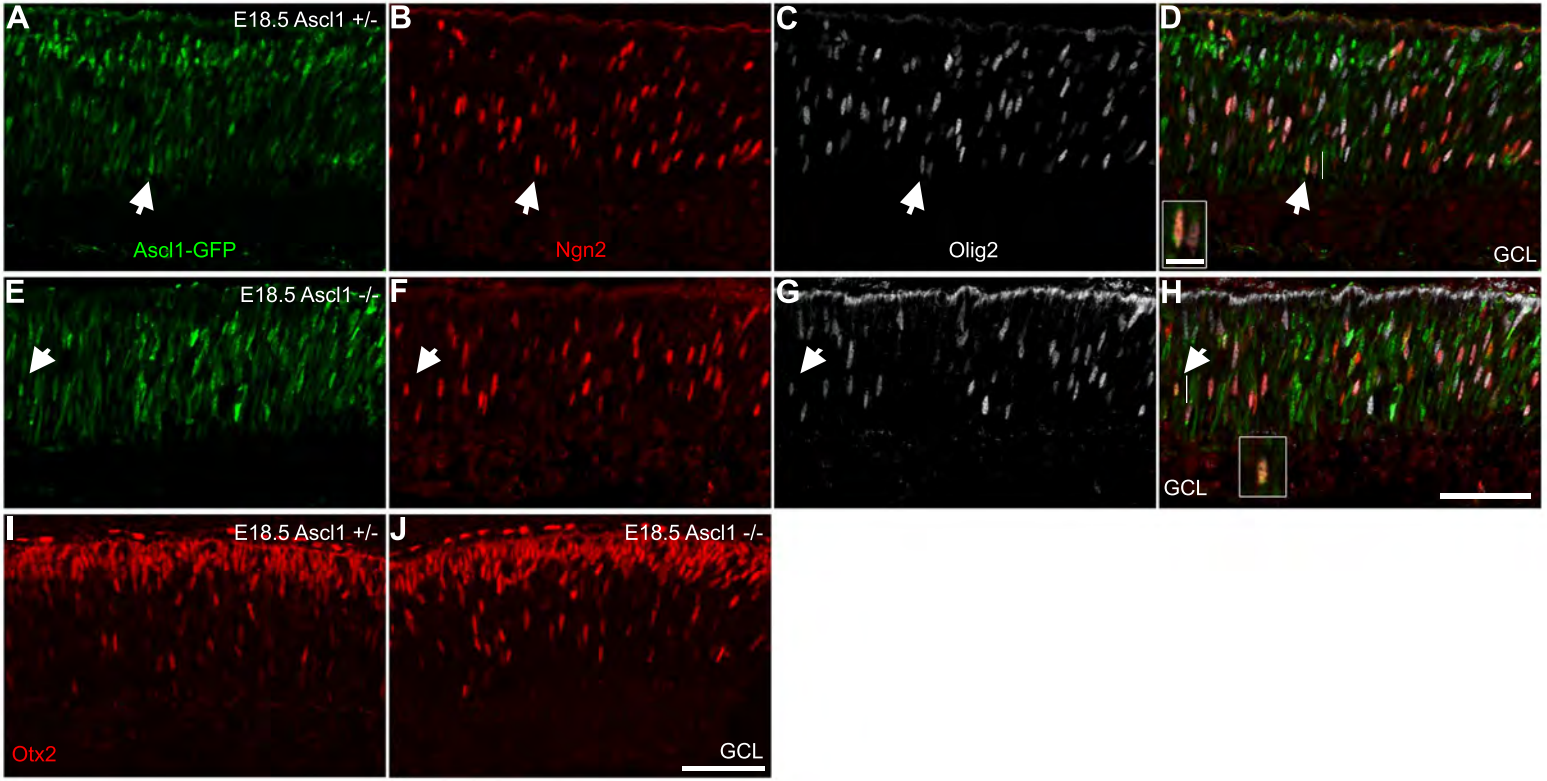
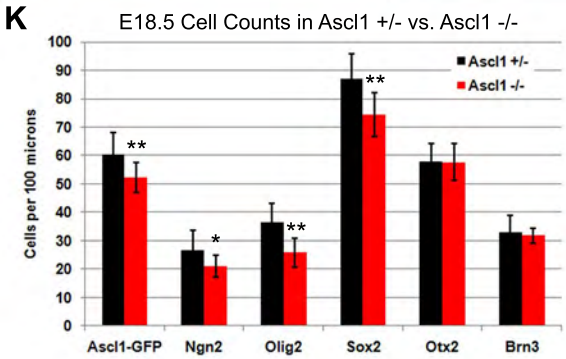
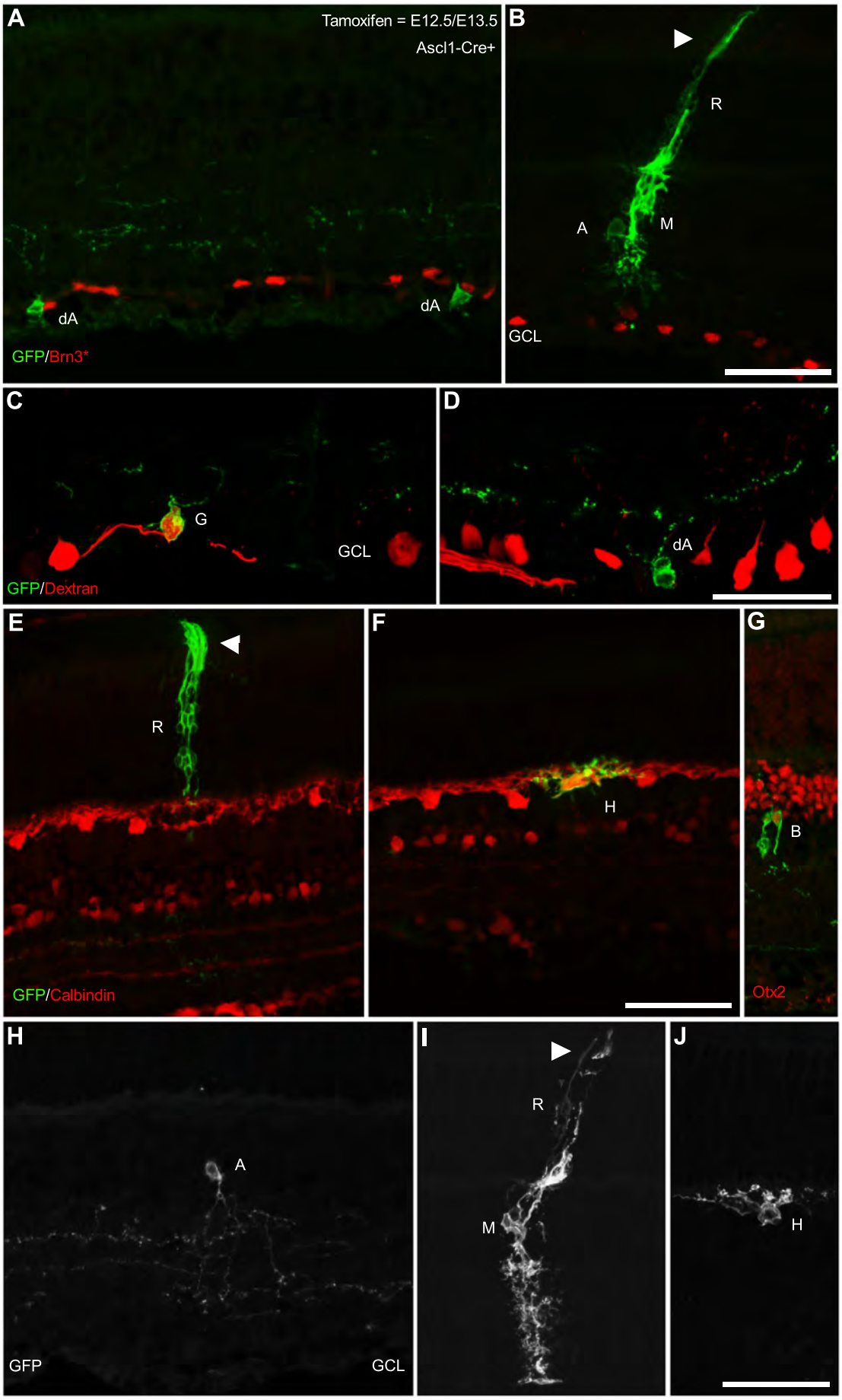
