Developmental-stage-specific proliferation and retinoblastoma genesis in RB-deficient human but not mouse cone precursors

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Supporting Information

www.pnas.org/cgi/doi/10.1073/pnas.1808903115

Figure S1

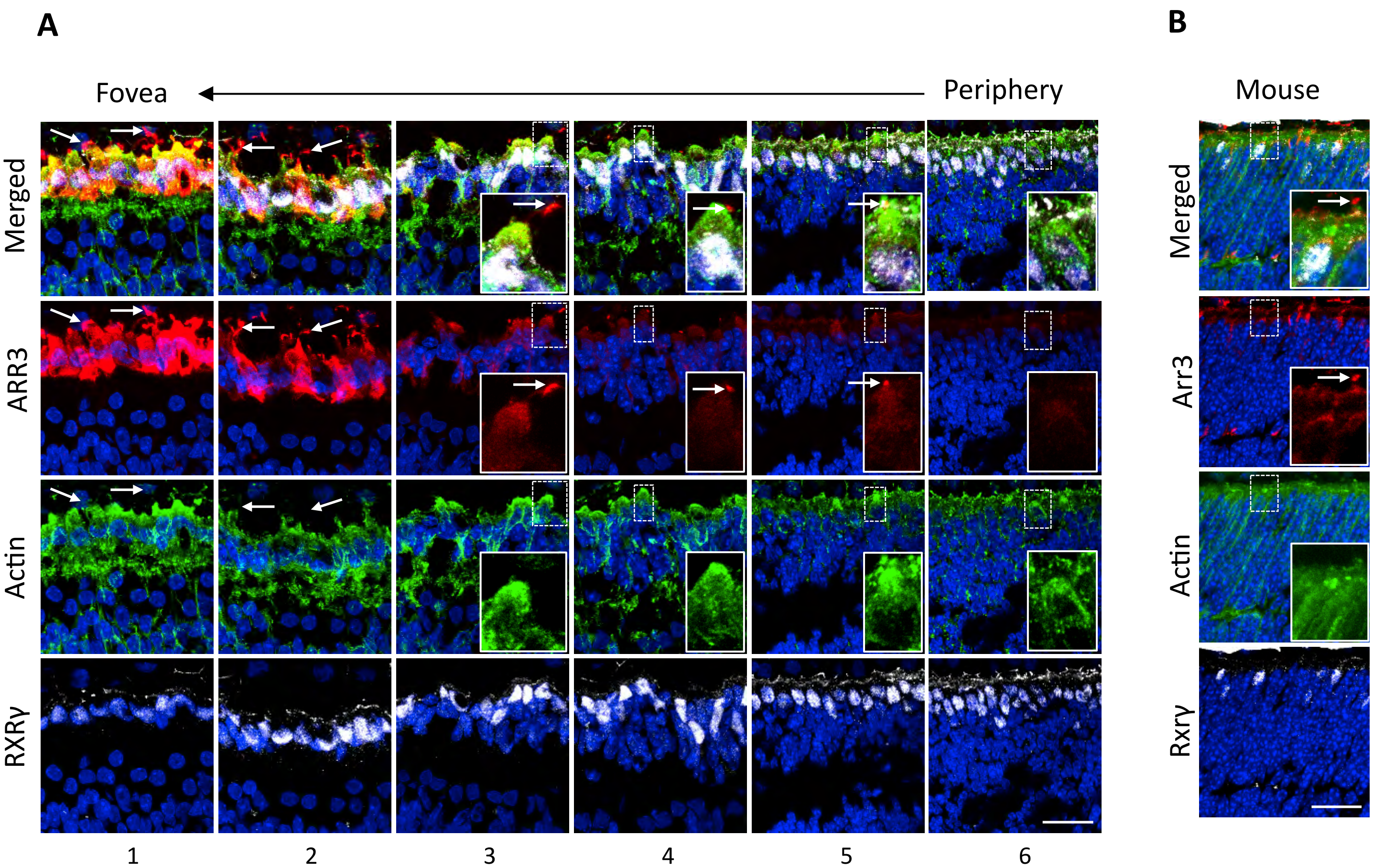

Figure S1. ARR3 expression coincides with the emergence of cone precursor outer segments. A. Human week 20 retina co-stained for ARR3 (red), Actin (green), and RXRγ (white). Arrow represents the increasing maturation gradient from the retinal periphery (position 6) to the fovea (position 1). Concentrated actin filaments in the apical portion of inner segments are thought to have a role in the development of photoreceptor outer segments and calycal processes (1-3). These concentrated actin filaments were detected at the apical surface in all RXRγ+ cone precursors in central retina (positions 1-4), in a minority of cone precursors at the more peripheral position 5, and in rudimentary cone precursor structures that did not reach the apical surface at the most peripheral position 6. RXRγ+ cone precursors with strong apical actin staining (at positions 4 and 5) had ~0.5 micron diameter outer segment buds that stained strongly for ARR3 (arrows), whereas cone precursors that lacked apical actin staining (at positions 5 and 6) lacked ARR3+ buds. **B**. Mouse P6 retina co-stained for Arr3, Actin, and Rxrγ. At P6, Arr3 was detected in Rxrγ+ cone precursor cell bodies and in ~0.5 micron outer segment buds (arrow) that first emerge at this age (3, 4) and were apical to highly actin-positive inner segments. Insets, enlarged view of actin clusters. Confocal images show maximum intensity projection of image stacks. Scale bars, 20 µm.

Figure S2

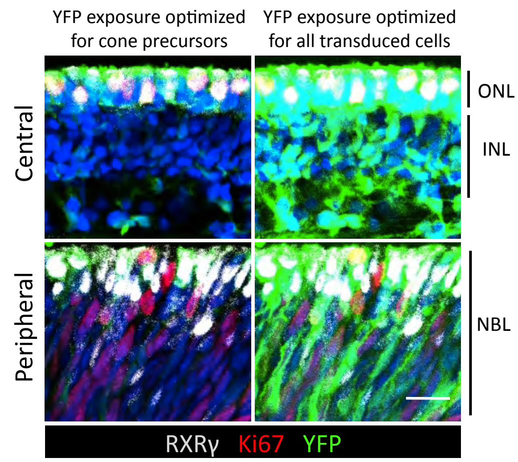

Figure S2. Lentiviral transduction of multiple layers of week 18 human retina. pLKO-YFP-sh*RB1* lentiviral transduction of cells throughout the inner nuclear layer (INL) and outer nuclear layer (ONL) of the central retina and throughout the neuroblastic layer (NBL) of the peripheral retina is evident with imaging optimized to detect all transduced cells. Cone precursors in the ONL shows brightest YFP signal and were best resolved with exposures optimized for this cell type. Scale bar, 20 µm.

Figure S3

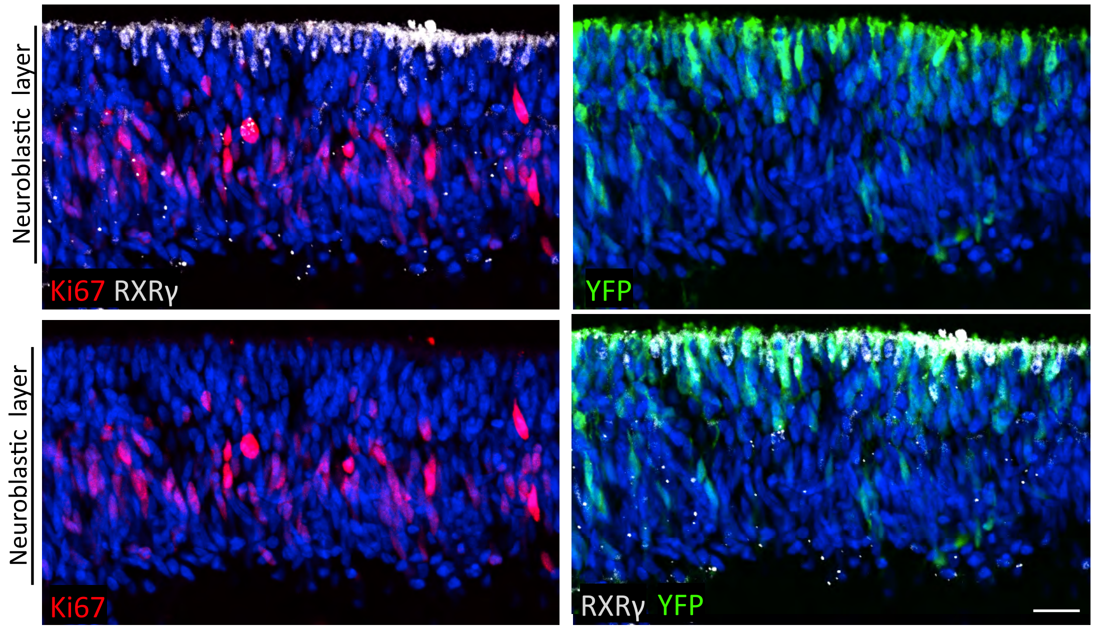

Figure S4

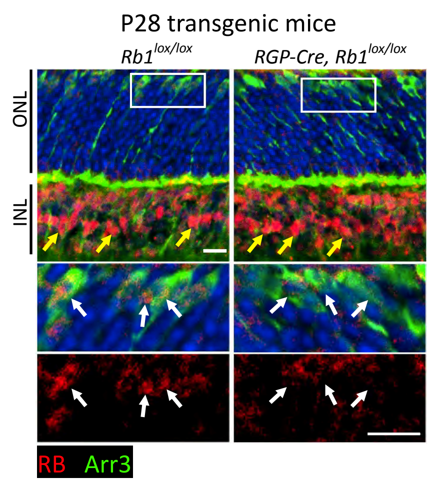

Figure S3. Proliferating RXRγ(-) cells in sh*SCR*-transduced human retina. sh*SCR* transduced retina at 12 DIC shows Ki67+,RXRγ(-) cells in the neuroblastic layer, similar to that observed in peripheral sh*Rb1*-transduced retina (Fig. 1C-E). Scale bar, 20µm.

Figure S4. **Cone-specific *Rb1* knockout in *RGP-Cre;Rb1$^{lox/lox}$* mouse retina at postnatal day P28.** Upper panels show sections traversing the outer nuclear layer (ONL) and inner nuclear layer (INL). Boxed ONL regions are enlarged in lower panels. White arrows show Arr3+ cones and yellow arrows show Müller glia. Residual signal in the P28 *RGP-Cre;Rb1$^{lox/lox}$* ONL is intercellular and likely background. Scale bar, 10µm.

Figure S5

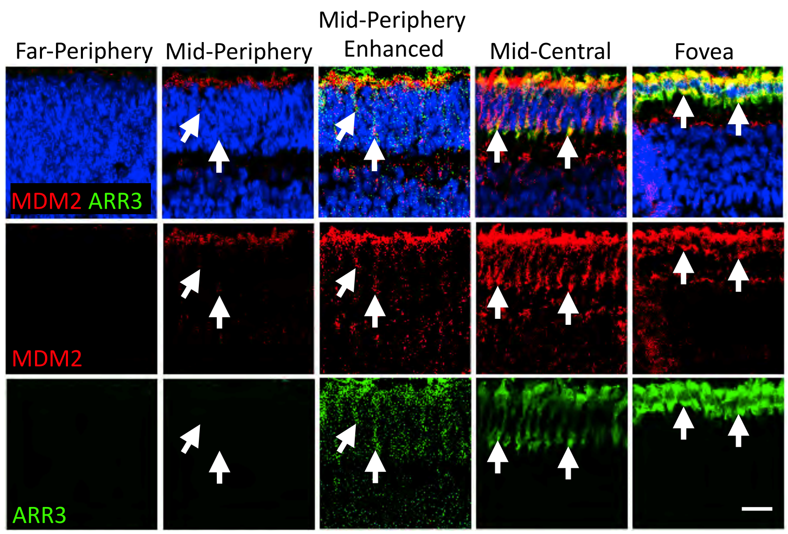

Figure S5. Coincident onset of MDM2 and ARR3 expression in developing human retina. Week 21 retina immunostained for MDM2 (red) and ARR3 (green) and imaged in a peripheral to central series of increasing maturity. Weak MDM2 and ARR3 co-expression was first detected in a mid-peripheral region that was elucidated by enhancing the same images in the adjacent column. Prominent MDM2 and ARR3 co-expression was observed in mid-central retina and fovea. Scale bar, 20 µm.

Figure S6

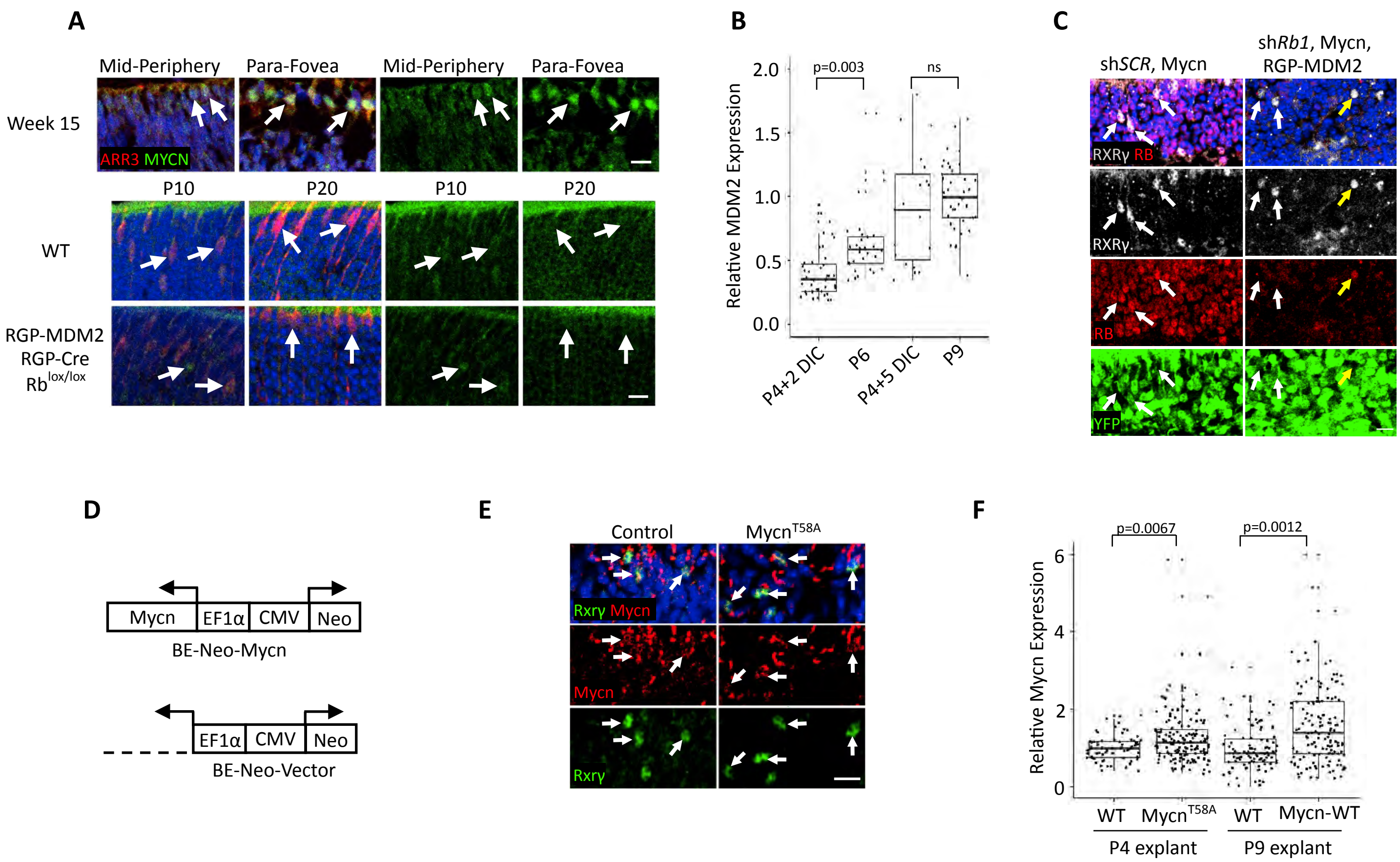

Figure S6. **MDM2 and Mycn overexpression in transgenic and lentivirus-transduced mouse retinae. A.** Developmental increase in MYCN expression in human but not in mouse cone precursors. *Upper panels,* ARR3+ cells in human week 15 retinae show increasing MYCN expression from the mid-periphery to parafovea. *Lower panels*, Mycn was weakly expressed in Arr3+ cells at P10 and not detected above background levels at P20 in both WT and *RGP-MDM2;RGP-Cre;Rb$^{lox/lox}$* mice retinae demonstrating that the MDM2 transgene did not alter endogenous Mycn expression. **B.** Quantitation of MDM2 expression in Rxrγ+ cone precursors in cultured *vs*. *in vivo* developed *RGP-MDM2* littermate retinae. MDM2 levels were compared between P6 retina and P4 retinal explants cultured for 2 days or between P9 retinae and P4 explants cultured for 5 days. Each dot represents a quantitatively imaged cell outlined by Rxrγ signal, and box plots show median (line inside box), upper and lower quartiles (box borders), and data range (whiskers). Significance was assessed by t-test. P values are as shown; ns, not significant. **C.** *Rb1* KD in a P4+7 DIC retinal explant co-stained for Rxrg, Rb, and YFP. White arrows, infected (YFP+) cells that are Rb+ after transduction with sh*SCR* but not with sh*Rb1*. Yellow arrow, uninfected (YFP-) cell with Rb expression. **D.** Lentiviral constructs for transduction of intact murine retina with ectopic Mycn or Mycn^{T58A} (BE-Neo-Mycn$^{(T58A)}$) or with the empty vector (BE-Neo). **E.** Mycn expression in Rxrγ+ cells of P4 retinal explants transduced with Mycn^{T58A} at 7 DIC. **F.** Quantitation of Mycn levels in Rxrγ+ cells (*left*) or Arr3+ (*right*) at 7 DIC in P4 and P9 explants, respectively. Box plots and significance tests are as in panel B. Scale bars in A, C, and E, 10 µm.

Figure S7

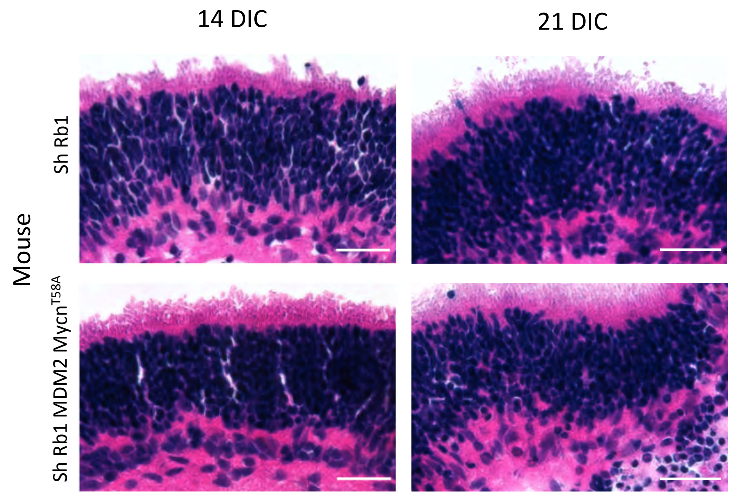

Figure S7. **Absence of rosettes and fleurettes in Rb-depleted cultured murine retinae**. Hematoxylin & eosin staining of explanted P4 wild type retina at 14 and 21 days post-Rb KD (*top*) and explanted P4 *RGP-MDM2* retina at 14 and 21 days post-Rb KD and Mycn overexpression (*bottom*). Scale bars, 20 µm.

Figure S8

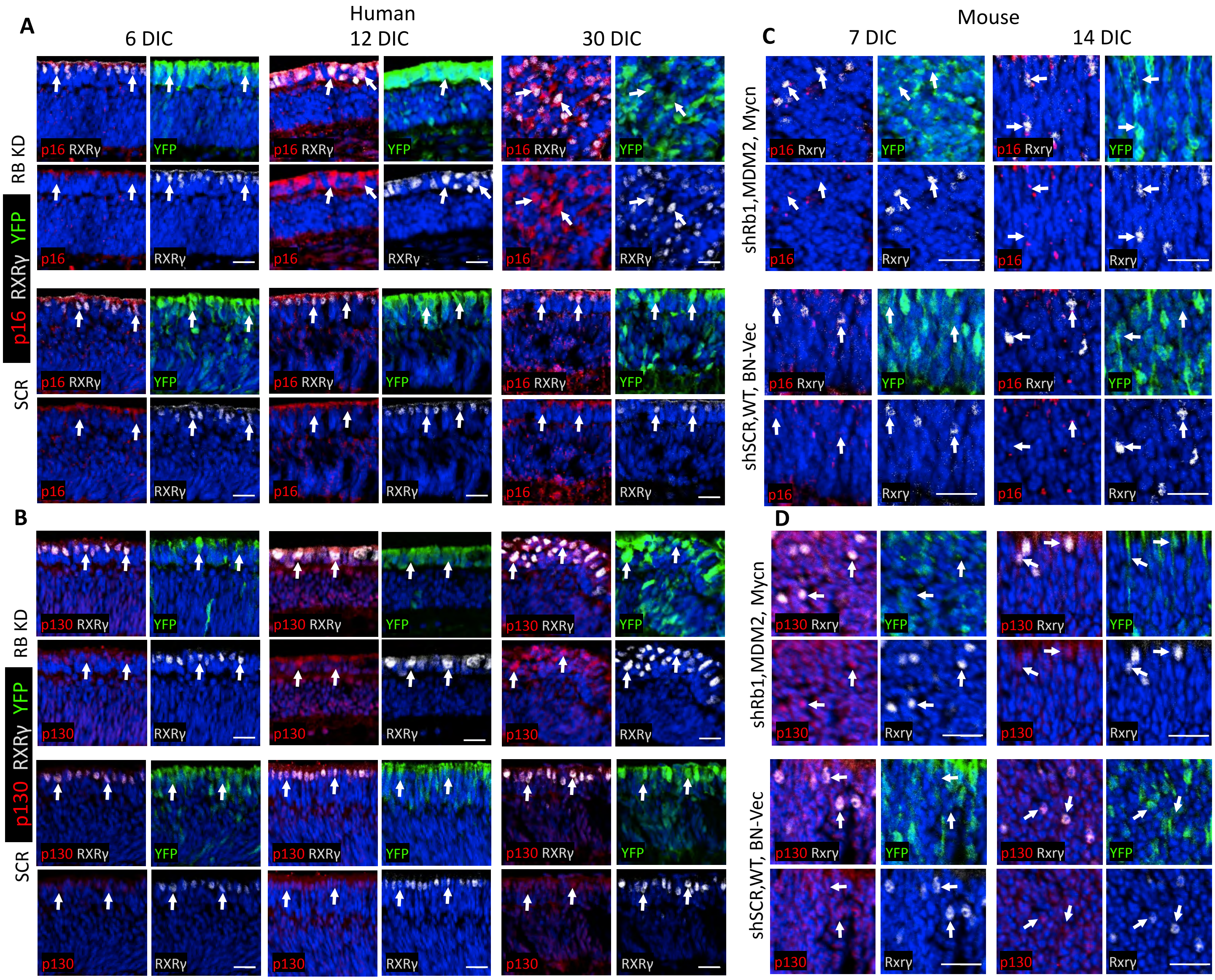

Figure S8. Cone precursor expression of the retinoma-related p16^{INK4A} and p130 in RB-depleted human but not mouse retinae. Immunofluorescence co-staining of p16 (A, C) and p130 (B, D) and co-staining of cone marker RXRγ and shRNA transdcution marker YFP in human retina at 6, 12, and 30 days post-transduction with sh*RB1* or sh*SCR* (A, B) and in P4 WT or *RGP-MDM2* mouse retinae at 7 and 14 days post-transduction with sh*SCR*, sh*Rb1*, BN vector, or BN-*Mycn*^{T58A} (C, D). Scale bars, 20 µm.

Table S1. Antibodies used in this study

Name	Clone	Species	Source	Catalog number	Titre
Actin	-	Goat	Santa Cruz	SC-1616	1:100
ARR3 (LUMIf-hCAR)	-	Rabbit	Cheryl Craft (5)		1:5000
ARR3	-	Rabbit	Millipore	AB15282	1:500
CCNB1	GNS1	Mouse	Santa Cruz	SC-245	1:100
GFP	-	Goat	Abcam	ab6673	1:500
Ki67	B56	Mouse	BD Biosciences	550609	1:200
L/M Opsin	-	Rabbit	Millipore	AB5405	1:500
MDM2	IF2	Mouse	Calbiochem	OP46	1:100
MDM2	SMP14	Mouse	Santa Cruz	SC-965	1:150
MYCN	NCM II-100	Mouse	Santa Cruz	SC-56729	1:100
p130	10/Rb2	Mouse	BD Biosciences	610261	1:100
p16	-	Rabbit	Proteintech (6)	10883-1-AP	1:100
PH3	-	Rabbit	Millipore	ab5176	1:200
RB	G3-245	Mouse	BD Biosciences	554136	1:100
RXRγ	-	Rabbit	Santa Cruz	sc-555	1:1000
RXRγ	G-6	Mouse	Santa Cruz	sc-514134	1:200

Supporting Information References

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