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Supplemental Information

Genetic Activation of Hedgehog Signaling Unbalances the Rate of Neural Stem Cell Renewal by Increasing Symmetric Divisions

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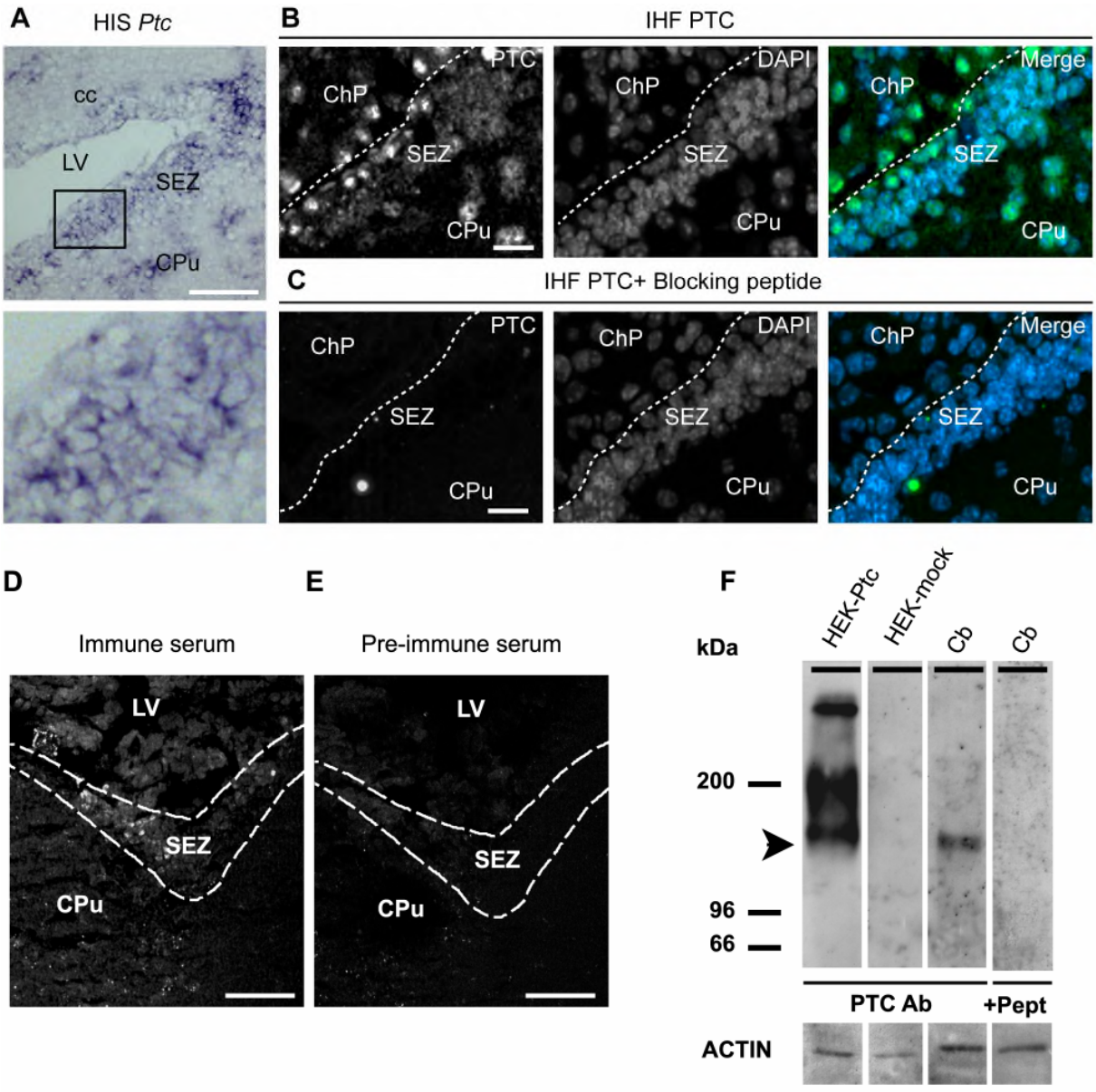
A
HIS Ptc
cc
LV
SEZ
CPu
B
IHF PTC
PTC
ChP
SEZ
CPu
DAPI
ChP
SEZ
CPu
Merge
ChP
SEZ
CPu
C
IHF PTC+ Blocking peptide
PTC
ChP
SEZ
CPu
DAPI
ChP
SEZ
CPu
Merge
ChP
SEZ
CPu
D
Immune serum
LV
SEZ
CPu
E
Pre-immune serum
LV
SEZ
CPu
F
HEK-Ptc
HEK-mock
Cb
Cb
kDa
200
96
66
PTC Ab
+Pept
ACTIN

