Supplementary Materials for

Fate-Restricted Neural Progenitors in the Mammalian Cerebral Cortex

Santos J. Franco, Cristina Gil-Sanz, Isabel Martinez-Garay, Ana Espinosa, Sarah R. Harkins-Perry, Cynthia Ramos & Ulrich Müller

correspondence to: umueller@scripps.edu

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Materials and Methods

Mice

Cux2-Cre (*7*), *Nestin-CreERT2* (*14*), *Rosa26* (*Gt(ROSA)26Sor*) (*21*), *Z/EG* (*Tg(ACTB-Bgeo/GFP)21Lbe*) (*22*), *Ai9* (*23*), *Brainbow* (*B6.Cg-Tg(Thy1-Brainbow1.0)HLich/J*) (*24*), and *FLPe* (B6.Cg-Tg(ACTFLPe)9205Dym/J) (*25*) mice have been previously described. *Brainbow* mice were crossed to *FLPe* mice for 4 generations to generate *Brainbow;FLPe*^{4}, which harbors only one copy of the Brainbow 1.0 transgene. *Cux2-CreERT2* mice were generated at inGenious Targeting Laboratory using an identical strategy as the *Cux2-Cre* strain (*7*), but using a modified *CreERT2* (*11*) gene with an engineered Kozak sequence. Tamoxifen induction of the *CreERT2* lines was performed by intraperitoneal injection of 2 mg tamoxifen (Sigma) dissolved in sunflower oil (Sigma) into pregnant mothers at the indicated times, except in some cases (Fig. 2, F and H; fig. S2, F and G) in which *Nestin-CreERT2* mice were induced with 0.02 mg tamoxifen to generate sparse labeling to facilitate quantification. Progesterone (Sigma) was co-administered at half the concentration of tamoxifen to prevent late abortions caused by the mixed-estrogen effects of tamoxifen. For postnatal analysis of tamoxifen-induced animals, pups were delivered by cesarean section at E19.5 and provided with a foster mother until analysis. Analysis was performed on 4 animals from 3 separate experiments for each condition.

In Situ Hybridization

In situ hybridization was carried out on 12 μm frozen sections as described (*26*). To generate the Cux2 sense and antisense probes, a 2.4kb fragment of the murine Cux2 gene was amplified from E15.5 mouse brain cDNA and cloned into pGEM-T (Promega) using the following primers: 5'-GCCCAGCGTGAGGTGGAAAG -3' and 5'-GGACCTCCTTGACTCTCTTGG -3'.

Immunohistochemistry

Immunostaining was performed as described (*7*). Sections used for BrdU immunostaining were first treated with 2N HCl for 20 minutes and washed twice with borate buffer pH 8.0 to equilibrate. Antibodies used for immunostaining were: anti-BrdU rat monoclonal (1:300; AbD Serotec), anti-Ki-67 mouse monoclonal (1:200; BD Pharmingen), anti-Ctip2 (25B6) rat monoclonal (1:500; Abcam), anti-Cux1 CDP (M-222) rabbit polyclonal (1:200; Santa Cruz Biotechnology), anti-FoxP2 rabbit polyclonal (1:500, Abcam), anti-GAD65/67 rabbit polyclonal (1:1000, Sigma), anti-nestin (Rat401) mouse monoclonal (1:10; Developmental Studies Hybridoma Bank, NICHD and University of Iowa Department of Biology), anti-Pax6 rabbit polyclonal (1:250, Covance), anti-Satb2 rabbit polyclonal (1:2000, Abcam), anti-Smi32 mouse monoclonal (1:2000, Abcam), anti-Tbr1 rabbit polyclonal (1:500; Abcam), anti-Tbr2 rabbit polyclonal (1:500; Abcam), anti-Tuj1 mouse monoclonal directly conjugated to Alexa-488 (1:2000, Covance), anti-βgal chicken polyclonal (1:2000; Abcam). Anti-RFP rabbit polyclonal (1:200, Abcam ab62341) was used to recover tdTomato signal only in sections treated with HCl. Nuclei were stained with ToPro3, YoYo1 or DAPI (1:5,000; Molecular Probes) and sections were mounted on slides with Prolong Gold mounting medium (Molecular Probes).

