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Supplemental Information

Notch-Dependent Pituitary SOX2^{+} Stem Cells Exhibit a Timed Functional Extinction in Regulation of the Postnatal Gland

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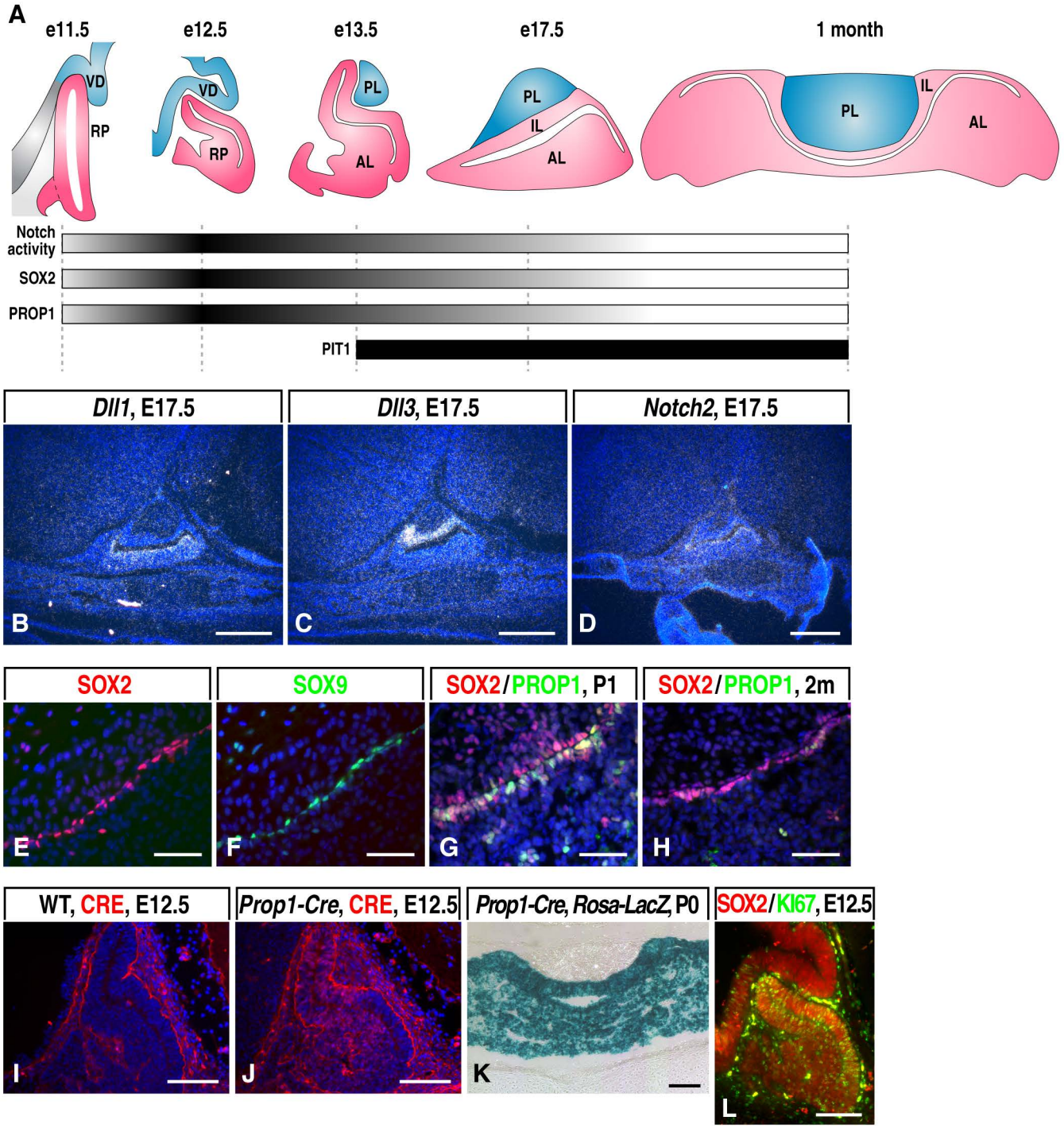