Figure S1

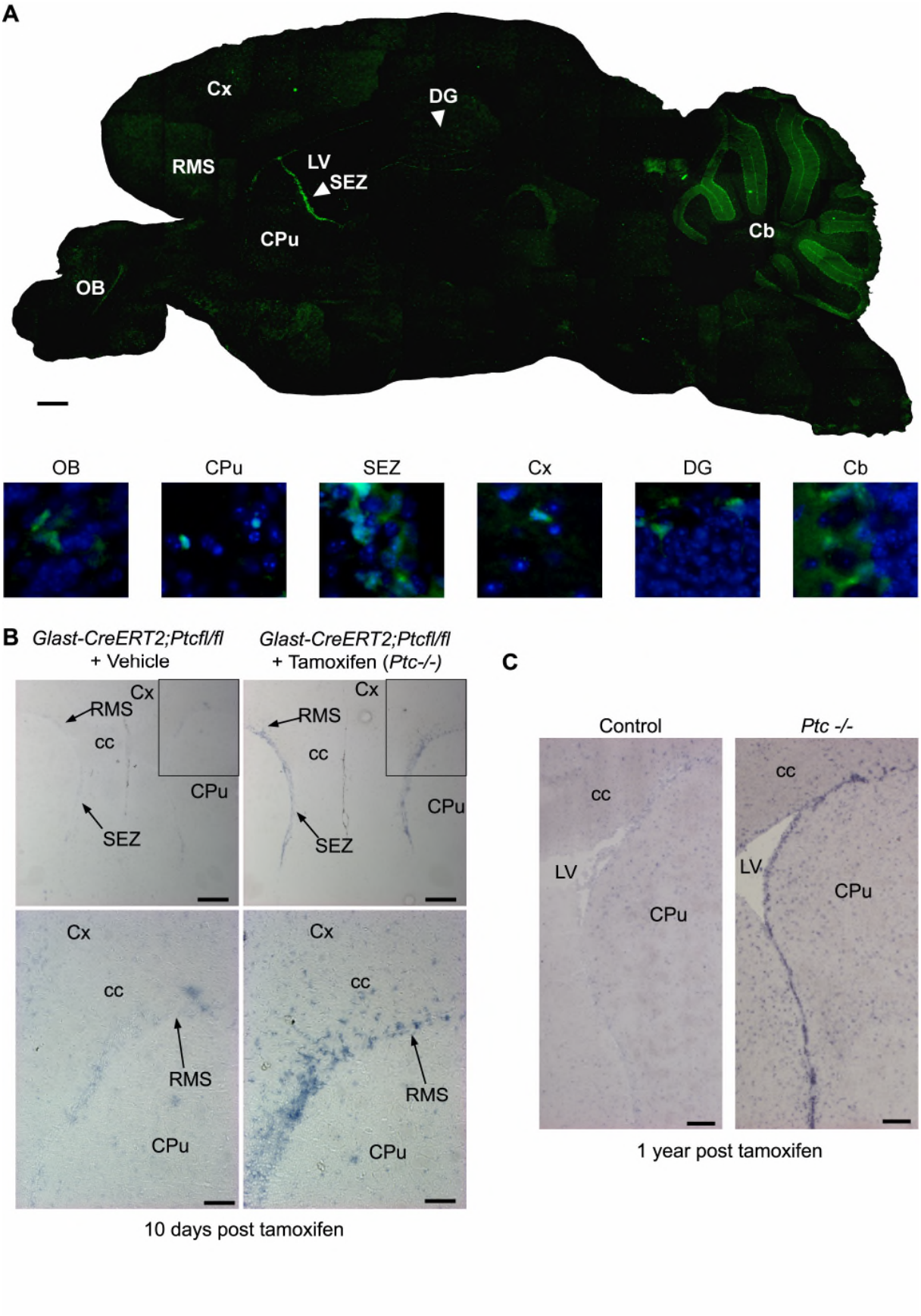
A
Cx
DG
RMS
LV
SEZ
CPu
Cb
OB
OB
CPu
SEZ
Cx
DG
Cb
B
Glast-CreERT2;Ptcfl/fl
+ Vehicle
Glast-CreERT2;Ptcfl/fl
+ Tamoxifen (Ptc-/-)
Cx
RMS
cc
CPu
SEZ
Cx
RMS
cc
CPu
SEZ
Cx
cc
RMS
CPu
Cx
cc
RMS
CPu
10 days post tamoxifen
C
Control
Ptc -/-
cc
LV
CPu
cc
LV
CPu
1 year post tamoxifen

