Supplementary Information

Pten coordinates retinal neurogenesis by regulating Notch signaling

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Contents

Supplemental Figure Legends

Figure S1. Split channel images of Pten and EGFP co-immunostaining results shown in Figure 1C. Scale bar in the picture represents 50μm.

Figure S2. Proximal expansion of *Pten*-deficient cells in *Pten-cko* retina. (A) Pten expression in P4 Pax6-aCre and Pten-cko littermate mouse retinas was examined by immunostaining with anti-Pten (red) with counterstaining of EGFP (green), which represents Pax6-aCre-positive cells. Pten was eliminated not only in *Pten-cko* distal retina (top row), but also in the proximal retina (bottom row). Asterisks (*) indicate Pten expressed in astrocytes or blood vessels. (B) The R26R-lacZ-positive cells were distributed in the entire P4 *Pten-cko* retina in contrast to the distally restricted lacZ expression in the *Pax6-aCre* retina, indicating that the *Pten*-deficient cells expanded to the proximal retinal area of *Pten-cko* mice. Scale bars in the pictures represent 50μm.

Figure S3. *Pten*-deficient retinal cells are resistant to apoptotic cell death. (A) TUNEL-positive apoptotic cells among cells expressing R26-lacZ, which encodes β-galactosidase gene at R26 locus, in P5 *Pax6-aCre;R26-lacZ* or *Pten-cko;R26-lacZ* retina was measured by co-staining with the anti-terminal deoxynucleotidyl transferase (TdT) antibody (red) marking apoptotic cells and anti-β-galactosidase antibody (green) detecting lacZ expression from R26R locus, respectively. Right column images are enlarged version of boxed areas in the left column. Arrows indicate TUNEL(+);R26-lacZ(-) cells, while arrowheads point TUNEL(+);R26-lacZ(+) cells. Dashed line indicates the retina-RPE border. (B) The TUNEL-positive apoptotic cells among the β-galactosidase-negative (TUNEL(+);lacZ(-)/lacZ(-); red in (A)) or the β-galactosidase-positive ((TUNEL(+);lacZ(+)/lacZ(+); yellow in (A)) were counted. The percentages of total TUNEL-positive cell counts among DAPI-stained total retinal cell counts were also shown in a graph (TUNEL(+)/Total). Scale bars in (A) represent 50μm. Error bars in (B) represent the standard deviations (SD). *, p-value <0.01, **, p-value <0.005.

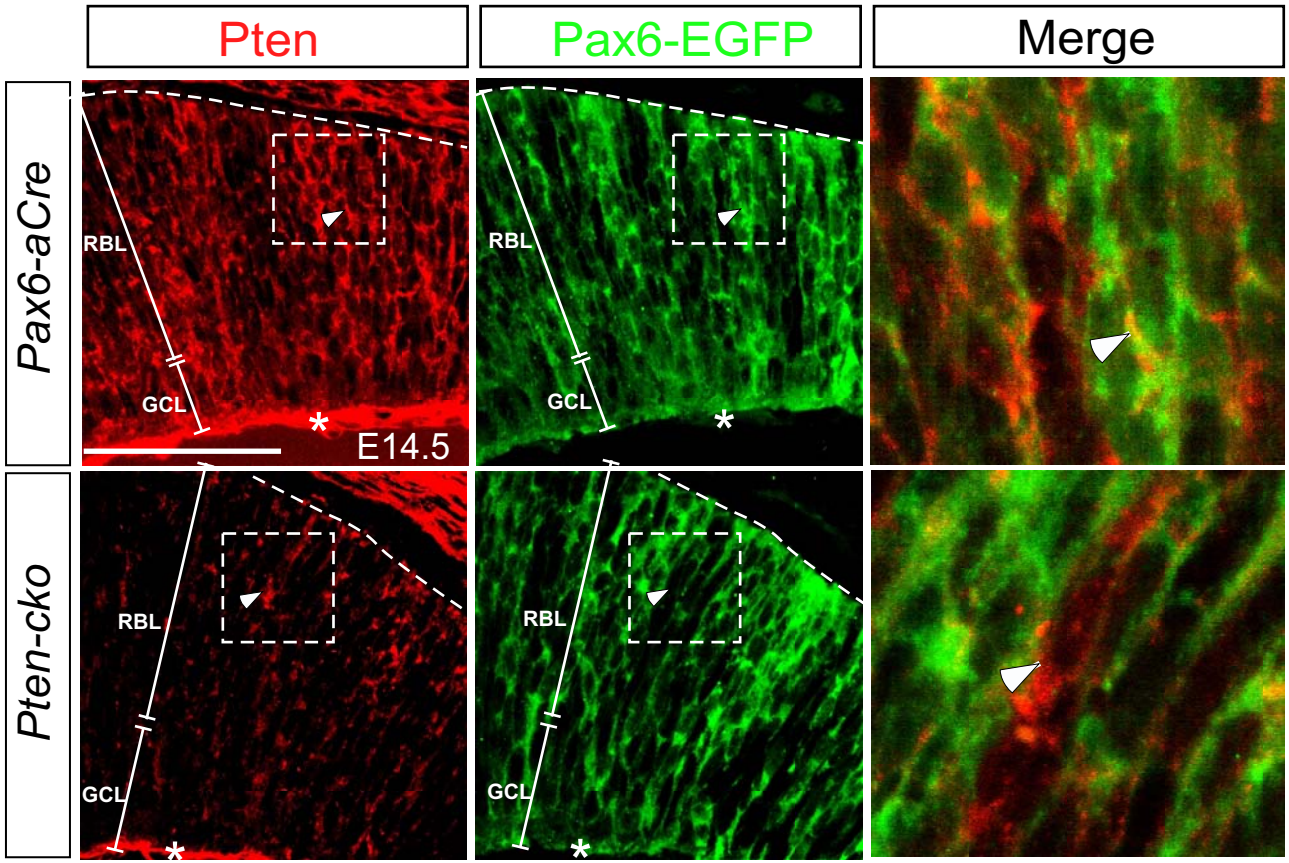
Figure S4. Enhanced mitotic index of Pten-cko retina. (A) The numbers of mitotic cells in P0 and P4 retinas were measured by immunostaining with anti-phospho-histone H3

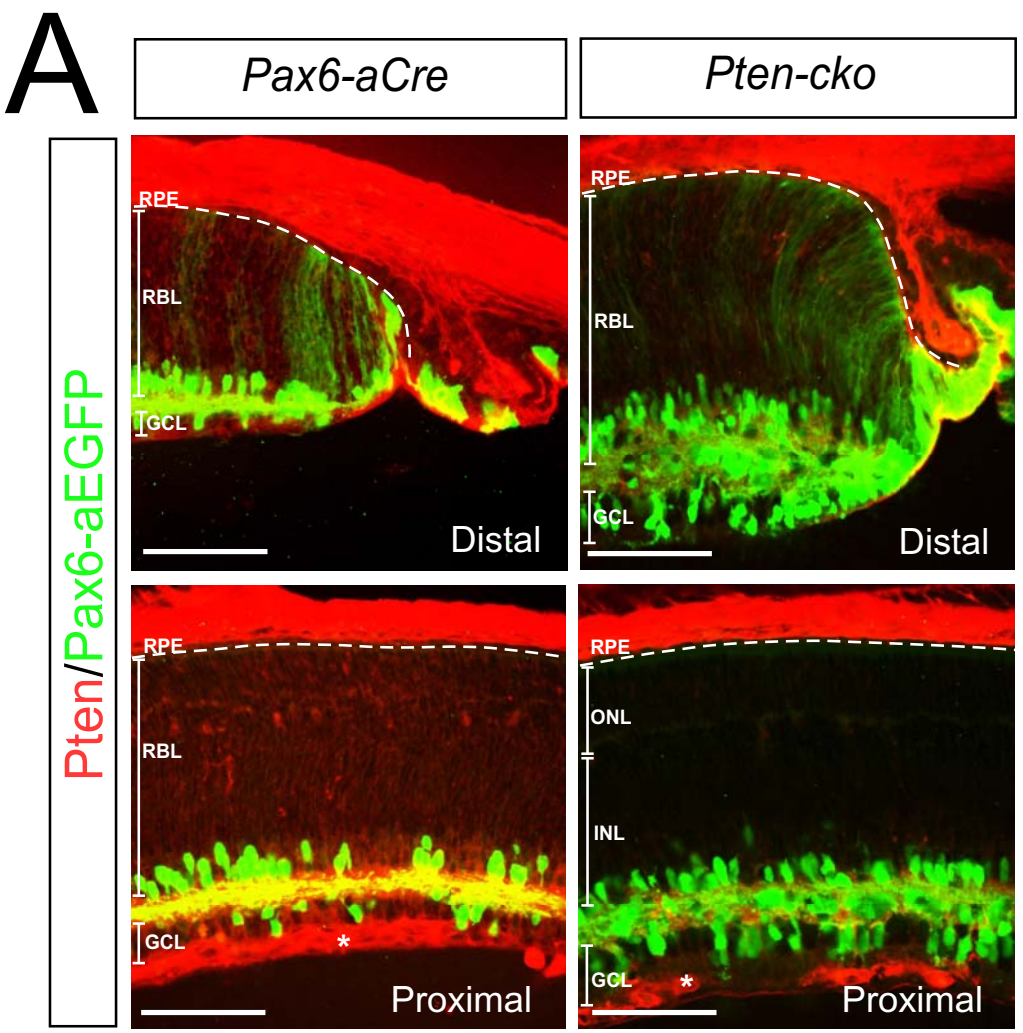
(pH3; red) antibody. Nuclei were counterstained with DAPI (blue). (B) The open squares and open triangles indicate cells in the distal and proximal areas of the *Pax6-aCre* retina, respectively, whereas the closed squares and solid triangles indicate cells in the distal and proximal areas of the *Pten-cko* retina. The open and closed arrows indicate the numbers of cells in *P6aCIEG* and *Pten-cko* retinas, respectively, at P0 and P4 stages. Y-axis values in graphs are the average numbers of pH3-positive cells per image; error bars denote SD of these values (n=4). Scale bars, 50 μm.

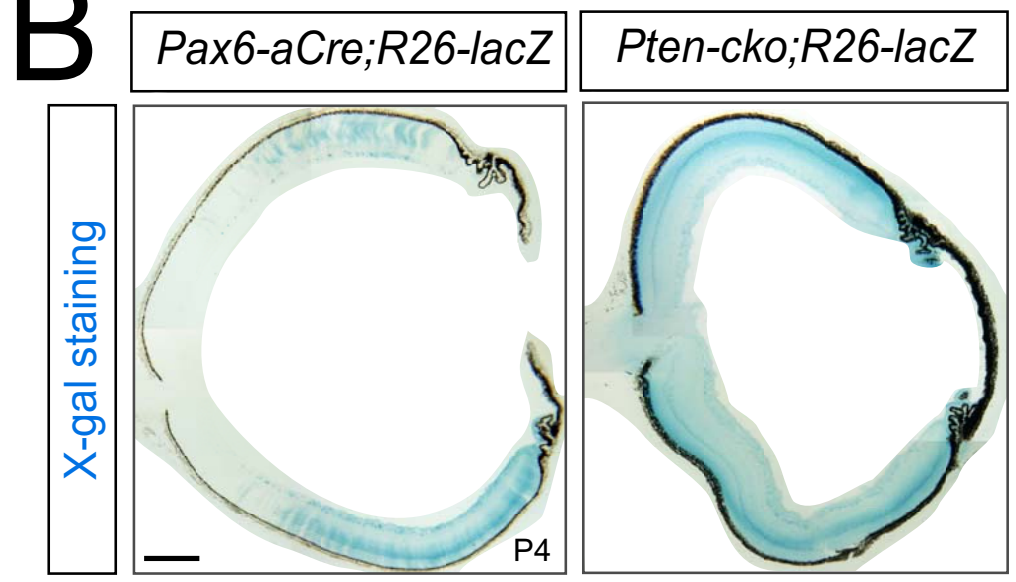
Figure S5. Accelerated cell cycle progression in *Pten-cko* retina. (A) To measure the speed of cell division, the pregnant *Pax6-aCre* or *Pten-cko* mice were injected with CldU (30mg/kg) at 13.5 dpc (days post coital), and kept for 16 hours prior to the injection with IdU (30mg/kg). The embryos were then obtained from the CldU/IdU-injected pregnant mice at 3 hours after the injection with IdU, and the sections (12 μm) were co-stained with rat anti-CldU (red) and rabbit anti-IdU (green) antibodies. Scale bar, 50μm. (B) Cells that are labeled by CldU, IdU, or both were counted, and shown as a graph. Open and filled bars indicate *Pax6-aCre* and *Pten-cko* results, respectively. (C) The cells labeled with both IdU and CldU were defined as cells that had reentered cell cycle during the period. The scores represent the average scores obtained from eight different samples of three independent litters. Error bars, SD. *, p-value <0.05, **, p-value <0.01.

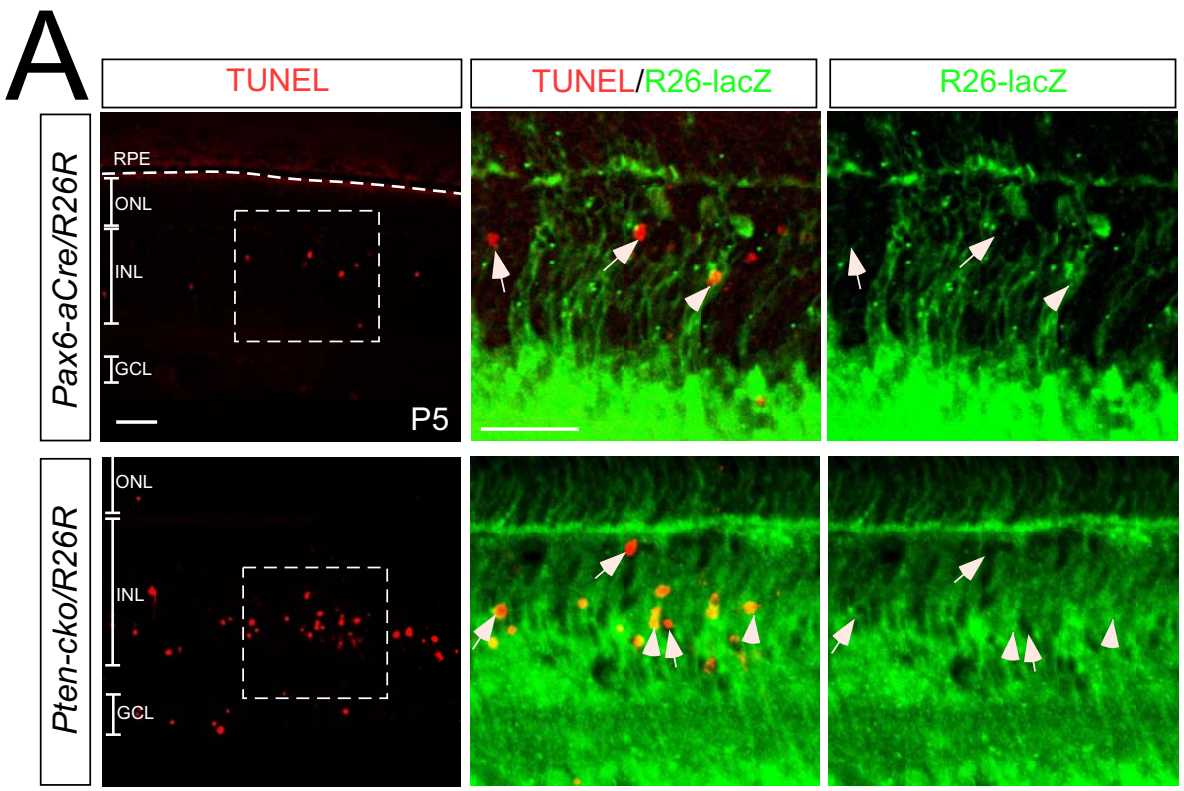
Figure S6. (A) Expression of the Notch signaling components, *Notch1 and Dll1,* in E14.5 or *Hes1* in E13.5 *Pax6-aCre* and *Pten-cko* retinas was examined by *in situ* RNA hybridization with DIG-labeled probes (see details in Materials and Methods). Dotted lines in (B) indicate basal margin of GCL.

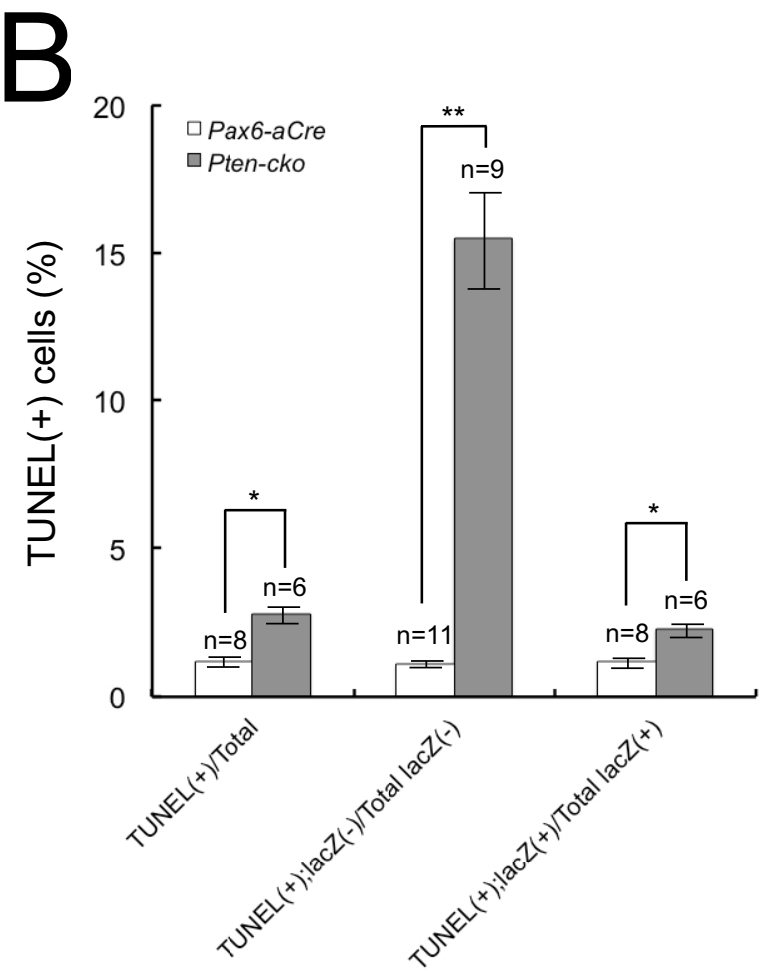
Figure S7. Akt inhibits Notch-dependent transcription. Rat retinal progenitor R28 cells were co-transfected with *Hes1*-luciferase reporter construct in combination with Flag-NICD, HA-Akt(CA), or HA-Akt(KM), and the Notch-induced expression of luciferase was examined by measuring the luciferase activity. n = 6; **, p-value <0.005; ***, p-value <0.001.

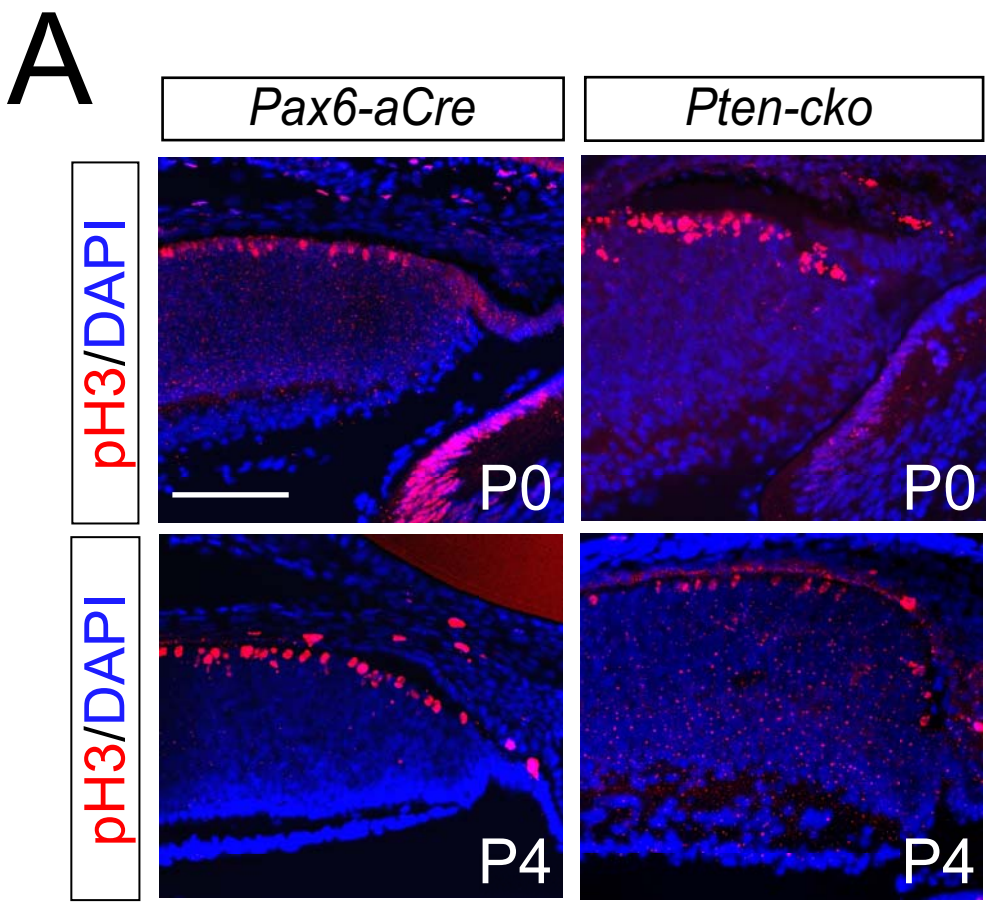

Pten
Pax6-EGFP
Merge
Pax6-aCre
Pten-cko
RBL
GCL
E14.5

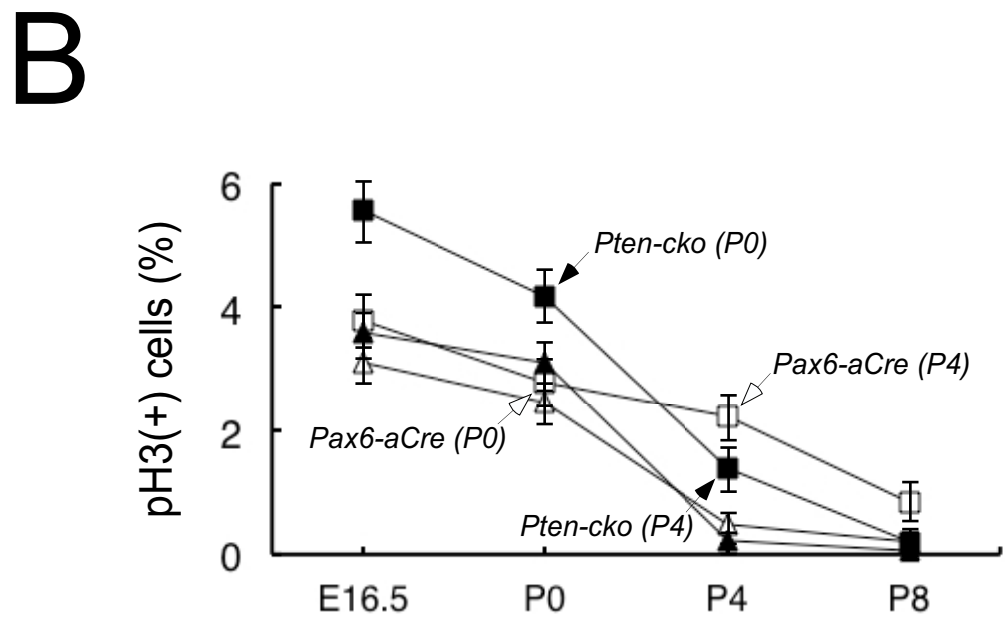

A
Pax6-aCre
Pten-cko
Pten/Pax6-aEGFP
RPE
RBL
GCL
Distal
RPE
RBL
GCL
Distal
RPE
RBL
GCL
*
Proximal
RPE
ONL
INL
GCL
*
Proximal