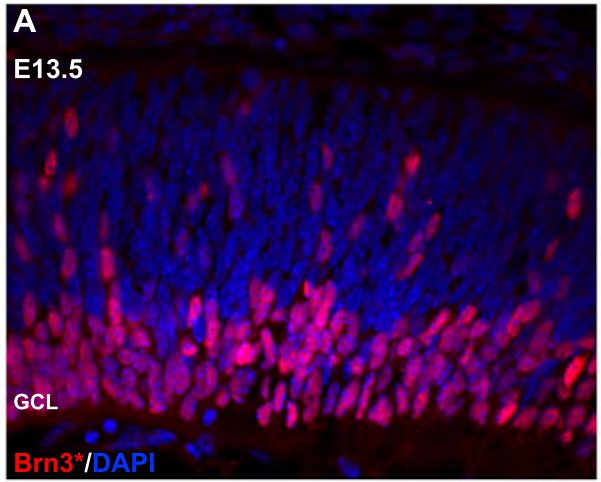
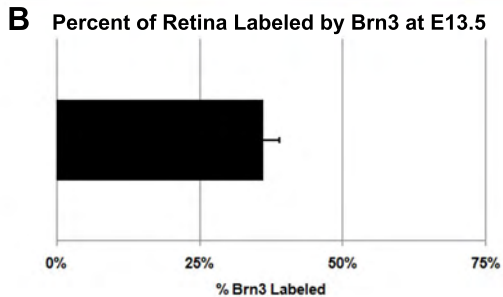
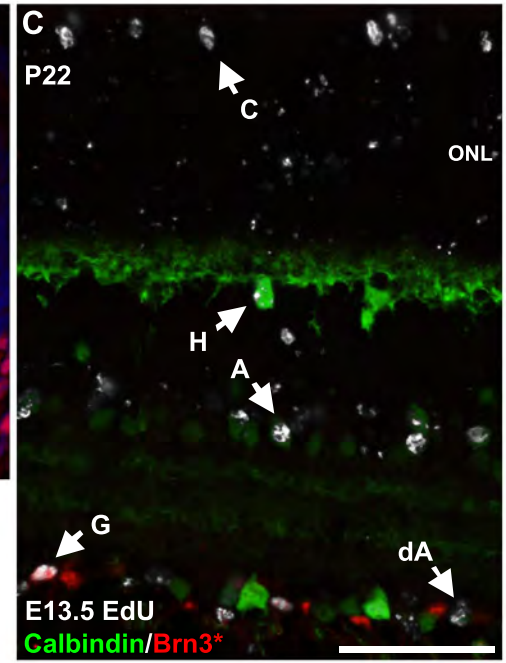