Cell Cycle Exit

BrdU was administered at E12.5 by IP injection at 100 mg/kg body weight into pregnant females resulting from *Cux2*$^{+/cre}$ x *Ai9*$^{+/fl}$ crosses. Embryos were dissected 24 hours after injection and brains were sectioned at 100 μm and processed for immunostaining for BrdU, Ki67 and tdTomato. For quantification of cell cycle exit, BrdU^{+} cells were first marked in a standardized cortical field from pia to ventricle. Next, BrdU^{+} cells were marked as either Ki67^{+} or Ki67^{-}. Finally, presence or absence of tdTomato signal was used to identify cells in the Cux2^{+} and Cux2^{-} lineage, respectively. Cell cycle exit was therefore quantified separately and blindly for Cux2^{+} and Cux2^{-} cells within the same sections, and shown as the percentage of BrdU^{+} cells that were Ki67- for each lineage. Analysis was performed on 4 animals from 3 separate experiments.

Expression Constructs

Control-iGFP (pCIG2) has been described (*27*). Dominant-negative TCF4 was amplified from E15.5 mouse brain cDNA using the primers 5'-ATCTCGAGTAAAATGAGCAGTGGGAAAAATGGACCAAC-3' (Bases 645 - 667 of NM_013685.2) and 5'-TCACATCTGTCCCATGTGATTCG-3' (Bases 2545 - 2567 of NM_013685.2) and cloned into pCIG2 to generate DN-TCF4-iGFP. Constitutively-active notch (notch intracellular domain) was amplified from pCAGGS-NICD (Addgene plasmid 26891) using primers 5'- CCTGAATTCGACCATGGACTACAAAG-3' and 5'-GTAGAATTCTTATTTAAATGCCTCTGGAATGTG-3' and cloned into pCIG2 to generate CA-notch-iGFP. Notch activity reporter constructs have been described (*17*). CBFRE-GFP (Addgene plasmid 17705) was used as is. Hes5p-GFP was generated by replacing dsRed2 with eGFP in Hes5p-dsRed (Addgene plasmid 26868). CβA-FLEx comprises: chicken β-actin promoter/intron (forward orientation), lox2372 (forward orientation), tdTomato (forward orientation), lox511 (forward orientation), GFP (reverse orientation), lox2372 (reverse orientation), lox511 (reverse orientation), and an SV40 polyA signal (forward orientation).

In Utero Electroporation

Timed pregnant mice were anesthetized and their uterine horns exposed. Endotoxin-free plasmid DNA (1-2 mg ml^{-1}) was injected into the embryos' lateral ventricles. For electroporation, 5 pulses separated by 900 ms were applied at 38 V for E12.5 embryos and at 40 V for E13.5 embryos. Embryos were allowed to develop *in utero* for the indicated time. For analysis of embryos, brains were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. For postnatal analysis, pups were fixed by transcardial perfusion with 4% PFA before dissection and post-fixation. Brains were sectioned coronally at 100 μm with a vibrating microtome (VT1200S; Leica). Electroporations were analyzed from 3 animals from 3 separate experiments for each condition.

In Vitro Cultures of Primary Cortical Cells

Cerebral cortices of *Cux2-Cre;Ai9* embryos were dissected and dissociated with trypsin at E13.5. Single cell suspensions were plated on LabTek II 1.5 borosilicate coverglass chambers (Thermo Scientific/Nunc) coated with Matrigel basement membrane matrix (1:100, BD Biosciences). Cells were cultured 24 or 72 hours in serum-free

medium (*28*) without additional growth factors. For 24-hour cultures, live cells were imaged using an inverted brightfield microscope 1 hour and 24 hours after plating to identify single cells that divided once to generate cell pairs. