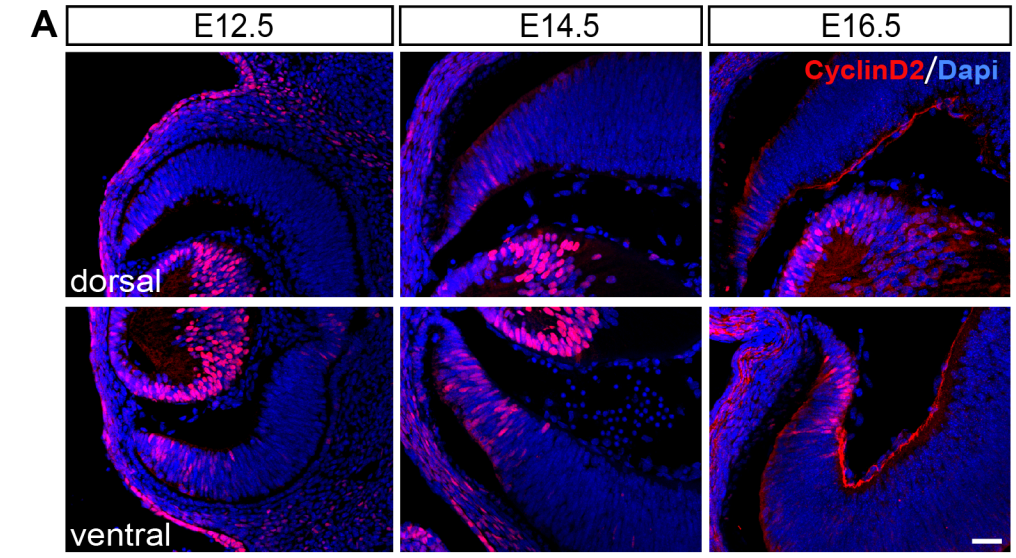
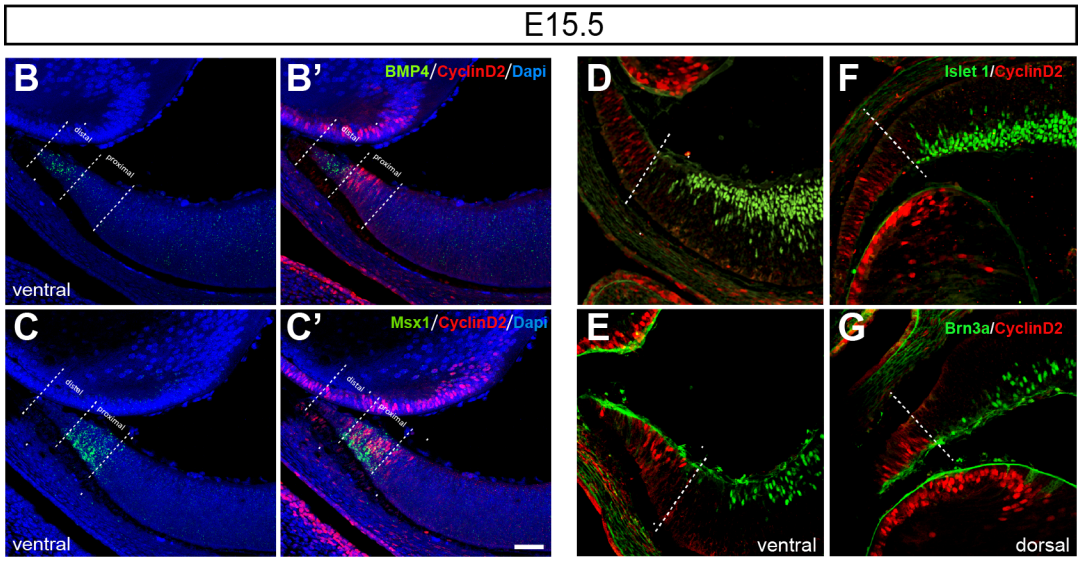
SUPPLEMENTAL INFORMATION

Supplemental data





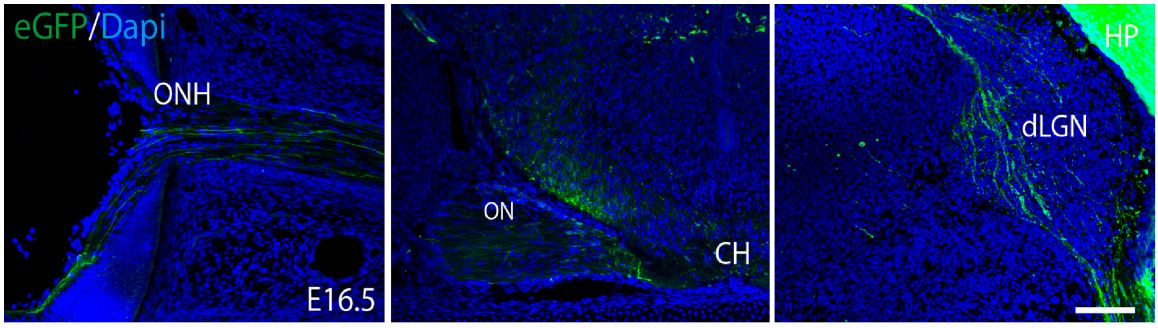
Supplemental Figure 1. Related to Figure 2: Cyclin D2 is highly expressed in the proximal ventral CMZ.

A. Cyclin D2 immunolabeling in coronal retinal sections from wild type embryos at ages indicated. Cyclin D2 is enriched in ventral retina at all stages analyzed. Cyclin D2 is also

enriched in ventral retina as early as E11.5 (Wang and Mason, submitted). Scale bar 50 μm.

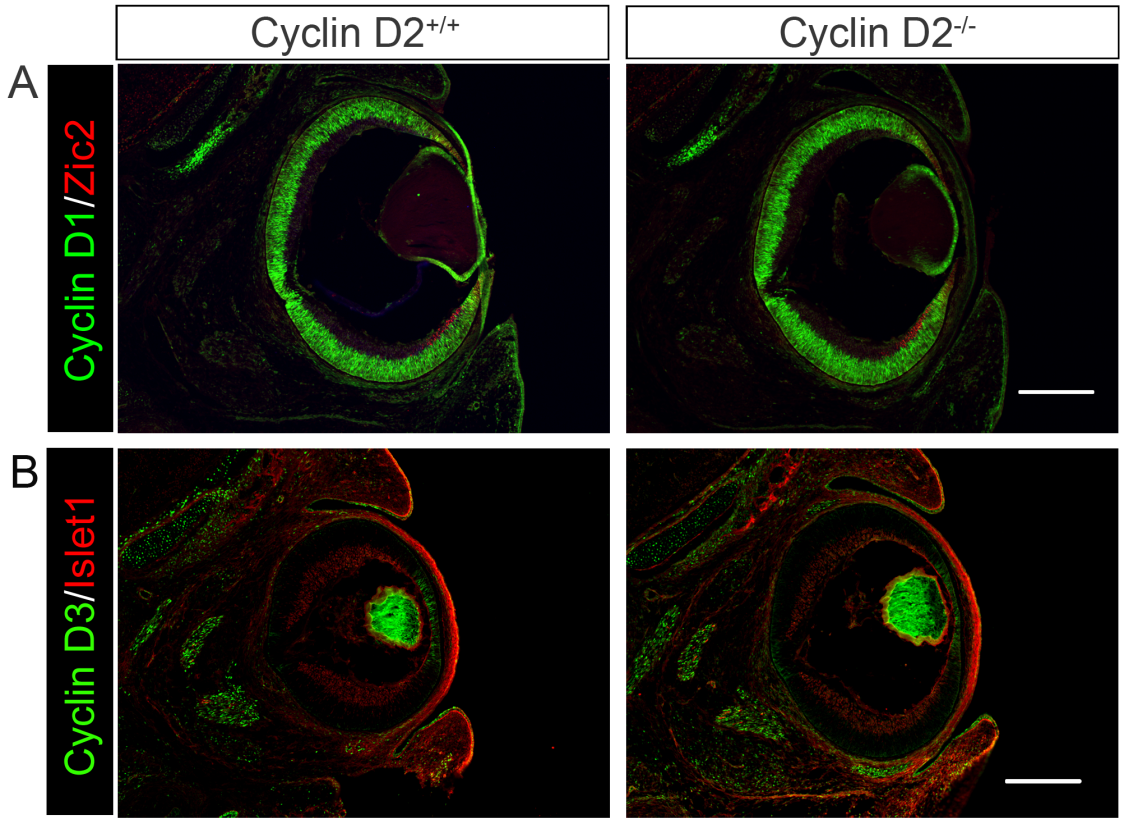
B-C′. *In situ* hybridization for BMP4 and Msx1 combined with immunolabeling for Cyclin D2 in coronal sections through the ventral retina. In the CMZ, the CyclinD2 expression zone is apposed to the $Msx1^{+}$ region. Scale bar 50 μm.

D-G. Coronal retinal sections from E14.5 wild type embryos double-immunolabeled with Cyclin D2 and Islet1 (upper panels) or Brn3a, specific for contralateral RGCs (lower panels). Islet1 and Brn3a label differentiated RGCs in the neural retina, located more centrally and in a zone distinct from that of CyclinD2, which is restricted to the CMZ in both the dorsal and the ventral retina. Note that the Brn3a antibody appears to label the inner limiting layer all along the inner (basal) side of the retina but this staining is nonspecific.



Supplemental Figure 2. Related to Figure 1: GFP^{+} RGCs in Tg(Zic2^{eGFP}) embryos extend axons from the eye to the brain.

At E16.5, GFP^{+} axons exit the retina through the optic nerve head (ONH). GFP^{+} axons can be visualized along the retinofugal pathway, passing through the optic nerve (ON) and the optic chiasm (CH). GFP^{+} axons also innervate the dorsal lateral geniculate nucleus (dLGN), one of the targets of RGCs. The strongly GFP+ region to the right of the micrograph is the hippocampus (HP), which expresses high levels of Zic2. Scale bar 100 μm.

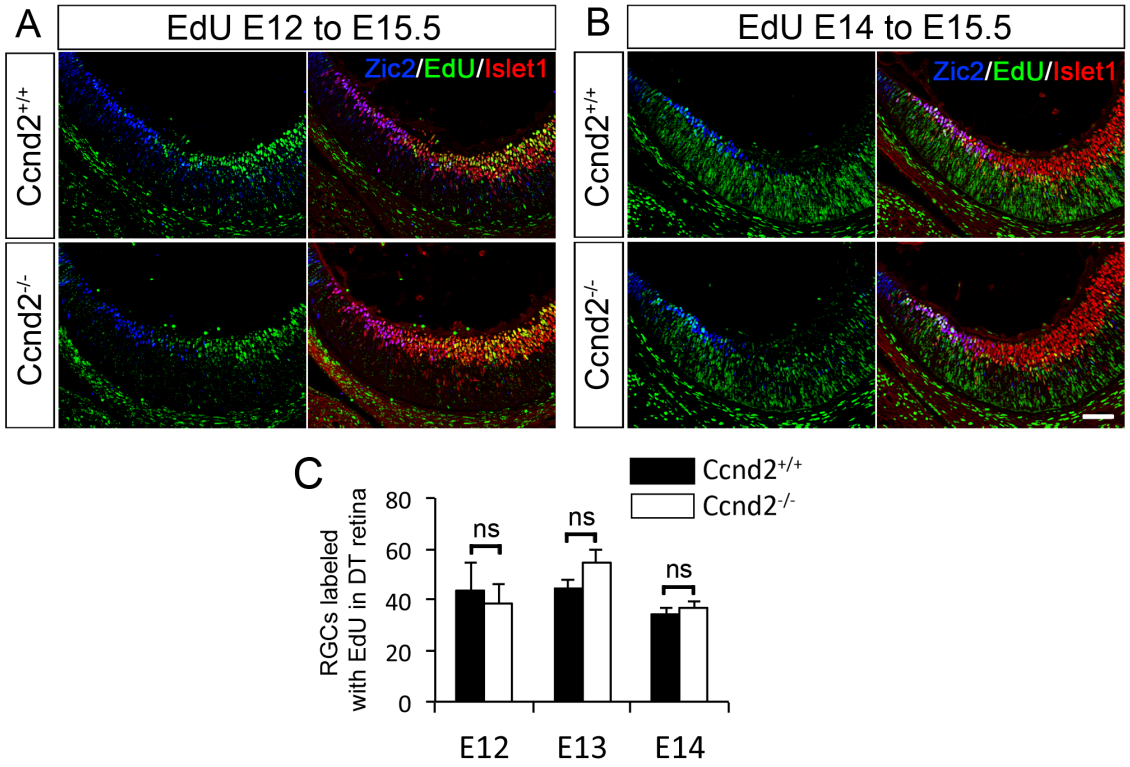


Supplemental Figure 3. Related to Supplemental Figure 1: Expression of Cyclin D1 and D3 in the retina is not affected in the absence of Cyclin D2.

A. Retinal sections of Cyclin $D2^{-/-}$ and wild type mice at E15.5 labeled with an antibody against Cyclin D1.

B. Retinal sections of Cyclin $D2^{-/-}$ and wild type mice at E15.5 labeled with an antibody against Cyclin D3.

Scale bar 250 µm.



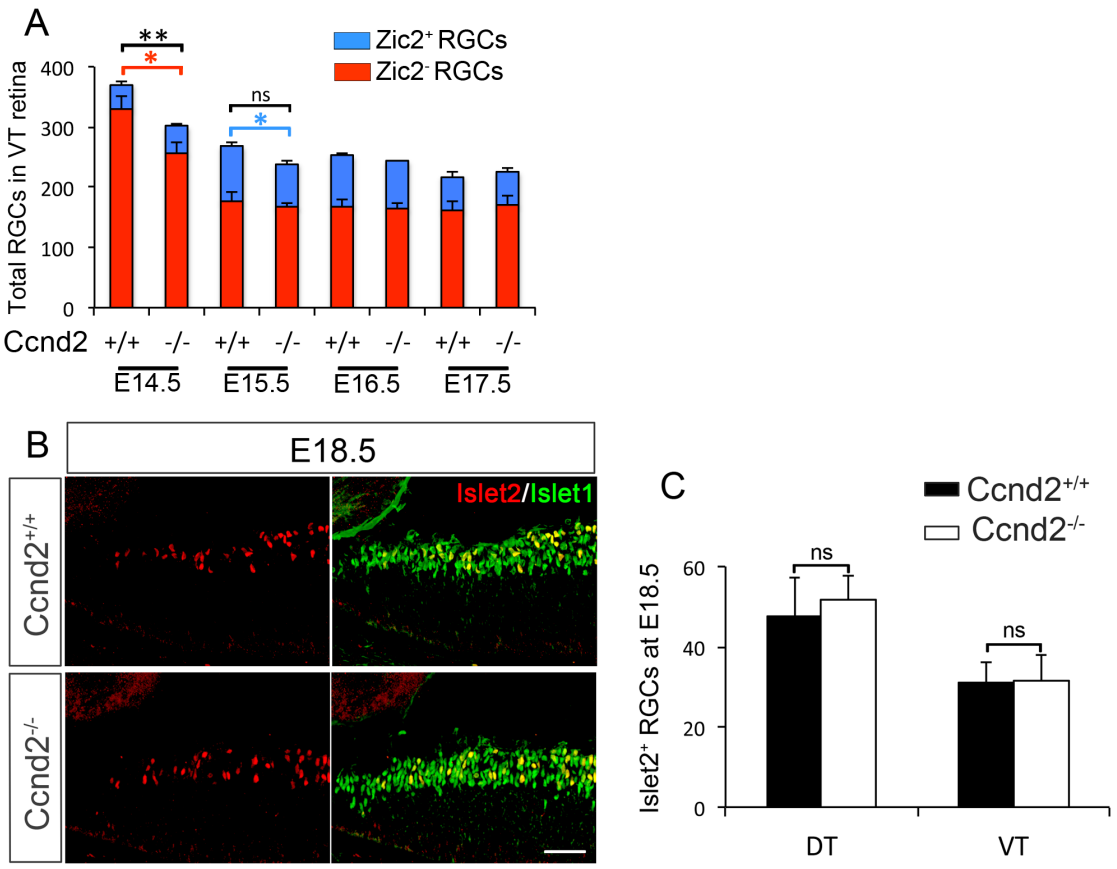
Supplemental Figure 4. Related to Figure 4: Fewer ipsilateral RGCs are born between E12-E14 in ventrotemporal (VT) retina of Cyclin D2$^{-/-}$ mice.

A and B. Representative retinal sections of Cyclin D2$^{-/-}$ and wild type littermates injected with EdU at E12 or E14, and the embryos sacrificed at E15. RGCs were labeled with Zic2 (blue), Islet1 (green) (by immunohistochemistry) and for EdU (green). The longer time interval between the ages of EdU injection and sacrifice allow for more rounds of cell division among retinal progenitors and result in a greater dilution of the EdU label (compare A with B).

C. Quantification of Islet1^{+}/EdU^{+} RGCs in DT retina of Cyclin D2$^{-/-}$ and wild type littermates. There were no differences in the time of birth of DT RGCs in Cyclin D2$^{-/-}$ and wild type littermates at the ages analyzed.

Student´s two-tailed unpaired t-test. Error bars signify ±SEM. ns, non-significant $p>0.05$.

Scale bar 40 μm.



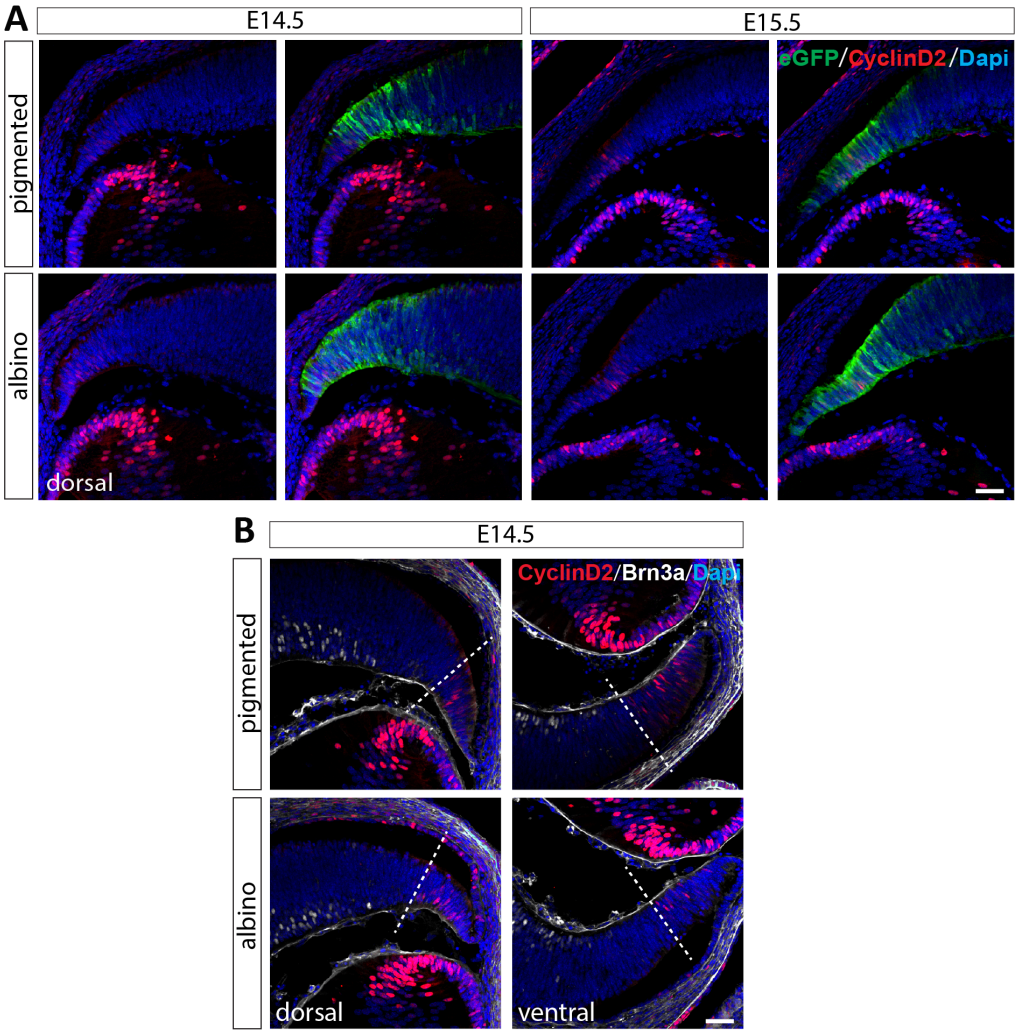
Supplemental Figure 5. Related to Figure 5:

A. Quantification of total RGC number in Cyclin D2 mutant mice. RGCs were divided into two subtypes based on their expression of the ipsilateral marker Zic2: $Zic2^{+,}$ or iRGCs; $Zic2^{-}$ or cRGCs. $Zic2^{+}$ and $Zic2^{-}$ RGCs were compared in the two genotypes at the ages indicated. At E14.5 both the total number of RGCs (black asterisks) and the number of $Zic2^{-}$ RGCs (red asterisk) decreased. In contrast, at E15.5 only the number of $Zic2^{+}$ RGCs (blue asterisk) decreased even though the total number of RGCs did not change (ns).

B and C. Generation of "late-born" contralateral RGCs in VT retina is independent of Cyclin D2 activity. B. Immunolabeling of wild type and mutant Cyclin D2 retinal

sections at E18.5 with antibodies against Islet2 (red), expressed by cRGCs in VT retina after E17.5), and antibodies against the differentiated RGC marker Islet1 (green). **C.** Quantification of $Islet2^{+}/Islet1^{+}$ cells indicates that the number of "late-born" contralateral RGCs does not change in the absence of Cyclin D2 at E18.5, during the late phase of axonal outgrowth (30.94±9.79 in $Ccnd2^{+/+}$ vs. 31.5±6.1 in $Ccnd2^{-/-}$ for VT retina; 47.63±4.98 in $Ccnd2^{+/+}$ vs. 51.8±6.57 in $Ccnd2^{-/-}$ for DT retina).

Student´s two-tailed unpaired t-test. Error bars, ±SEM. ns, non-significant. Scale bar 40 μm.


A
E14.5
E15.5
pigmented
albino
eGFP/CyclinD2/Dapi
dorsal
B
E14.5
pigmented
albino
CyclinD2/Brn3a/Dapi
dorsal
ventral
C
E13.5
Pigmented
Albino
dorsal
NR
NR
NR
CMZ
ventral
PH3
E16.5
dorsal
NR
NR
NR
CMZ
ventral
PH3
D
Neural Retina
Ciliary Marginal Zone
pH3+ cells/10 3µm2 E13.5
pH3+ cells/10 3µm2 E16.5
ns
***
Dorsal
Ventral
Pigmented
Albino

Supplemental Figure 6. Related to Figure 6:

A. Pigmented and albino dorsal retina at E14.5 and E15.5 immunostained with antibodies against Cyclin D2. There is no change in the number of Cyclin $D2^+$ cells in the dorsal aspect of albino compared to pigmented retina at E14.5 (10.66±0.72 dorsal pigmented retina vs. 8.8±0.351 dorsal albino retina) and at E15.5 (14.71±2.04 dorsal pigmented retina vs. 10.33±1.41 dorsal albino retina).

B. Cyclin D2 and Brn3a immunolabeling in coronal retinal sections of pigmented and albino mice at E14.5 indicates that Cyclin D2 and Brn3a expression do not overlap and are localized to different compartments of the retina (CMZ and the neural retina, respectively).

C. PH3 staining in dorsal and ventral retinal sections of pigmented and albino mice at E13.5 and E16.5 with squares delimiting the CMZ and the neural retina as in Figure 6C.

D. Quantification of $PH3^+$ cells in the peripheral and the neural retina of albino and pigmented E13.5 embryos indicates more proliferating cells in the ventral CMZ of both pigmented and albino embryos than in the dorsal retina (0.13±0.04 dorsal pigmented CMZ vs. 0.3±0.04 ventral pigmented CMZ; 0.2±0.05 dorsal albino CMZ vs. 0.35±0.04 ventral albino CMZ). At this age the number of PH3 cells in the neural retina is similar in both cases (0.7±0.05 dorsal pigmented retina vs. 0.6±0.04 dorsal albino retina; 0.6±0.04 ventral pigmented retina vs. 0.55±0.03 ventral albino retina). Quantification of $PH3^+$ cells in the CMZ and the neural retina of albino and pigmented E16.5 embryos revealed an increase of mitotic cells in the dorsal and the ventral neural retina of albino embryos compared with pigmented littermates (0.4±0.02 pigmented dorsal retina vs. 0.5±0.03 pigmented ventral retina; 0.6±0.03 albino dorsal retina vs. 0.65±0.04 albino ventral

retina). In contrast, at this stage, the number of proliferating cells in the CMZ was similar in pigmented and albino retinas (0.03±0.01 pigmented dorsal CMZ vs. 0.03±0.006 pigmented ventral CMZ; 0.06±0.01 albino dorsal CMZ vs. 0.06±0.01 albino ventral CMZ).

NR, Neural Retina; CMZ, Ciliary marginal zone; ns, non significant; Student´s unpaired t-test; Error bars mean ±SEM, *$p<0.05$; ***$p<0.001$). Scale bar 100 mm.

Supplemental movie:

Time-lapse imaging from E14.5 Tg(Zic2^{eGFP}) retinal slides. Images were recorded every 20 minutes over 17 hours. Cells moving laterally were labeled in red, green, blue and pink. Cells moving in an apico-basal direction were labeled in yellow. eGFP cells were tracked using IMARIS Surpass software in order to rotate the image and visualize each cell trajectory in 3D. Cell trajectories were corrected for physiological tissue growth by applying Correct 3D Drift (See Material and Methods for further details). The movie is played at 2 frames per second. Scale bar 50 μm.

Supplemental Experimental Procedures

Tissue preparation

Tg(Zic2^{EGFP}) and albino animals at E12.5-E13.5 were fixed by immersion with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH7.4) and E14.5 and E15.5 embryos were intracardially perfused. All brains were post-fixed in the same fixative for 4 hours, and washed in PBS three times. The brains were cryoprotected in 30% (w/v) sucrose in PBS and frozen in dry ice. Coronal sections (20 μm thick) were cut with a cryostat (SLEE medical GmbH, Mainz) and stored at -20º C until used.

Cyclin D2 mice at E15.5 and younger were harvested and heads were fixed by immersion with 4% PFA for 1 hour. Cyclin D2 mice E16.5 and older were intracardially perfused with 4% PFA and postfixed by immersion in 4% PFA for an additional hour. After fixation, tissue was washed with PBS, cryoprotected with 10% sucrose in PBS for 24 to 72 hours at 4°C, and frozen in dry ice. Coronal sections (14 μm) were cut with a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) and collected on Fisherbrand Frosted microscope slides.

In situ Hybridization

Briefly, cryostat sections were post-fixed with 4% PFA for 5 min, washed in PBS 1X and hybridized with DIG-labeled cRNA probe overnight in hybridization solution [50% formamide (Ambion), 10% dextran sulfate, 0.2% tRNA (Invitrogen), 1× Denhardt's solution (from a 50× stock; Sigma-Aldrich), 1× salt solution (containing 0.2 M NaCl, 0.01 M Tris, 5 mM NaH2PO4, 5 mM Na2HPO4, 5 mM EDTA, pH 7.5)]. After

sections were washed, alkaline phosphatase-coupled anti-digoxigenin or anti-digoxigenin-POD Fab fragments were applied. Color development in the presence of NBT and BCIP (4-nitroblue tetrazolium chloride, 5-bromo-4-chloro-3 indolylphosphate) or TSA fluorescein respectively was performed in the dark at room temperature using the same reaction time for all samples.

Immunohistochemistry

For immunohistochemistry, embryos from all mouse strains and genotypes were sectioned and slides were rinsed in PBS, and antigen retrieval was performed: slides were incubated in Sodium Citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 20 min at 96°C, followed by 20 min at room temperature. Slides were then washed in PBS for 5 min and blocked in 10% NGS, 0.2% Tween in PBS for 1 hour, incubated in primary antibody in 0.2% Tween, 1% NGS, in PBS overnight at 4°C, washed 3 × 10 minutes in PBS at room temperature, incubated in secondary antibody for 2 hours at room temperature, washed in PBS 3 × 10 minutes at room temperature, and mounted with Fluor-Gel.

Antibodies

The following primary antibodies were used: rabbit anti-CyclinD2 (sc-593; Santa Cruz Biotechnology, 1:500); chicken anti-GFP (Aves Labs, 1:1000); goat anti-Brn3 (Santa Cruz, 1:200); mouse anti-Brn3a (MAB1585; Chemicon, 1:50); mouse anti-Phospho-Histone H3 (Ser10)(6G3) (PH3; Cell Signalling Technology, 1:800); rabbit anti-Phospho-Histone H3 (Millipore, 1:100); rabbit anti-Zic2 [Herrera lab, 1:1000 used for

experiments in Tg(Zic2^{EGFP}) and albino mice]; rabbit anti-Zic2 (gift of Stephen Brown, University of Vermont, 1:10000, used for experiments on Ccnd2 mice); mouse anti-Islet1 (1:100); rabbit anti-Islet2 (1:3000). Brn3 antibody recognizes Brn3a, b and c, in differentiated RGCs. Antibodies against Islet1 and Islet2 were gifts of Susan Morton and Thomas Jessell, Columbia University. The Islet1 antibody was made against Islet1 (expressed in postmitotic RGCs) but also recognizes Islet2 (expressed in a subset of cRGCs) (Pak et al., 2004, Pan et al., 2008). For immunofluorescence detection, Alexa 488, Alexa 546, Alexa 594 and Alexa 647 (Invitrogen, Molecular Probes) secondary antibodies were used. A DAPI staining solution was used to visualize nuclei (2 μg/mL). Fluorescent sections were mounted with coverslips in Moviol or Fluor-Gel.

Cell number analysis of retinal sections

The number of Cyclin D2 positive cells in pigmented and albino mouse retina was quantified by examining coronal sections and counting the total number of labeled cells within comparable regions of the CMZ or the neural retina of different genotypes. Depending on the stage, 2 or 3 sections spanning the rostral to caudal axis were analysed from a minimum of three animals per age and genotype. For all of the counts, 20x Z-stack images obtained dorso-ventrally were examined in three consecutive antero-posterior sections (20 μm thick) in each eye.

For quantification of Zic2, Islet1, Islet2, Brn3 and Brn3a cell number in sections of Cyclin D2 mice, a sector of 240 μm long (as measured in the superficial aspect of the retina) was traced starting at the most peripheral Islet1-positive RGCs in dorsotemporal and/or ventrotemporal retina. Cell number was determined within the 240 μm sector.

For quantification of PH3-positive cells in sections from Cyclin D2, pigmented and albino mice, cell number was determined in the ciliary marginal zone (CMZ), and two sectors from the neural retina: To delineate the region corresponding to the CMZ, a sector of 65 μm was traced ending at the most peripheral Islet1-positive RGC of dorsotemporal and ventrotemporal retina. To delineate the regions corresponding to the neural retina, dorsotemporal and ventrotemporal retina were divided into 4 (E13.5) or 5 (E14.5) consecutive sectors of 65 μm in length radially (in the apical to basal axis of the retina), with the first sector traced starting at the most peripheral Islet1-positive RGC (and at the border of the CMZ). For E13.5 embryos the first and 4th sectors, and for E14.5 embryos the first and 5th sectors were counted and considered as neural retina. Cell numbers were recorded blind to genotype within the assigned sectors using Meta Imaging Series Metamorph 7.0 (Molecular Devices, Sunnyvale, CA, USA). For PH3 immunolabeling, images were thresholded with Metamorph such that only the most strongly labeled cells were counted. For Zic2, Islet1, Islet2 and Brn3a immunolabeling, no threshold was necessary as background staining not noticeable. Depending on the embryonic age, 2 to 4 sections after the optic nerve per animal were averaged for analysis. A minimum of 5 animals, from at least 3 different litters, was taken into account for statistical analysis of wild-type and mutant groups or pigmented versus albino mice.

Retina and lens area were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Birthdating analysis

EdU (5-Ethynyl-2'-deoxyuridine, 2.5 mg/ml, InVitrogen) was injected into the

pregnant mother intraperitoneally three times, at 10 am, 2 pm, and 6 pm, in animals at E11, 12, E13 or E14. EdU, similar to BrdU and [^{3}H]thymidine, is incorporated into DNA and labels cells undergoing S-phase (Salic and Mitchison PNAS 2008; Bhansali Neural Development 2014). Embryos were sacrificed and collected at E15.5. Embryos were fixed and processed for IHC as per the procedure described above for retinal sections. After immunostaining for Zic2 and Islet1, sections were permeabilized in 0.5% Tween in PBS for 30 minutes, washed in PBS 2 × 5 minutes, and the EdU histochemistry performed as described (Bhansali et al., 2014, Salic and Mitchison, 2008), using a fluorescent azide through a Cu(I)-catalyzed cycloaddition reaction (Click-iT EdU imaging kit, Invitrogen, Carlsbad, CA, USA), for 30 minutes at room temperature. After the EdU reaction was visible, slides were washed 3 × 10 minutes in PBS at room temperature. Sections were coverslipped with Fluoro-Gel. Imaging, quantification and analysis were performed as described above. The EdU signal in retinae injected at E11, E12 or E13 and collected at E15.5 was thresholded such that only the cells that became post-mitotic at E11-13 (brightest) were counted, using the Meta Imaging Series software Metamorph 7.0. The EdU signal in retinae injected at E14 and collected at E15.5 did not require thresholding because the window between the age of injection and collection was too short for multiple rounds of division, and therefore had less variability in EdU signal strength amongst postmitotic RGCs.

Preparation of retinal slices for time-lapse imaging

E14.5 pigmented or albino embryos were placed in ice-cold Krebs buffer and embed in 3% low melting point agarose. Brains were cut into 150 μm thick coronal sections and sections were collected in ice-cold Krebs buffer supplemented with antibiotics. Slices were transferred to a Millipore Millicell Membrane (PICMORG650) in a petri dish with 1.5 ml of supplemented MEM and incubated 1h at 5% CO_2, 37°C. The Millicell Membrane was then transferred to a microwell dish with 1.2 ml of supplemented Neurobasal medium.