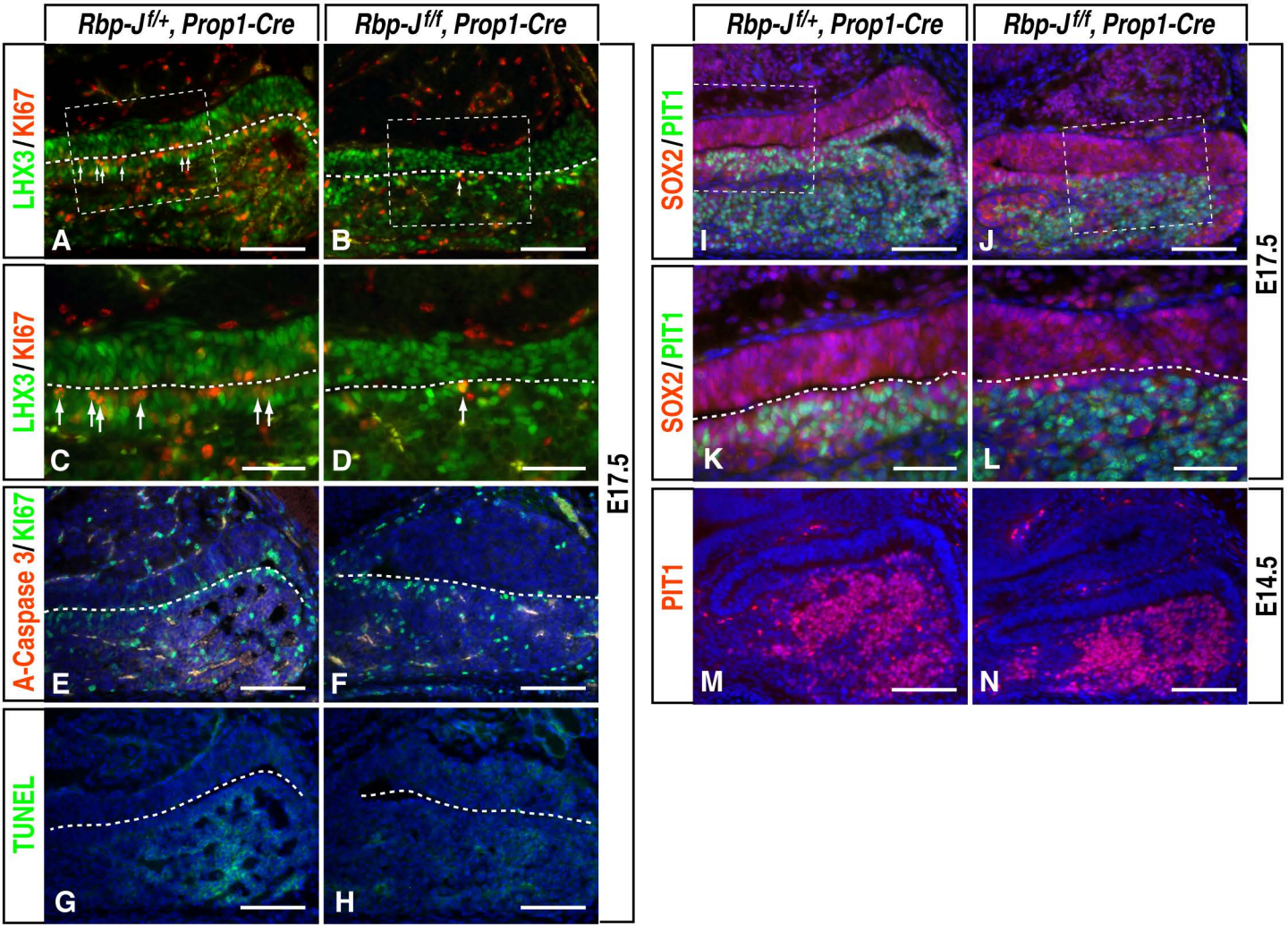
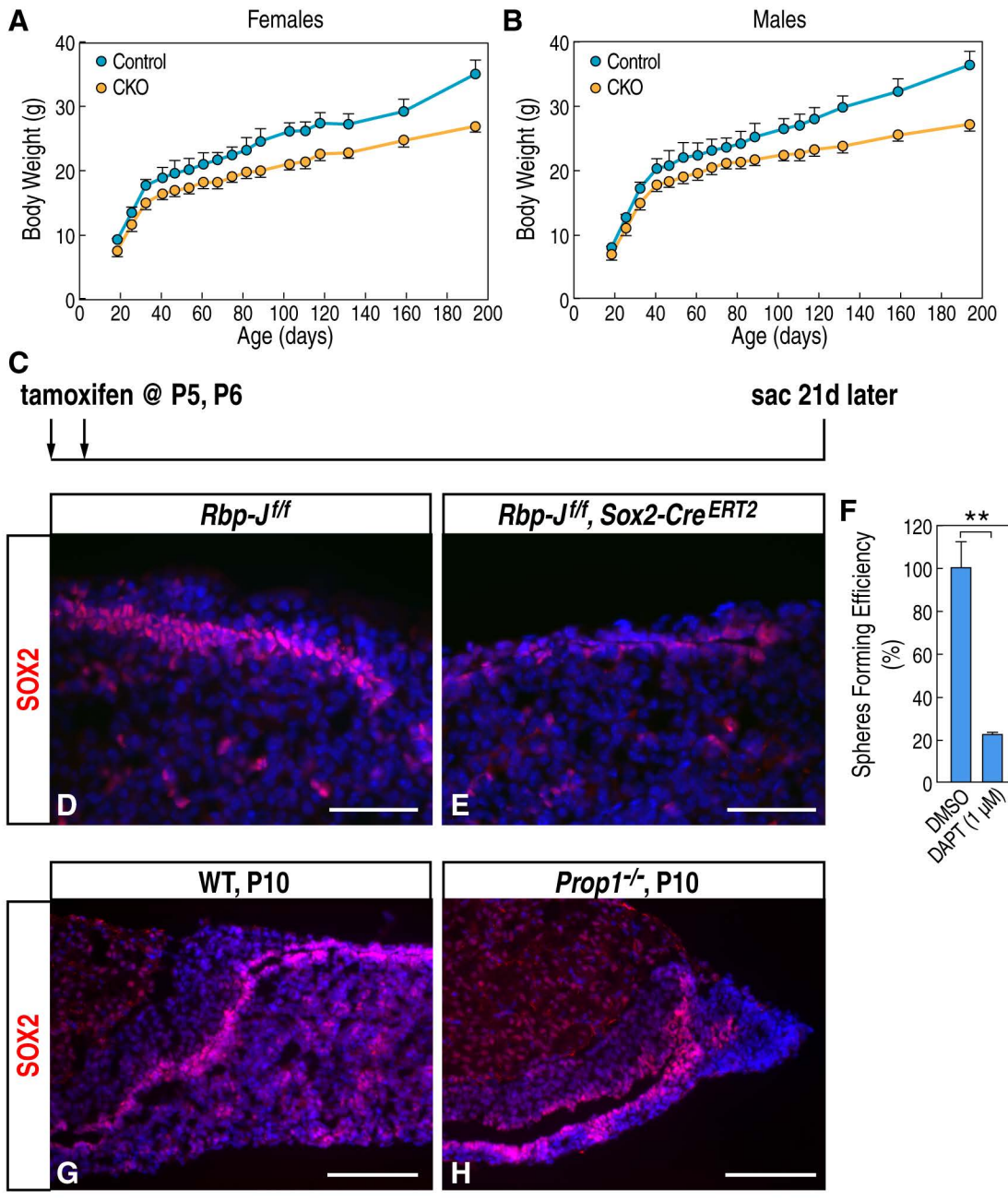
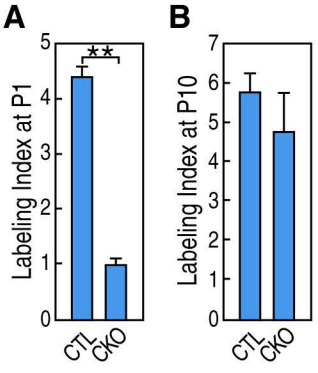