Figure S2

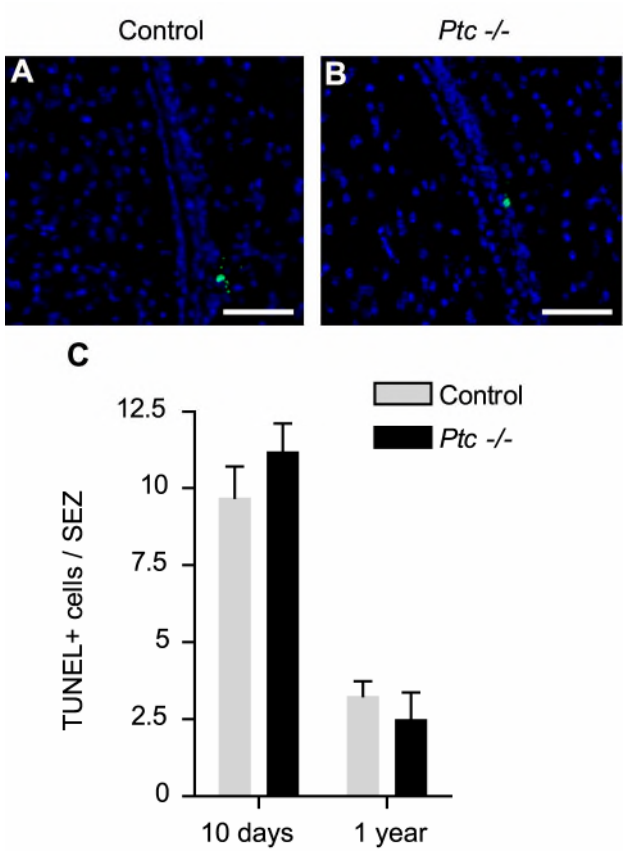
Control
Ptc -/-
A
B
C
Control
Ptc -/-
TUNEL+ cells / SEZ
12.5
10
7.5
5
2.5
0
10 days
1 year

Figure S3

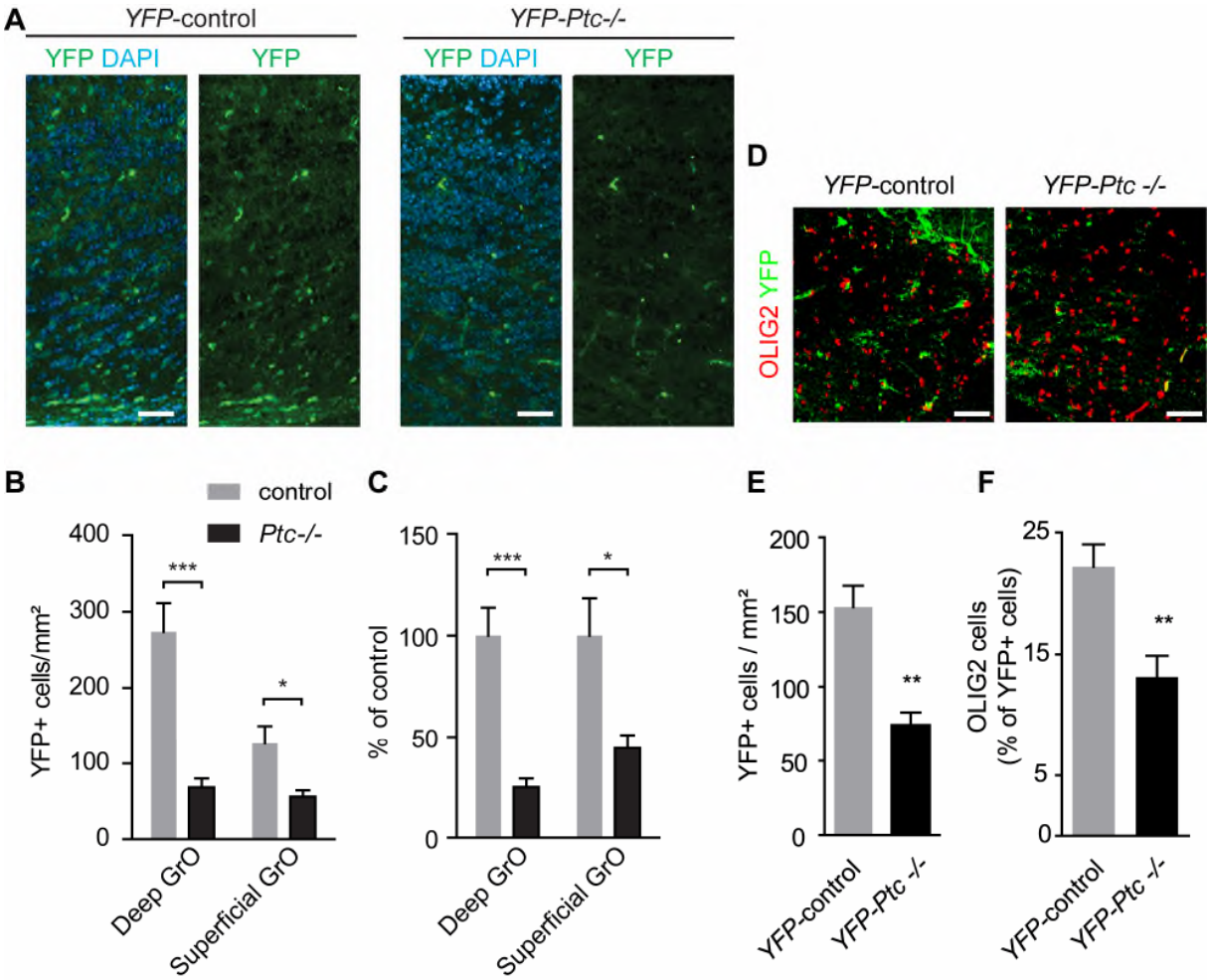
A
YFP-control
YFP-Ptc-/-
YFP DAPI
YFP
YFP DAPI
YFP
D
YFP-control
YFP-Ptc -/-
OLIG2 YFP
B
control
Ptc-/-
YFP+ cells/mm²
400
300
200
100
0
***
*
Deep GrO
Superficial GrO
C
% of control
150
100
50
0
***
*
Deep GrO
Superficial GrO
E
YFP+ cells / mm²
200
150
100
50
0
**
YFP-control
YFP-Ptc -/-
F
OLIG2 cells
(% of YFP+ cells)
25
15
5
0
**
YFP-control
YFP-Ptc -/-

Figure S4

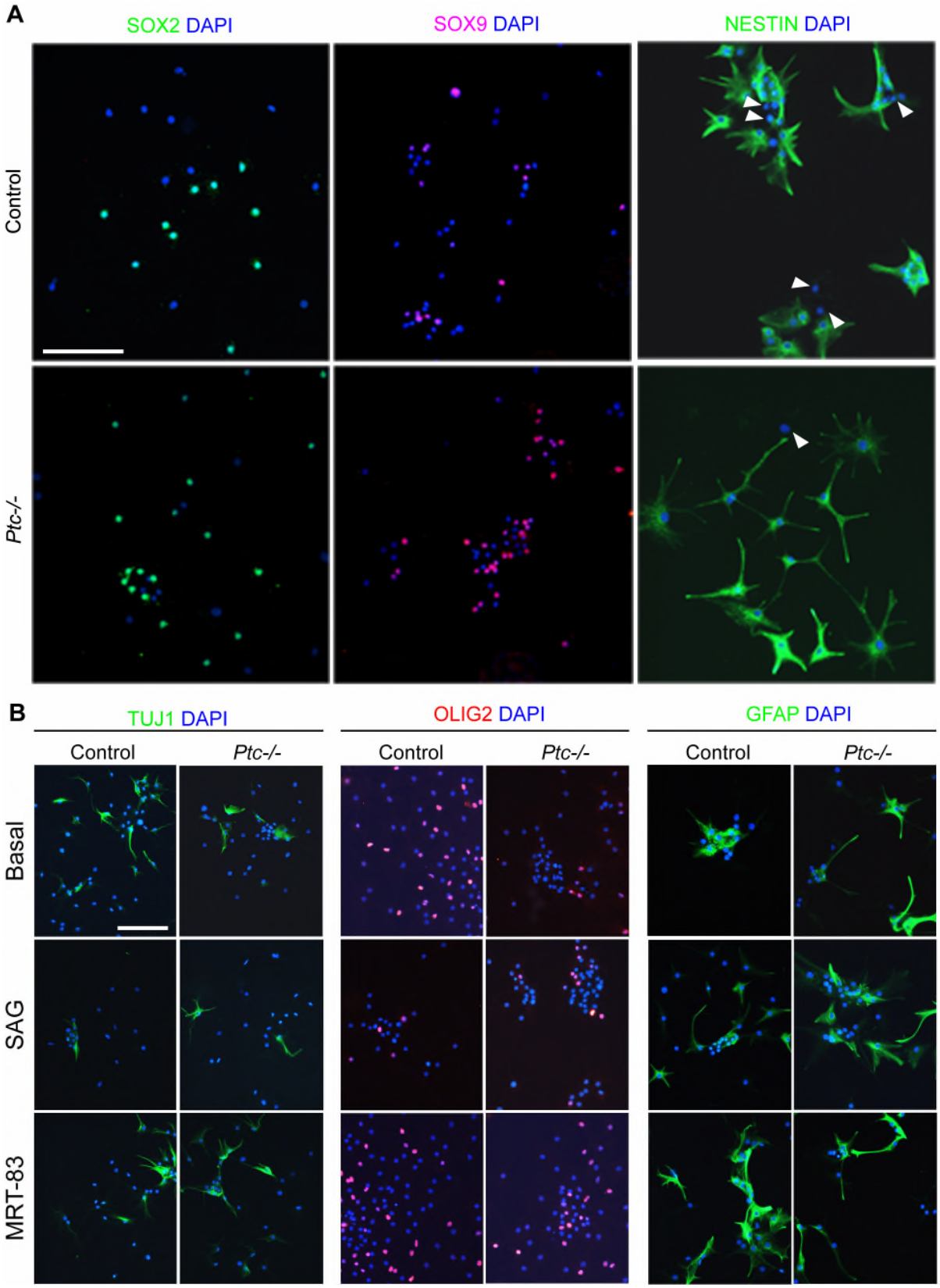
A
SOX2 DAPI
SOX9 DAPI
NESTIN DAPI
Control
Ptc-/-
B
TUJ1 DAPI
OLIG2 DAPI
GFAP DAPI
Control
Ptc-/-
Control
Ptc-/-
Control
Ptc-/-
Basal
SAG
MRT-83

Figure S5

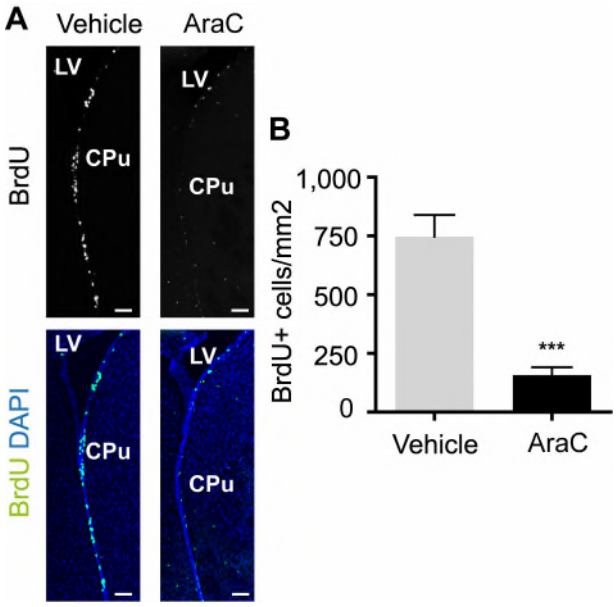
A
Vehicle
AraC
LV
CPu
BrdU
BrdU DAPI
B
BrdU+ cells/mm2
1,000
750
500
250
0
***
Vehicle
AraC

Figure S6

Figure S1. Expression of PTC in the adult SEZ, Related to Figure 1. (A), *In situ* hybridization of *Ptc* transcripts in the dorsal SEZ. The bottom panel is a magnification of the boxed area. (B,C), IHF staining performed at the level of the dorsal SEZ using the polyclonal Ab130 PTC antiserum in the absence (B) or in the presence (C) of the fusion protein used for antibody production. Signals associated to PTC^+ cells are observed in the SEZ, but are absent in preadsorption experiments. **(**D,E). IHF staining performed at the level of the SEZ using the polyclonal Ab130 PTC antiserum identified signals associated to PTC^+ cells that were not evidenced when the experiments were performed with the corresponding pre-immune serum. (F), Immunoblot analysis of whole cell lysates (4 µg of proteins) from HEK293 cells transiently transfected with an empty vector (mock) or with a vector containing the full length DNA encoding mouse PTC was performed by SDS-PAGE using the PTC antiserum Ab130. The arrowhead indicates a major band of 140 kDa detected in HEK-PTC but not in HEK-mock. A second signal of 200 kDa is also detected in HEK-PTC and might reflect post-translational modifications. P5 cerebellum (Cb) proteins (30 µg) were analyzed using the same PTC antiserum leading to a signal of 140 kDa which is blocked in preadsorption experiments. Abbreviations: ChP, choroid plexus; CPu, caudate putamen. Scale bars (µm): 100 (A, D, E); 25 (B, C).

Figure S2. Characterization of Cre-mediated recombination in the newly generated transgenic mouse lines, Related to Figure 1. (A) *YFP*-control animals received tamoxifen for 5 days and were analyzed 2 months after induction. Successful recombination was monitored by direct visualization of YFP fluorescence. Bottom panels are magnifications of the indicated region. (B) ISH performed with the *Gli1* riboprobe on slices derived from *Glast-CreERT2/*$Ptc^{fl/fl}$ animals treated by vehicle (left) or tamoxifen (right; $Ptc^{-/-}$) 10 days before analysis. Induction of the target gene *Gli1* is detected upon tamoxifen but not vehicle

treatment. The boxed areas are magnified in the corresponding bottom panels. (C) *Gli1* up-regulation is still strongly detected in slices derived from *Ptc*$^{-/-}$ and control mice treated with tamoxifen 1 year before. Abbreviations: Cb, cerebellum; cc, corpus callosum; CPu, caudate putamen; Cx, cerebral cortex; DG, dentate gyrus; LV, lateral ventricle; OB, olfactory bulb; RMS, rostral migratory stream; SEZ, subependymal zone. Scale bars (µm); 500 (A), 400 (B, top), 200 (C), 100 (B, bottom).

Figure S3. Conditional *Ptc* deletion in GLAST^{+} cells of the SEZ does not modify cell apoptosis, Related to Figure 2. Detection of TUNEL^{+} nuclei in the SEZ of control (A) or *Ptc*$^{-/-}$ (B) animals and quantification (C) of these nuclei 10 days and 1 year after tamoxifen injection. Data are mean ± SEM from 3-4 animals. Scale bars: 100µm.

Figure S4. Recombination in OB granular cells and oligodendroglial lineage, Related to Figure 3. (A) Visualization of the YFP reporter in the superficial (top) and deep (bottom) granular layers of the OB in *YFP*-control and *YFP-Ptc*$^{-/-}$ mice. (B,C) Histograms showing the quantification of YFP^{+} cells expressed as cell number / mm^{2} (B) and as percentage of YFP^{+} cells quantified in *YFP*-control animals (total number of counted cells, n=223 for *YFP*-control, n=73 for *YFP-Ptc*$^{-/-}$). (D) OLIG2 IHF performed at the level of the corpus callosum (cc) of *YFP*-control and *YFP-Ptc*$^{-/-}$ mice. YFP-expressing cells likely correspond to both endogenous astrocytes expressing GLAST and the progeny of GLAST^{+} NSC born in the SEZ which have migrated and undergone differentiation into cells of the oligodendroglial lineage. (E,F), Histograms showing the quantification of the density of YFP^{+} cells (E) and the percentage of YFP^{+} cells which co-express OLIG2 in *YFP*-control and *YFP-Ptc*$^{-/-}$ cc (total number of counted cells, n= 187 for *YFP*-control, n= 74 for *YFP-Ptc*$^{-/-}$). Values are the mean ± SEM from 3-4 animals. *, p≤0.05, **, p≤0.01, ***, p≤0.005 Scale bars : 50 µm.

Figure S5. Visualization of SEZ-derived cell cultures from control and *Ptc*$^{-/-}$ mice, Related to Figure 4. (A) IHF using SOX2, SOX9 and NESTIN antibodies are performed on control (top) and *Ptc*$^{-/-}$ (bottom) precursor cell cultures maintained in the absence of growth factors for 12 h before the experiment. Nuclei are labeled with DAPI (blue). (B) IHF using TUJ1, OLIG2 and GFAP antibodies are performed on control (left) or *Ptc*$^{-/-}$ (right) precursor cell cultures maintained in the absence of growth factors for 7 days before the experiment in the presence or in the absence of the HH agonist SAG (0.3 µM) and HH antagonist MRT-83 (3 µM). Nuclei are labeled with DAPI (blue). Scale bars: 100µm.

Figure S6. Depletion of BrdU-incorporating cells in the SEZ of wild-type mice, Related to Figure 6. (A), Coronal views of the SEZ showing BrdU-incorporating cells (top) together with DAPI^{+} nuclei (bottom) at the end of AraC or vehicle infusion (t=0) in wild–type mice. (B), Quantification of BrdU^{+} cells indicates a marked reduction in cell proliferation. Values are the mean ± SEM from 3 animals. ***, $p \leq 0.0001$. Scale bars: 200µm.

Supplemental experimental procedures

Histology and immunostaining. Animals were or not perfused by 4% paraformaldehyde and tissue sections were sectioned using a cryostat. Antibodies were as follows: BrdU, GFAP, NEUN, PSA-NCAM, β-GAL as described in (Angot et al., 2008), goat C-FOS (1/300; Santa Cruz), mouse KI67 (1/300; BD Pharmingen), guinea pig GLAST (1/100; Millipore), mouse NESTIN (1/500; Millipore), rabbit OLIG2 (1/1000; Millipore), rabbit EGFR (1/400; Millipore), rabbit SOX2 (1/400; Millipore), goat SOX9 (1/400; R&D Systems), mouse TUJ1 (1/500; Abcam), rabbit calbindin (1/500, Swant). The secondary antibodies were goat anti-mouse Alexa 546 (Molecular Probes), goat anti-rabbit Alexa 555 (Life Technologies), donkey anti-goat Alexa 546 (Life Technologies), goat anti-mouse FITC (Sigma), anti-guinea pig

DyLight 488 (Jackson immunoresearch), goat anti-rat Alexa 488 (Jackson Immunoresearch), biotinylated goat anti-rat (Vector Laboratories), donkey anti-mouse, -goat or -rabbit DyLight 649 conjugated antibodies (Jackson Immunoresearch). Cell nuclei were visualized using DAPI (Vector). TUNEL analysis was performed by using Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) experiments (ApoptagR Peroxidase In situ Apoptosis Detection Kit; Millipore) according to supplier's protocol. For hematoxylin and eosin staining, hematoxilin (Vector) and eosin (Polysciences) were applied.

***In situ* hybridization.** ISH protocol and synthesis of *Ptc*, *Gli1* and *Gfap* specific riboprobes were as previously described (Traiffort et al., 1999). *Ptc del* riboprobe corresponds to mouse *Ptc* nucleotides 1025-1305 (Genbank accession NM_008957.2). The *vGlut2* probe was a kind gift from Dr O. Kiehn (Borgius et al., 2010).