A
E18.5 Ascl1 +/-
Ascl1-GFP
B
Ngn2
C
Olig2
D
GCL
E
E18.5 Ascl1 -/-
F
G
H
GCL
I
E18.5 Ascl1 +/-
Otx2
J
E18.5 Ascl1 -/-
GCL


K
E18.5 Cell Counts in Ascl1 +/- vs. Ascl1 -/-
Cells per 100 microns
100
90
80
70
60
50
40
30
20
10
0
Ascl1 +/-
Ascl1 -/-
**
*
**
**
Ascl1-GFP
Ngn2
Olig2
Sox2
Otx2
Brn3

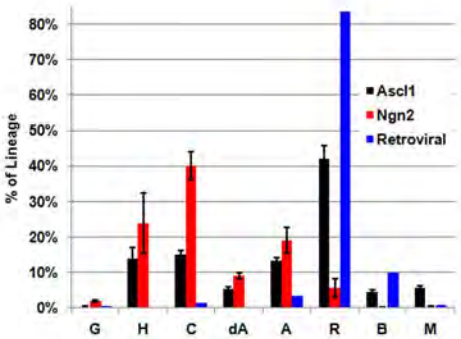

A
Tamoxifen = E12.5/E13.5
Ascl1-Cre+
dA
dA
GFP/Brn3*
B
R
A
M
GCL
C
G
GCL
GFP/Dextran
D
dA
E
R
GFP/Calbindin
F
H
G
B
Otx2
H
A
GFP
GCL
I
R
M
J
H


A
E13.5
GCL
Brn3*/DAPI

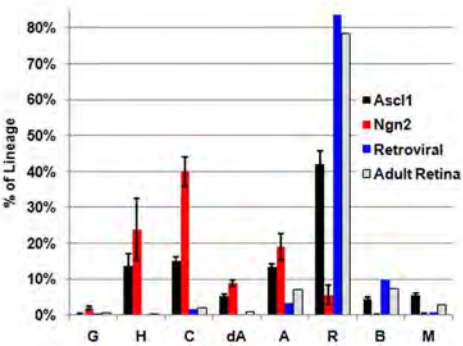

B
Percent of Retina Labeled by Brn3 at E13.5
0%
25%
50%
75%
% Brn3 Labeled


C
P22
C
ONL
H
A
G
dA
E13.5 EdU
Calbindin/Brn3*

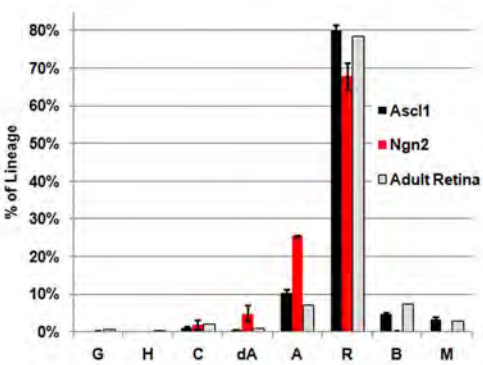
A Fate Distribution from E12.5/E13.5 Lineage Traces



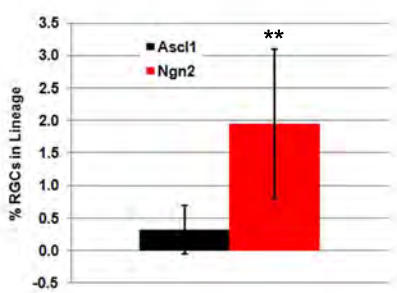
B Fate Distribution from E12.5/E13.5 Lineage Traces

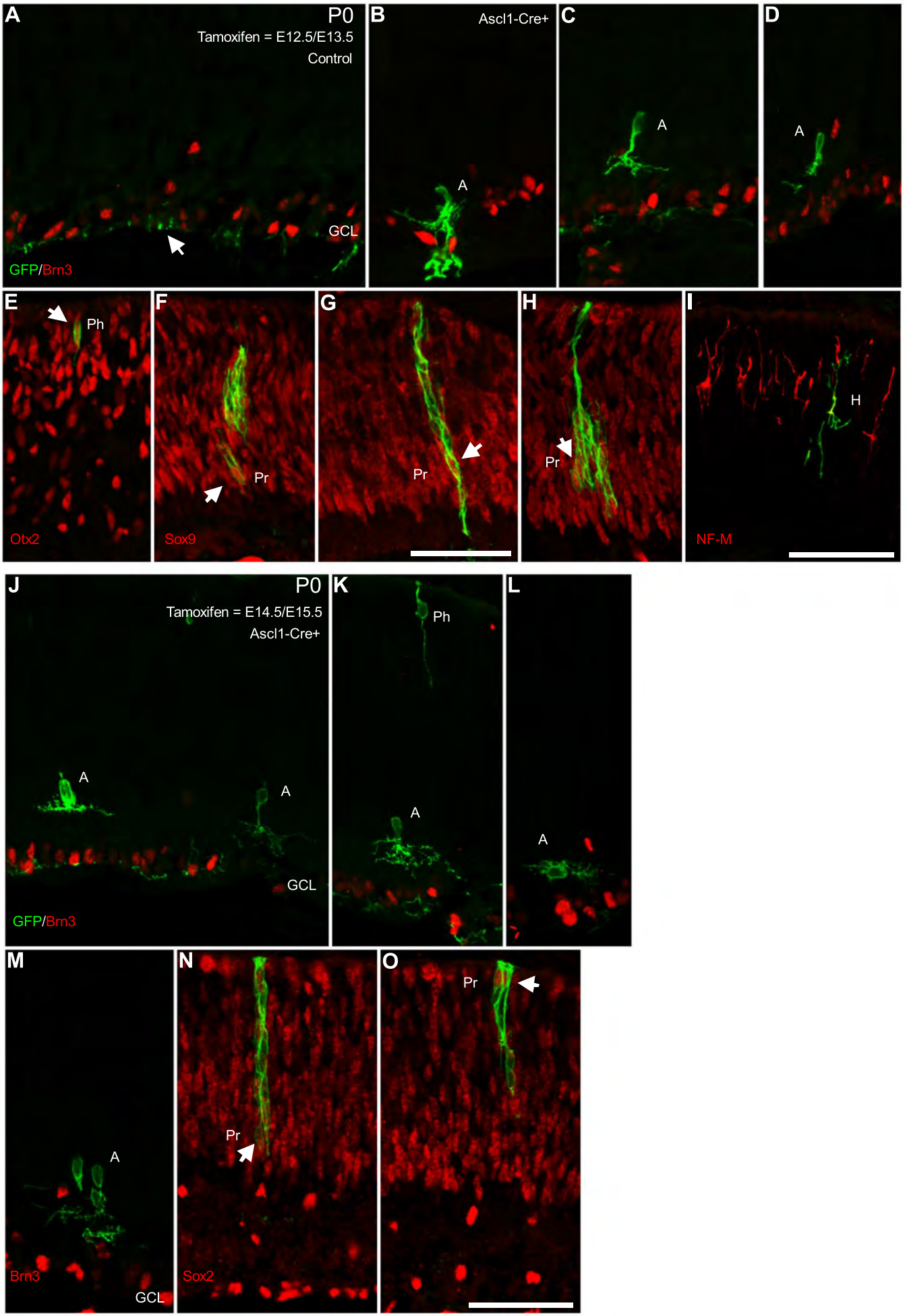


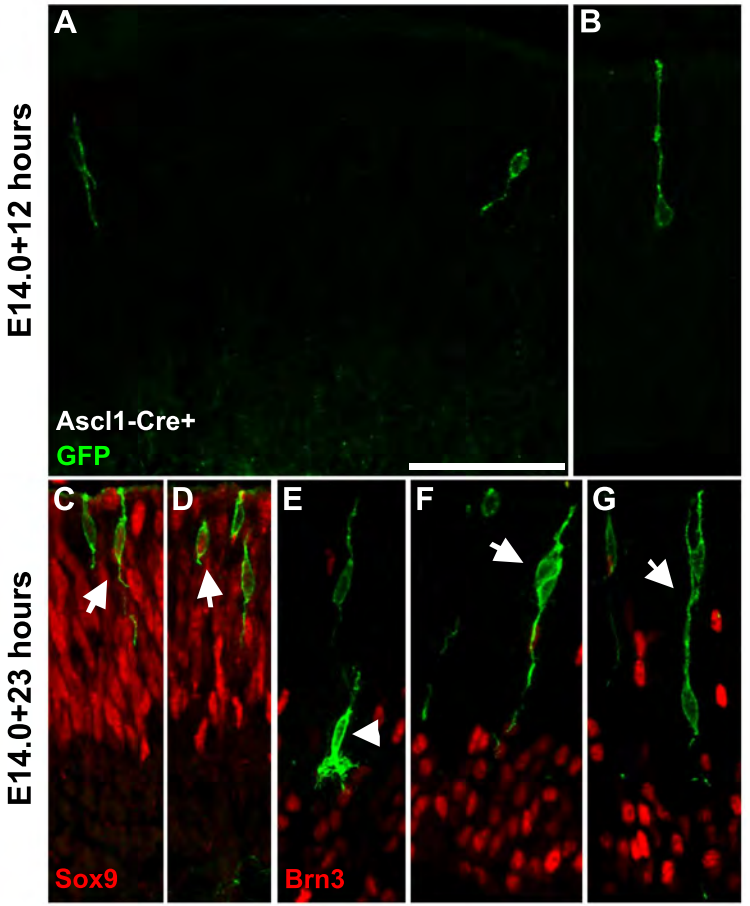
C Fate Distribution from E17.5 Lineage Traces