A
e11.5
e12.5
e13.5
e17.5
1 month
VD
RP
VD
RP
PL
AL
PL
IL
AL
PL
IL
AL
Notch activity
SOX2
PROP1
PIT1
Dll1, E17.5
Dll3, E17.5
Notch2, E17.5
B
C
D
SOX2
SOX9
SOX2/PROP1, P1
SOX2/PROP1, 2m
E
F
G
H
WT, CRE, E12.5
Prop1-Cre, CRE, E12.5
Prop1-Cre, Rosa-LacZ, P0
SOX2/KI67, E12.5
I
J
K
L

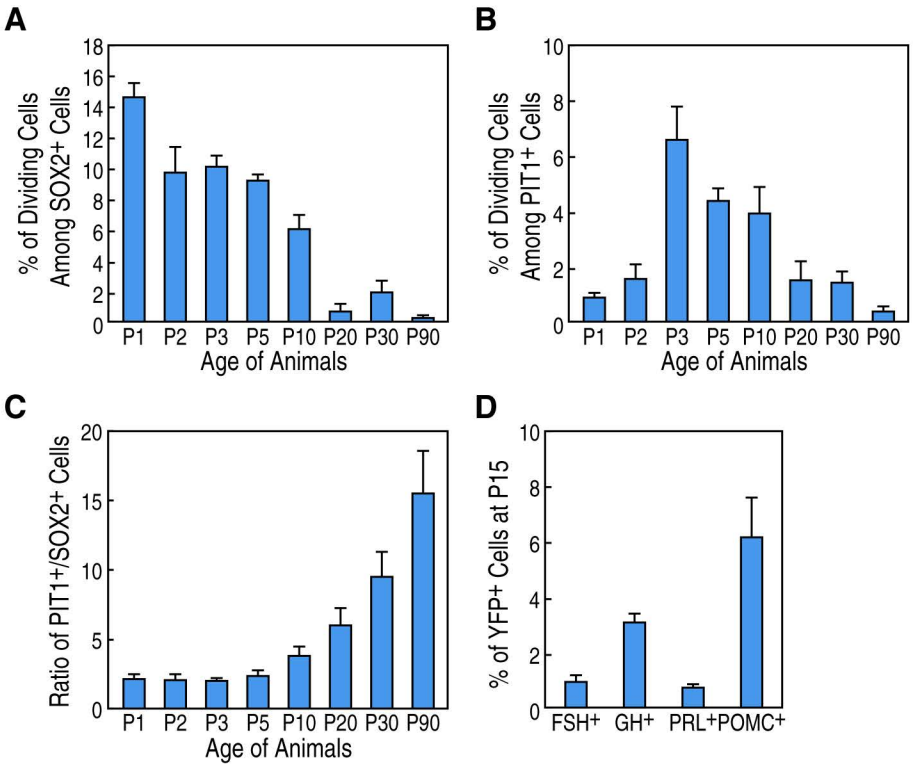