Neurosphere cultures. The telencephalic SEZ was dissected from adult wild-type, $Ptc^{-/-}$ or control animals (Pastrana et al., 2011). SEZ fragments were digested in papain (Sigma) and trypsin (Invitrogen) solution for 20 min at 37°C and mechanically dissociated by passing through a 26-gauge needle. If not stated otherwise, single cells were seeded at 5000 cells per well in 24-well plates in serum-free media consisting of DMEM supplemented with B27, penicillin/streptomycin, sodium pyruvate and in the presence of 20 ng/ml EGF and 10 ng/ml basic fibroblast growth factor (FGF, Invitrogen). After 6–9 d in cultures, spheres were collected, incubated in 0.05% Trypsin (Invitrogen) for 30–60 min at 37°C, mechanically dissociated and reseeded as single cells as described above. If not indicated otherwise, the cells were derived from animals treated 2 months earlier with tamoxifen and the experiments involving single cells or neurospheres (either treated with drugs or untreated) were performed after one or two passages or on primary spheres.

For the multipotency experiments, 5-10 spheres were cultured on poly-D-lysine (Sigma) coated coverslips in the above mentioned medium devoid of growth factors for 7 days. For the immunofluorescence studies, cells were fixed in 4% PFA at 4°C for 30 min, before blocking for 30 min at room temperature in 0.5% BSA in PBS. The fixed cells were then incubated with the primary antibodies at room temperature for 90 min, followed by incubation with secondary antibodies for 60 min and mounting of the coverslips.

For the paired cell assay, single cells were plated directly after SVZ microdissection on coated coverslips at a cell density of about 50,000 cells per well. The cultures were maintained for 24-36 h in the presence of 10 ng/ml of EGF. IHF analyses were performed as described above. About 60 pairs were evaluated in each mutant and wild type sample.

The labeling index allowing the evaluation of the cell cycle length corresponds to the percentage of $KI67^{+}$ precursors that were labeled by a single pulse of BrdU. The dissociated spheres were cultured on poly-d-lysine coated coverslips in the presence of growth factors, treated with 10 µM BrdU for the indicated time frames and processed further for anti-BrdU and KI67 IHF.

Drugs. SAG and MRT-83 were synthesized as described (Roudaut et al., 2011).

Semi-quantitative RT-PCR. Total RNA was isolated using the Trizol kit (Invitrogen) from sphere cultures derived from animals treated with tamoxifen 2 months before. RNA was reverse transcribed in 20 µl reactions with Superscript II reverse transcriptase (Invitrogen). PCR reactions were performed using Go Taq polymerase (Promega). *Actin* was used to assess input RNA levels. The sequences of the primers which were used are as follows:

Notch1, Fwd: 5'CCAGCATGGCCAGCTCTGG3'

Rev: 5'CATCCAGATCTGTGGCCCTGTT3'

Hes1, Fwd: 5'AAAGACGGCCTCTGAGCACA3'

Rev: 5'TCATGGCGTTGATCTGGGTCA3'

Hes5, Fwd: 5'AAGTACCGTGGCGGTGGAGATGC3'

Rev: 5'CGCTGGAAGTGGTAAAGCAGCTT3'

Gli1, Fwd: 5'TTCGTGTGCCATTGGGGAGG3'

Rev: 5'CTTGGGCTCCACTGTGGAGA3'

Western blot analysis

Cerebellum from P5 OF1 mice (Charles Rivers, Saint-Germain sur l'Arbresle, France) were homogenized. Proteins derived from the pellet obtained after a 20 min centrifugation at 9000 g were electrophoresed on 8% polyacrylamide gels as described previously (Coulombe et al., 2004). Immunoreactivity was detected using the polyclonal antiserum (130Ab, 1:1000; Bidet et al., 2011) to the mouse PTC. Blocking experiments were performed by overnight preincubation of the antiserum with the recombinant mouse glutathione-S-transferase (GST)–PTC purified fusion protein (15 µg/ml).

Quantification and statistical analysis. Immunofluorescent signals were detected by a conventional epifluorescence microscope (DMRXA2; Leica Microsystems) equipped with a CDD camera (Photometrics) or scanned for Z stacks colocalizations by an Apotome micoscope (ZEISS) and AxioImager software. Optical sections were set as 1 µm intervals. Cells displaying an immunoreactive or ISH signal and areas were quantified using ImageJ software. Cell counts are presented as an average of several SEZ or olfactory bulb slices from at least three different mice per group. Statistical analysis was performed using the unpaired bilateral Student's t test. Statistical significance was considered for $p<0.05$.

Supplemental references

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