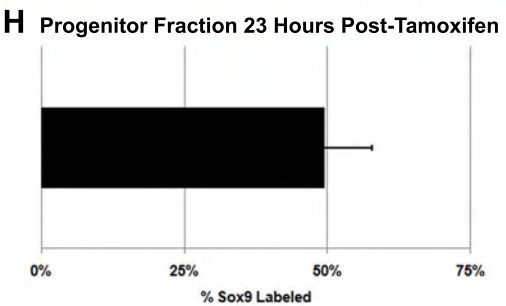


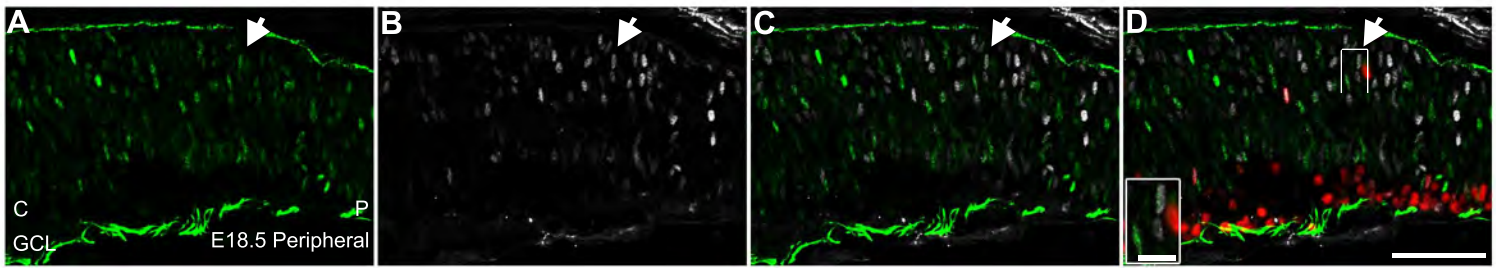
D RGCs from E12.5/13.5 Lineage Traces

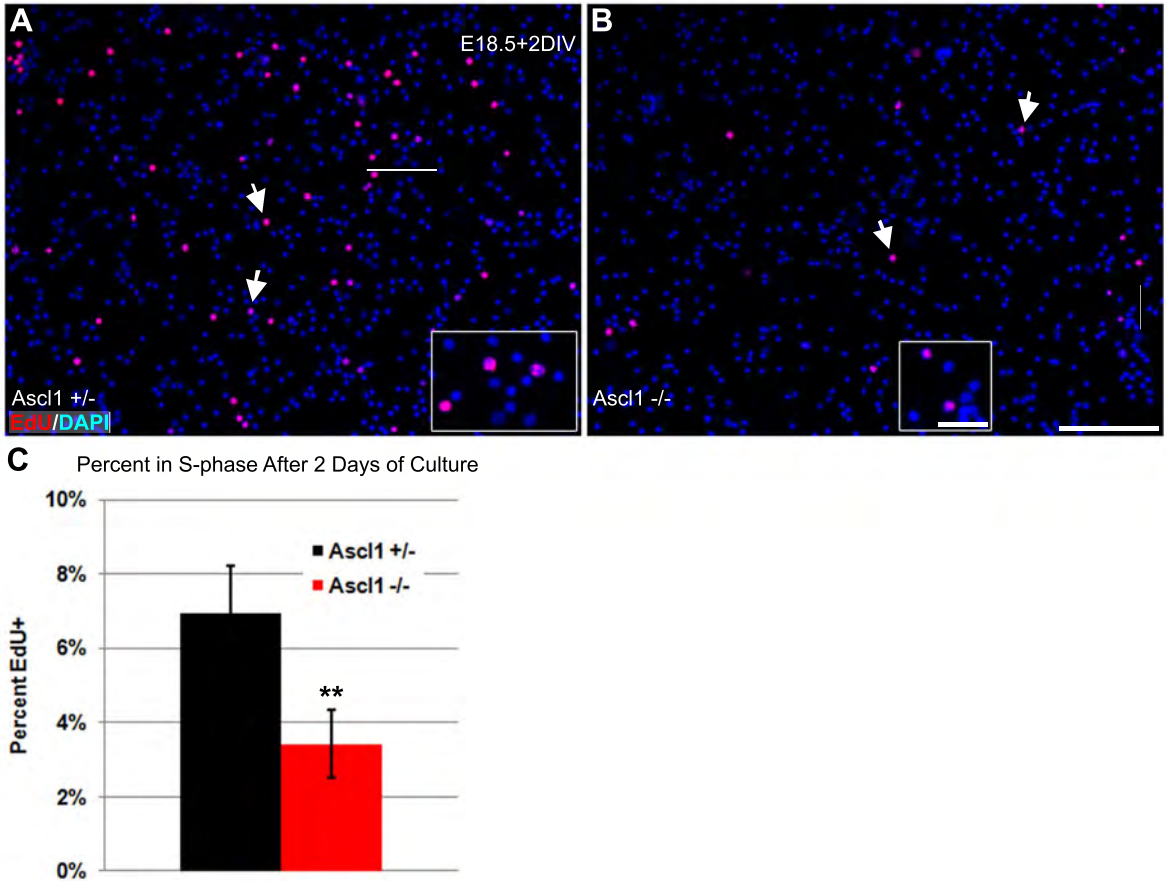


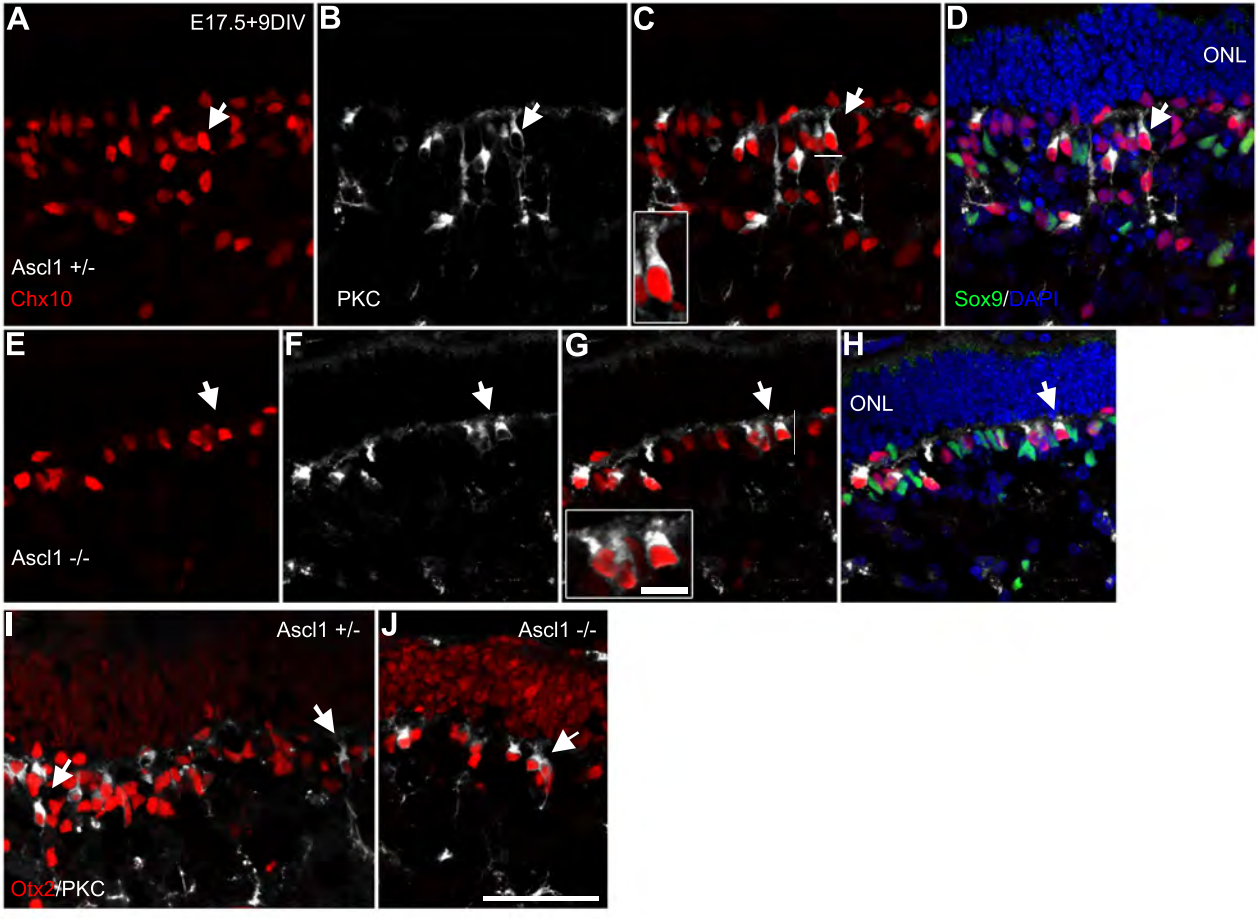

A
P0
Tamoxifen = E12.5/E13.5
Control
GCL
GFP/Brn3
B
Ascl1-Cre+
A
C
A
D
A
E
Ph
Otx2
F
Pr
Sox9
G
Pr
H
Pr
I
H
NF-M
J
P0
Tamoxifen = E14.5/E15.5
Ascl1-Cre+
A
A
GCL
GFP/Brn3
K
Ph
A
L
A
M
A
Brn3
GCL
N
Pr
Sox2
O
Pr


A
B
E14.0+12 hours
Ascl1-Cre+
GFP
C
D
E
F
G
E14.0+23 hours
Sox9
Brn3


H
Progenitor Fraction 23 Hours Post-Tamoxifen
0%
25%
50%
75%
% Sox9 Labeled


A
B
C
D
C
P
GCL
E18.5 Peripheral


A
E18.5+2DIV
Ascl1 +/-
EdU/DAPI
B
Ascl1 -/-
C
Percent in S-phase After 2 Days of Culture
10%
8%
6%
4%
2%
0%
Percent EdU+
Ascl1 +/-
Ascl1 -/-
**


A
E17.5+9DIV
Ascl1 +/-
Chx10
B
PKC
C
D
ONL
Sox9/DAPI
E
Ascl1 -/-
F
G
H
ONL
I
Ascl1 +/-
Otx2/PKC
J
Ascl1 -/-