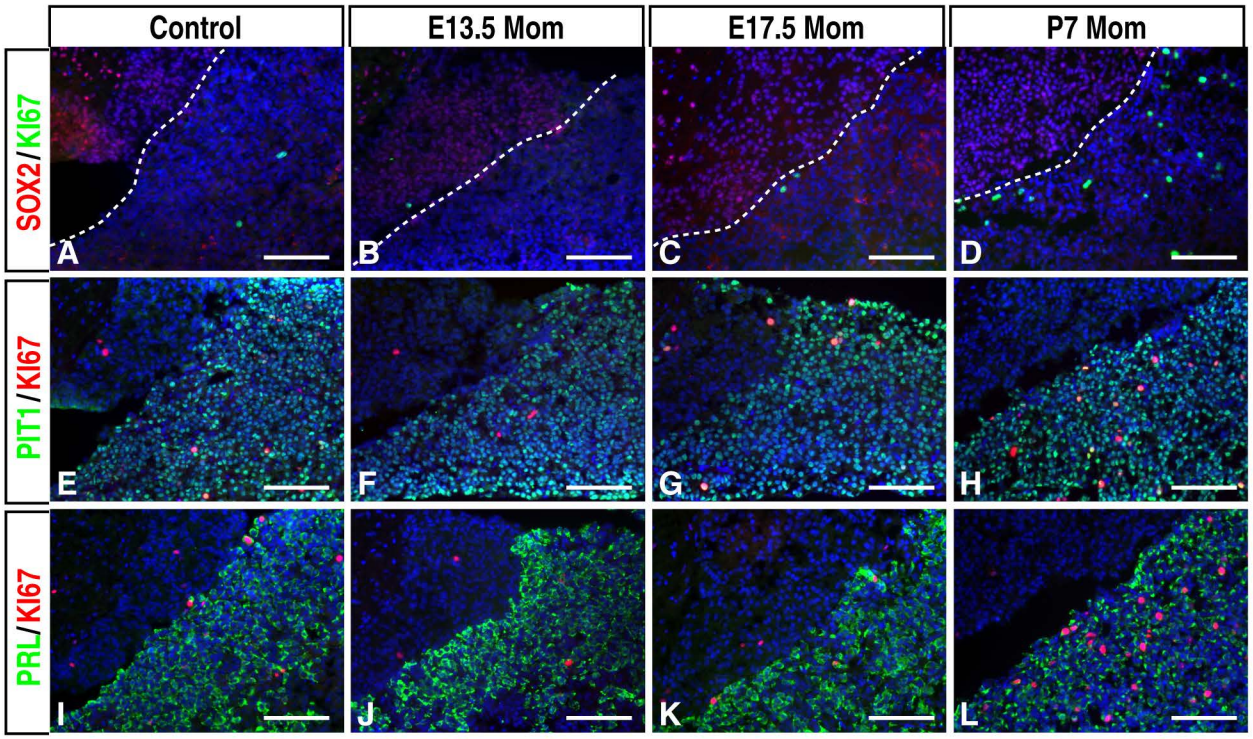
Zhu et al., 2015 Sup2


A
Females
Control
CKO
Body Weight (g)
Age (days)
B
Males
Control
CKO
Body Weight (g)
Age (days)
C
tamoxifen @ P5, P6
sac 21d later
Rbp-J f/f
Rbp-J f/f, Sox2-Cre ERT2
SOX2
D
E
F
Spheres Forming Efficiency (%)
**
DMSO
DAPT (1 μM)
WT, P10
Prop1 -/-, P10
SOX2
G
H

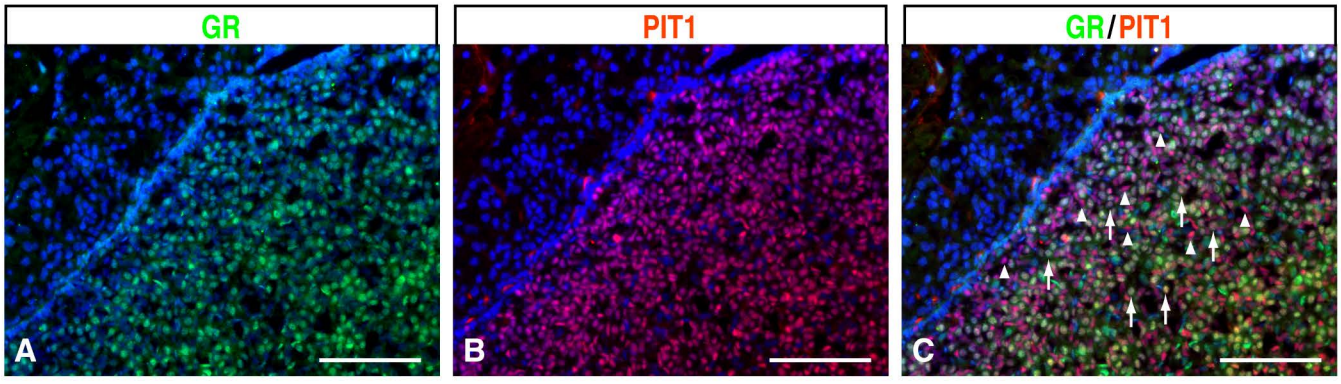


Zhu et al., 2015 Sup4


A
% of Dividing Cells Among SOX2+ Cells
0
2
4
6
8
10
12
14
16
18
P1 P2 P3 P5 P10 P20 P30 P90
Age of Animals
B
% of Dividing Cells Among PIT1+ Cells
0
2
4
6
8
10
P1 P2 P3 P5 P10 P20 P30 P90
Age of Animals
C
Ratio of PIT1+/SOX2+ Cells
0
5
10
15
20
P1 P2 P3 P5 P10 P20 P30 P90
Age of Animals
D
% of YFP+ Cells at P15
0
2
4
6
8
10
FSH+ GH+ PRL+ POMC+



Zhu et al., 2015 Sup6



Zhu et al., 2015 Sup7

Supplemental Figure Legends:

Figure S1. Characterization of pituitary stem cells and *Prop1-Cre*, Related to Figure 1.

(A) Ontogeny of Notch signaling, SOX2, PROP1, and PIT1 during pituitary organogenesis. VD: ventral diencephalon; RP: Rathke's pouch; AL: anterior lobe; IL: intermediate lobe; PL: posterior lobe. (B-D) *in situ* hybridization of *Dll1*, *Dll3* and *Notch2* in pituitary gland at E17.5. (E-F) Immunofluorescence labeling of SOX2 (E) and SOX9 (F) in 2-month-old pituitary gland. (G-H) Double immunofluorescence labeling of SOX2 and PROP1 in pituitary gland at P1 (G) and 2 months (H). (I-J) Immunofluorescence labeling of CRE in control and *Prop1-Cre* RP at E12.5. (K) LacZ reporter analysis of the *Prop1-Cre*, *Rosa-LacZ* at P0. (L) Double immunofluorescence labeling of SOX2 and KI67 in E12.5 embryonic RP. Scale bars: B-D, 400 μm; E-H, 50 μm; I-L, 100 μm.

Figure S2. Characterization of embryonic pituitary gland in wild-type controls and *Rbp-J$^{f/f}$*, *Prop1-Cre* mutants, Related to Figure 2.

(A-D) Double immunofluorescence labeling of LHX3/KI67 at E17.5 showed that LHX3 is expressed in the intermediate lobe and periluminal region in the anterior lobe, and there are reduced number of LHX3^{+}KI67^{+} cells in the mutant gland at periluminal region. Arrows indicate representative double-positive cells. (E-F) Double immunofluorescence labeling of activated Caspase 3 and KI67 at E17.5. (G-H) TUNEL assay at E17.5. (I-L) Double immunofluorescence labeling of SOX2/PIT1 showed expression of SOX2 and PIT1 is mutually exclusive and there are reduced periluminal SOX2^{+} cells in the mutant anterior gland at E17.5. (M-N) Immunofluorescence staining of PIT1 at E14.5 showed that differentiation of PIT1^{+} cells is not

affected in the mutant gland. Dashed line: lumen between intermediate and anterior lobes. Dashed area in A, B, I, and J (200 x) are presented in C, D, K, and L (400 x) respectively. Scale bars: A, B, E-H, I, J, M, N, 100 μm; C, D, K, L, 50 μm.

Figure S3. Notch signaling is required in SOX2$^+$ cells and SOX2 acts upstream of PROP1, Related to Figure 3.

(A, B) Growth curves of wild-type controls and *Rbp-J$^{f/f}$*, *Prop1-Cre* mutants showed reduced body weights of mutants in both females and males in comparison to the wild-type controls. Data are represented as mean ± SEM, n = 3 mice. C. Scheme of inactivating Notch signaling by tamoxifen induction in *Rbp-J$^{f/f}$*, *Sox2-Cre^{ERT2}* mice. (D, E) Immunofluorescence labeling of SOX2 in control and *Rbp-J$^{f/f}$*, *Sox2-Cre^{ERT2}*. (F) Quantification of pituitary spheres cultured from 3-month-old wild-type pituitary gland in the presence of DMSO or DAPT (1uM). Data are represented as mean ± SEM, n = 3 mice, ** $p < 0.01$. (G, H) Immunofluorescence labeling of SOX2 in control and *Prop1$^{-/-}$* pituitary gland at P10. Scale bars: D, E, 50 μm; G, H, 100 μm.

Figure S4. Labeling index of wild-type control and *Rbp-J$^{f/f}$*, *Prop1-Cre* mutant at P1 and P10, **Related to Figure 4.** Data are represented as mean ± SEM, n = 3 mice, ** $p < 0.01$.

Figure S5. Characterization of cell division of in wild–type pituitary glands at different postnatal stages and postnatal fate-mapping of SOX2$^+$ cells, Related to Figure 5.

(A-B) Quantification of actively dividing cells among SOX2$^+$ cells and PIT1$^+$ in the anterior lobe of wild-type pituitary gland. (C) Relative ratio of the numbers of PIT1$^+$ cells to SOX2$^+$ cells in the anterior lobe. (D) Percentage of distinct cells types among YFP$^+$ cells at P15 after fate mapping of SOX2$^+$ cells in *Sox2-Cre^{ERT2}*; *ROSA26-EYFP* mice. Data are represented as mean ± SEM, n = 3 mice.

Figure S6. Characterization of proliferating cells in 3-month-old *Rbp-J$^{f/f}$*, *Prop1-Cre* mice during pregnancy and lactation, Related to Figure 6.

(A-L) Double immunofluorescence labeling of SOX2/KI67 (A-D), PIT1/KI67 (E-H) and PRL/KI67 (I-L) at different stages of pregnancy and lactation. Dashed line: lumen between intermediate and anterior lobes. Scale bar: 100 μm.

Figure S7. GR is expressed in PIT1$^+$ cells, **Related to Figure 7.**

Expression of GR (A), PIT1 (B) and GR/PIT1 (C) in the wild-type anterior pituitary gland. Arrows and arrowheads indicate representative PIT1$^+$GR$^+$ and PIT1$^+$ cells, respectively. Scale bar: 100 μm.

Table 1: Primer Sequences Used in RT-QPCR

Primer name	Sequence (5'→3')
Rbp-J-F	TGCATTGCTTCAGGAACGAAGGTG
Rbp-J-R	TGACAGTCTGCCCGTAATGGATGT
Sox2-F	TGCAGTACAACTCCATGACCAGCTC
Sox2-R	TGGAGTGGGAGGAAGAGGTAACCA
E-Cadherin-F	AACAACTGCATGAAGGCGGGAATC
E-Cadherin-R	CCTGTGCAGCTGGCTCAAATCAAA
Hey1-F	TTCCTTCAGCTCCTTCCACCTACT
Hey1-R	CCGGGAGCTATCTTTCTTAAGTG
Hes1-F	CTATCATGGAGAAGAGGCGAAG
Hes1-R	CCGGGAGCTATCTTTCTTAAGTG
Prop1-F	TCAGCCTTTGGGAGGAACCAGTAT
Prop1-R	TTCTGGCAAGAAGCCAGAGAAGGT
POMC-F	TAGAGTTCAAGAGGGAGCTGGAAG
POMC-R	CGCGTTCTTGATGATGGCGTTCTT
Ki67-F	GCACCAAAGCAGGAAGCAACAGAT
Ki67-R	ACAGTGCTAACCTGCTCTTCCACA
Gr-F	ATCATGGTAGCCTTCGGGAGCTTT
Gr-R	AAGCCAACCCTTTCCTGAAGACCT
Gapdh-F	TCAACAGCAACTCCCACTCTTCCA
Gapdh-R	TCTTACTCCTTGGAGGCCATGTA

Supplemental Experimental Procedures:

In situ Hybridization, Immunofluorescence, and TUNEL Assay

In situ hybridization was carried out as previously described (Zhu et al., 2006). Immunofluorescence (IF) staining was performed as previously described (Zhu et al., 2006) with some modifications. The animals were transcardially perfused with 4% paraformaldehyde in PBS and the pituitary glands were dissected and postfixed for 1 hour, cryoprotected with 30% sucrose, embedded in OCT, and sectioned at 10 um. The primary antibodies used in this study were: rabbit anti-SOX2 (Millipore, AB5603, 1:500; Stemgent, 09-0024, 1:100); goat anti-SOX2 (Neuromics, GT15098, 1:500); E-Cadherin (Zymed, 13-1900, 1:1000); Hes1 (kindly provided by Dr. Nadean Brown, Cincinnati Children's Hospital Medical Center, 1:800); SOX9 (Sigma, HPA001758, 1:100); PROP1 (kindly provided by Dr. Aimee Ryan, McGill University 1:500); EGFP (Invitrogen, A6455, 1:500); KI67 (BD Biosciences, 550609, 1:100); LHX3 (Millipore, AB3202, 1:500); PIT1 (BD transduction laboratory, 610223, mouse, 1:100), PIT1 (in house, 1:100); chicken anti-Nestin (Novus Biologicals, NB100-1604, 1:1000); rat anti-BrdU (GeneTex, clone BU1/75, GTX26326, 1:50); mouse anti-BrdU (BD Biosciences, clone B44, 347580, 1:50); cleaved caspase-3 (Cell Signaling, 9661, 1:100); GR (Abcam, ab3578, 1:200); CRE (Millipore, MAB3120, 1:500); Prolactin (DAKO, A0569, 1:200); POMC (Fitzgerald, 10C-CR1096M2, 1:1000). Other antibodies against pituitary hormones were obtained from the National Hormone & Peptide Program (NHPP), NIDDK and Dr. A. F. Parlow. Alexa conjugated secondary antibodies were from Invitrogen. All images were captured with a Hamamatsu camera. TUNEL assay was performed according to manufacturer's instruction (Roche, Cell death detection kit). All the images were presented in the same orientation as Figure 1A and Figure 1B for embryonic stages and postnatal stages respectively unless otherwise indicated.

LacZ Activity Assay

For the whole mount X-gal staining, mouse embryos were fixed in 4% paraformaldehyde for 30 min on ice, permeabilized in PBS containing 0.02% Na deoxycholate and 0.01% NP-40 and were then subjected to 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) (5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM MgCl2, 0.02% NP-40, 1mg/ml X-gal in PBS) staining at 37^{o}C for overnight. Embryos were dehydrated in an ethanol series, and cleared in a 1:2 mix of benzyl alcohol and benzyl benzoate. For the tissue section X-gal staining, the mouse embryos were fixed in 4% paraformaldehyde for 30 min on ice, cryoprotected with 30% sucrose, and sectioned at 16 um. The sections were postfixed briefly and then subjected to X-gal staining for overnight.

Quantitative RT-PCR

Total RNA was isolated from dissected pituitary glands using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using SuperScript III (Invitrogen). Quantitative RT-PCR using Sybr Green was performed on an Mx3000P QPCR System (Stratagene). The primers are listed in Supplemental Table 1.

Pituitary Sphere Culture

Pituitary sphere culture was established as previously described (Fauquier et al., 2008) with some modifications. The anterior lobe of dissected pituitary gland was dissocaited in 0.4% Collegenase II and 200 ug/ml DNase I at 37^{0}C for 30 minutes with rotation followed by pipeting into single cells and passing through 40 um filter. The dissocaied cells were then cultured in DMEM/F12 containing 0.5% BSA, B27, N2 supplemented with 20 ng/ml FGF and EGF. DMSO or 1 uM DAPT were added the first day of primary culture. Spheres were counted 6 days later.

Bilateral Adrenalectomy

3-month-old mice were anaesthetized by intraperitoneal injection of Ketamine/Xylazine according to the UCSD guidelines. A small incision was made with dissection scissors on the skin and underneath body wall respectively, and the adrenal gland was completely removed using the forceps. The body wall was then be sewn up using a suture needle and the skin was closed with a wound clip system applicator. Following surgery, the mice were provided with drinking water containing 0.9% sodium chloride. Sex-matched and age-matched animals were used for the surgery. CldU were injected on the third and forth day after surgery and pituitaries were harvested on the fifth day.