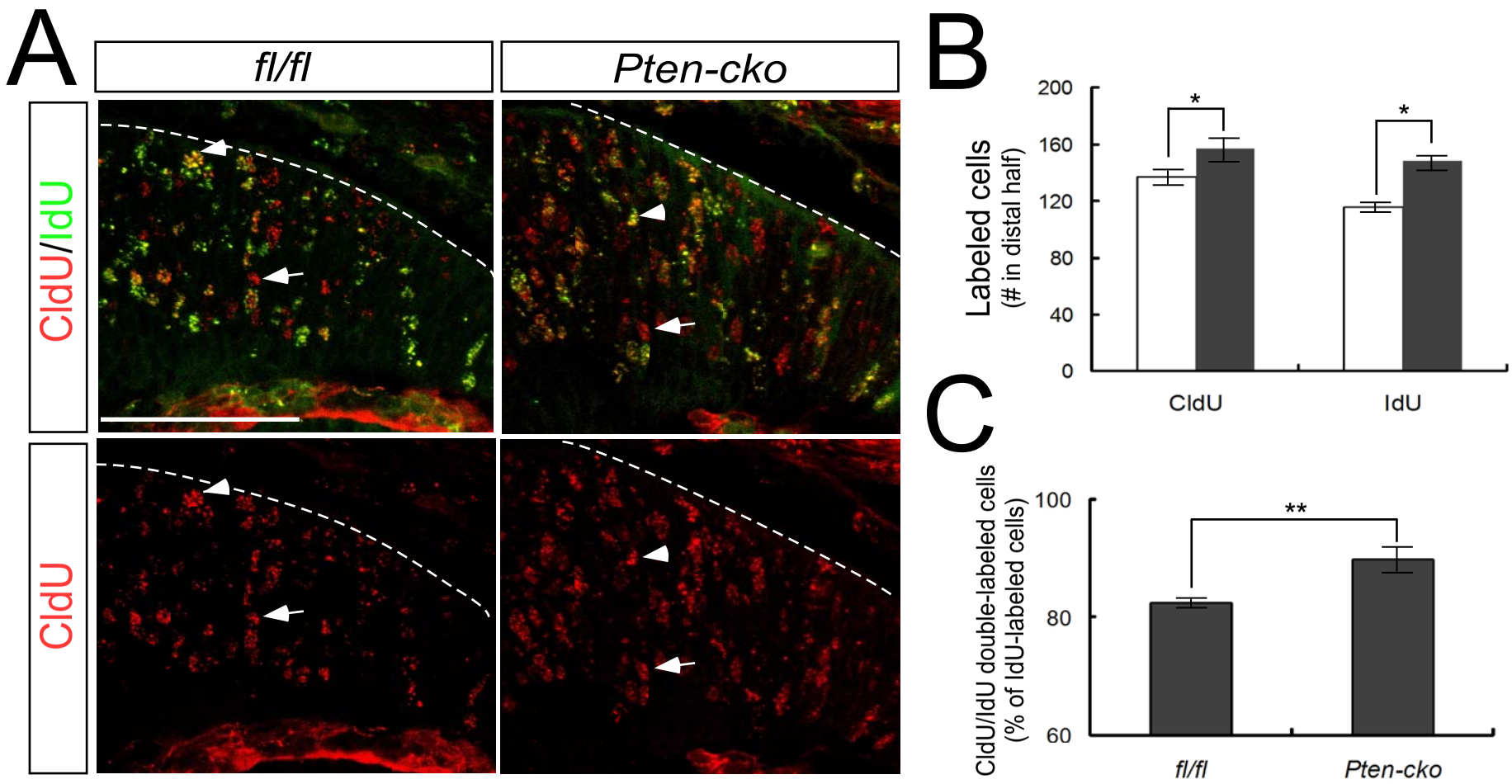

B
Pax6-aCre;R26-lacZ
Pten-cko;R26-lacZ
X-gal staining
P4

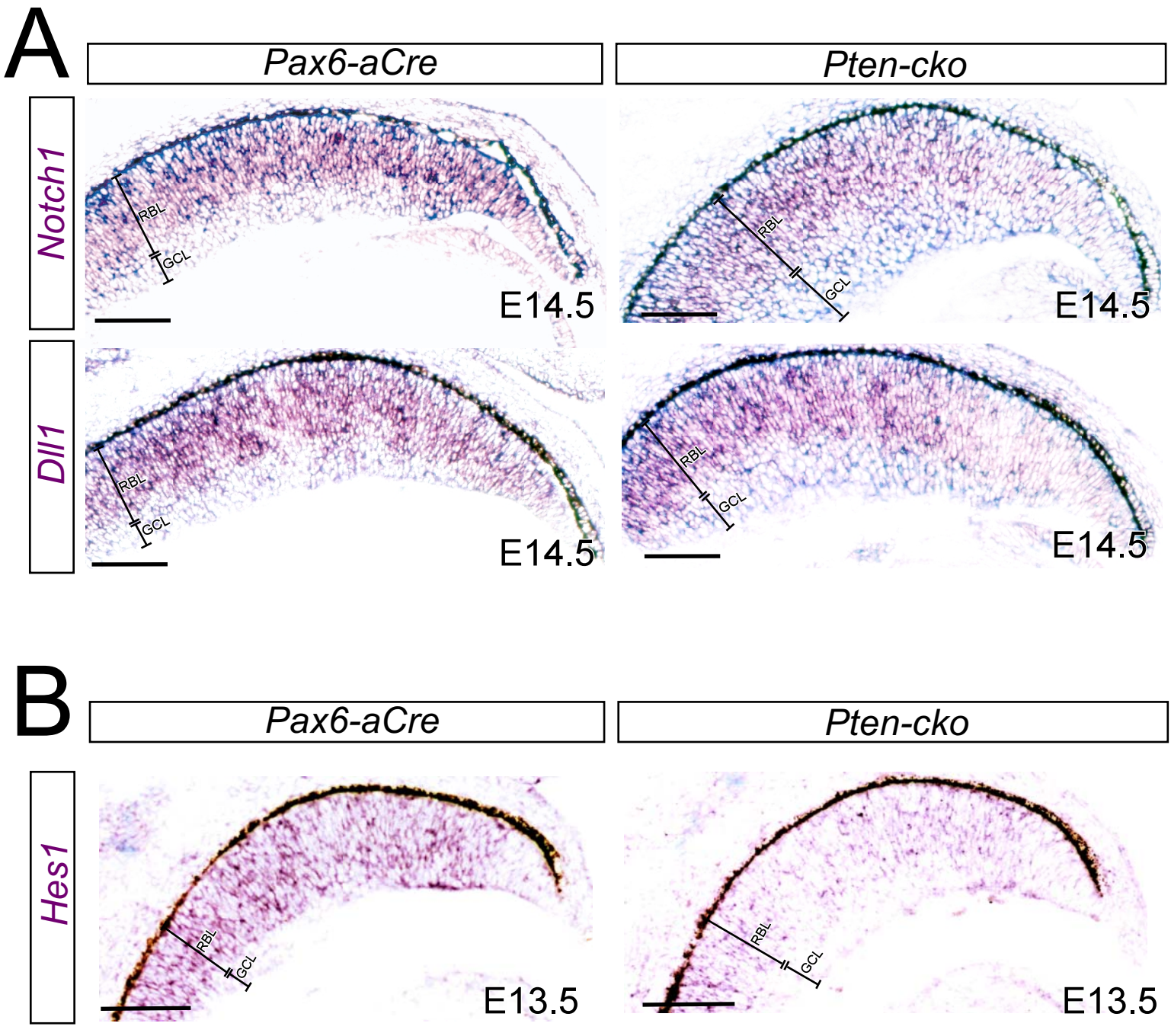

A
TUNEL
TUNEL/R26-lacZ
R26-lacZ
Pax6-aCre/R26R
Pten-cko/R26R
RPE
ONL
INL
GCL
P5


B
TUNEL(+) cells (%)
Pax6-aCre
Pten-cko
n=8
n=6
n=11
n=9
n=8
n=6
*
**
*
TUNEL(+)/Total
TUNEL(+);lacZ(-)/Total lacZ(-)
TUNEL(+);lacZ(+)/Total lacZ(+)


A
Pax6-aCre
Pten-cko
pH3/DAPI
pH3/DAPI
P0
P0
P4
P4


B
pH3(+) cells (%)
6
4
2
0
E16.5
P0
P4
P8
Pten-cko (P0)
Pax6-aCre (P4)
Pax6-aCre (P0)
Pten-cko (P4)


A
fl/fl
Pten-cko
CldU/IdU
CldU
B
Labeled cells
(# in distal half)
200
160
120
80
40
0
*
*
CldU
IdU
C
CldU/IdU double-labeled cells
(% of IdU-labeled cells)
100
80
60
**
fl/fl
Pten-cko


A
Pax6-aCre
Pten-cko
Notch1
Dll1
RBL
GCL
E14.5
E14.5
E14.5
E14.5
B
Pax6-aCre
Pten-cko
Hes1
RBL
GCL
E13.5
E13.5

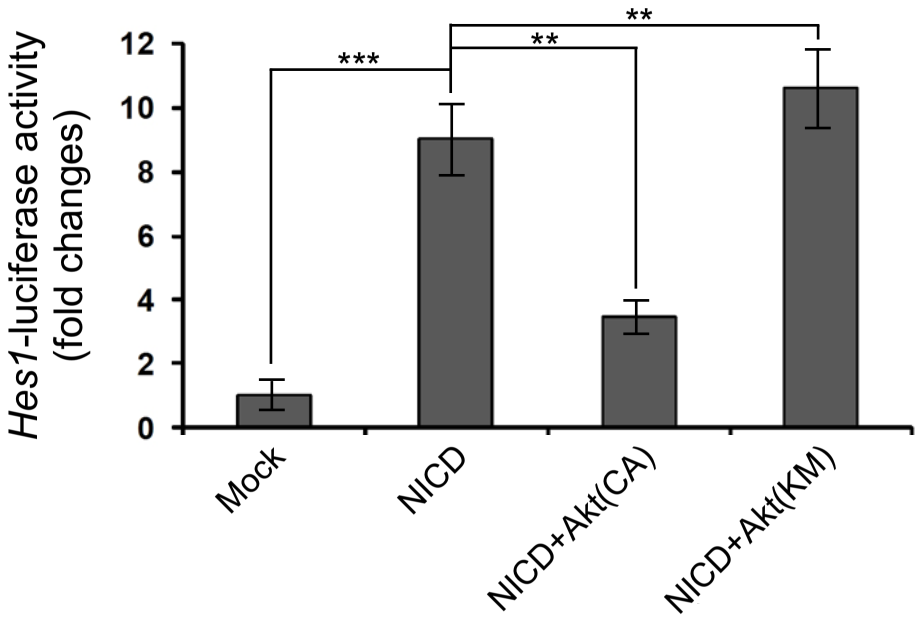

Hes1-luciferase activity
(fold changes)
12
10
8
6
4
2
0
***
**
**
Mock
NICD
NICD+Akt(CA)
NICD+Akt(KM)