A E12.5/E13.5 Ascl1, Ngn2, and Retroviral Lineages by Clump/Clone Size

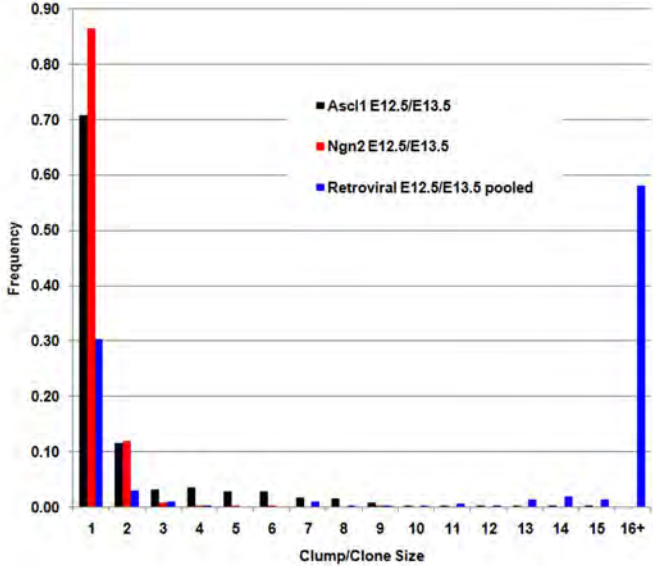


Table S1. Ascl1, Ngn2 and retroviral lineages

Lineage	G*	H*	C*	dA*	A*	R*	B*	M*	Clumps†
E12.5/E13.5 Ascl1	7 (0.22%)	302 (9.70%)	483 (15.51%)	149 (4.78%)	447 (14.35%)	1416 (45.46%)	154 (4.94%)	157 (5.04%)	1591
E12.5/E13.5 Ngn2	22 (1.75%)	245 (19.54%)	527 (42.03%)	122 (9.73%)	265 (21.13%)	66 (5.26%)	3 (0.24%)	4 (0.32%)	1075
E12.5+E13.5 retroviral (all clones)‡	43 (0.43%)	8 (0.08%)	152 (1.52%)	NA	337 (3.36%)	8390 (83.67%)	987 (984%)	85 (0.85%)	312
E12.5+E13.5 Retroviral (clones <16 cells)§	23 (5.7%)	6 (1.5%)	75 (18.7%)	NA	26 (6.5%)	234 (58.2%)	33 (8.2%)	5 (1.2%)	130
E17.5 Ascl1	0 (0.00%)	3 (0.06%)	52 (1.09%)	18 (0.38%)	489 (10.30%)	3799 (80.00%)	230 (4.84%)	158 (3.33%)	2528
E17.5 Ngn2	1 (0.09%)	0 (0.00%)	20 (1.84%)	52 (4.78%)	276 (25.37%)	738 (67.83%)	1 (0.09%)	0 (0.00%)	827
Adult WT retina¶	0.6%	0.5%	2.2%	0.9%	7%	78.5%	7.4%	2.9%	NA

*Number of cells observed (% of the total).
†Number of clumps or clones counted.
‡From Turner et al. (Turner et al., 1990). The two datasets are pooled.
§Cell fate distribution of clones containing 1-15 cells from the pooled datasets.
¶From Jeon et al. (Jeon et al., 1998).
G, retinal ganglion cell; H, horizontal; C, cone; dA, displaced amacrine; A, amacrine; R, rod; B, bipolar; M, Müller glia; WT, wild type.
NA, not available. All of the ganglion cell layer-labeled cells are in the retinal ganglion cell (RGC) category.

Table S2. E13.5 Birthdating

Cell type	Number birthdated	Total counted*	Quit fraction (%)†	Percentage born on E13.5‡
RGC	45	150	14.2	30.0
Hz	11	87	3.5	12.6
dA	50	225	15.7	22.2
Cones	75	ND	23.6	ND
INL amacrine	66	ND	20.8	ND
Rod	71	ND	22.3	ND

Hz, horizontal cell; dA, displaced amacrine; INL, inner nuclear layer amacrine cells; RGC, retinal ganglion cell.
ND, not determined.
*Counted from central third of the retina. RGCs were defined with pan-Brn3, and horizontals by calbindin immunostaining. Brn3-negative non-endothelial cell nuclei in the ganglion cell layer were counted as displaced amacrines.
†All intensely EdU-positive cells were summed, representing the total quit population at E13.5. The quit fraction was calculated as the number of EdU+ cells of a given type divided by all EdU+ cells.
‡The number of birthdated cells divided by the total number of cells counted of each type.

Table S3. Probability of finding ≤7 retinal ganglion cells (RGCs) in the 3115 cells sampled

Condition	Frequency	*P**
Horizontal†	0.097	0.0000000
Displaced amacrine†	0.048	0.0000000
Adult RGC‡	0.006	0.0018114

*Probability of seeing ≤7 RGCs in the Ascl1 lineage (3115 total cells) given the frequency at which they are expected to occur using the binomial distribution.
†Frequency observed in the E12.5/E13.5 Ascl1 lineage trace.
‡From Jeon et al. (Jeon et al., 1998).

Table S4. E13.5 Ascl1-GFP/Math5-Cre/retinal ganglion cell (RGC) distribution

	Ascl1-GFP+	Math5-Cre+	Brn3+	Ascl1-GFP+/Brn3+/Math5-Cre+
Count	239	886	987	1

Table S5. Probability of finding ≤1 Ascl1-GFP+ retinal ganglion cell (RGC) at E13.5

Condition	Frequency*	Ascl1 *P*[†]	RGC *P*[‡]
RGC E13.5[§]	0.360	0.0000000	0.0000000
RGC quit[¶]	0.142	0.0000000	0.0000000

*Frequency of RGCs used for calculations.
[†]Probability of seeing 0 or 1 Ascl1-GFP RGCs given the total number of Ascl1 cells seen (239) and the frequency at which RGCs are expected to occur using the binomial distribution.
[‡]Probability of seeing 0 or 1 Ascl1-GFP RGCs given the total number of RGCs seen (987) and the frequency at which RGCs are expected to occur using the binomial distribution.
[§]Percentage of cells in the E13.5 retina that are Brn3+ RGCs.
[¶]The fraction of all exiting cells that adopted RGC fate on E13.5.