Table S1. Summary of retinal development depending on Notch and Akt activity

Genotype	Notch activity	Akt activity	RPC population	Cell division speed	Retinal phenotype
Pax6-aCre	0	0	0	0	Normal development
Pten-cko	-	++	-	++	Precocious RPC depletion
Pax6-aCre;R26-NICD	++	+	+	-	Extended RPC maintenance
Pten-ckoR26-NICD	++	++	++	++	Retinal hyperplasia

Supplementary Table 2. List of antibodies used in this study

Antigen	Type	Target	Source
Akt (5G3)	Mouse monoclonal		Cell Signaling Technology
β-actin	Mouse monoclonal	Western blot control	Abchem
b-galactosidase (40-1a)	Mouse monoclonal		Dvelopmental Study Hybridoma Bank
b-galactosidase	Rabbit polyclonal		Cappel
G/R opsin	Rabbit monoclonal	Cone photoreceptors (cPR)	Chemicon (Milipore)
BrdU(BU1/75(ICR1))	Rat monoclonal	Proliferating cells (Used also for CldU staining)	Abcam
BrdU(clone B44)	Mouse monoclonal	Proliferating cells (Used also for IdU staining)	BD Pharmingen
Brn3b	Goat polyclonal	Retinal ganglion cell (RGC)	Santa Cruz Biotechnology
Calbindin D-28	Rabbit polyclonal	Horizontal cell (HZ); Amacrine cell (AC) subset	Chemicon (Milipore)
CBF1/RBPj-kappa	Goat polyclonal		R&D systems
Chx10	Guinea pig polyclonal	Retinal progenitor cell (RPC), Bipolar cell (BP)	Gift from Dr. Sam Pfaff (Salk Institute)
Crx	Rabbit polyclonal	Photoreceptors precursor	Santa Cruz Biotechnology
cyclin A1	Mouse monoclonal	S phase cell cycle marker	Santa Cruz Biotechnology
cyclin B1	Mouse monoclonal	G2/M phase cell cycle marker	Santa Cruz Biotechnology
EGFP	Mouse monoclonal		Invitrogen
EGFP	Rabbit polyclonal		Invitrogen
Flag	Rabbit polyclonal		Cell Signaling Technology
Flag(M2)	Mouse monoclonal		Stratagene
Glutamine synthetase (GS)	Rabbit polyclonal	Muller glia (MG)	Sigma
HA (HA-7)	Mouse monoclonal		Sigma
HA (Y-11)	Rabbit polyclonal		Santa Cruz Biotechnology
Hes1	Rabbit polyclonal	RPC	Gift from Dr. Ryoichiro Kageyama (Osaka Univ.)
Islet1	Guinea pig polyclonal	Post-mitotic retinal neurons	Gift from Dr. Sam Pfaff (Salk Institute)
Maml1	Rabbit polyclonal	Notch transcription complex	Cell Signaling Technology
Math5	Rabbit polyclonal	Post-mitotic retinal neurons	Chemicon (Milipore)
Myc	Rabbit polyclonal		Cell Signaling Technology
Myc(9E10)	Mouse monoclonal		Sigma
NeuroD1	Rabbit polyclonal	Post-mitotic retinal neurons	Chemicon (Milipore)
NICD	Rabbit polyclonal	gamma-secretase cleaved Notch1 in immunosta	Abcam
NICD	Rabbit polyclonal	gamma-secretase cleaved Notch1 in Western bl	Cell Signaling Technology
Notch1	Rabbit polyclonal	C-termius of Notch1	Cell Signaling Technology
p27	Mouse monoclonal	G1 phase cell cycle marker	BD
Pax6	Mouse monoclonal	RPC; AC; RGC	Dvelopmental Study Hybridoma Bank
phosphor-Akt(Thr308)	Rabbit polyclonal	Active Akt	Cell Signaling Technology
phosphor-Akt(Thr473) (587F11)	Mouse monoclonal	Active Akt	Cell Signaling Technology
phosphor-Akt(Thr473) (IHC-specific)	Rabbit polyclonal	Active Akt	Cell Signaling Technology
phosphor-histone H3	Rabbit polyclonal	Mitotic nuclei	Upstate
Protein kinase C alpha1	Mouse monoclonal	BP	Calbiochem
Prox1	Rabbit polyclonal	RPC; HZ; AC	Chemicon (Milipore)
PTEN	Rabbit polyclonal	PTEN	BD Pharmingen
PTEN	Mouse monoclonal	PTEN	R&D systems
Recoverin	Mouse monoclonal	Photoreceptors (rod/cone)	Chemicon (Milipore)
Rhodopsin	Mouse monoclonal	Rod photoreceptor (rPR)	Chemicon (Milipore)
Sox2	Goat polyclonal	RPC & AC subset	Santa Cruz Biotechnology
Sox9	Rabbit polyclonal	RPC (embryonic) & MG precursor & MG	Santa Cruz Biotechnology
Tuj1 (4H1)	Mouse monoclonal	Neuron-specific tubulin beta-3	BAbCo
Ubiquitin	Mouse monoclonal		Chemicon (Milipore)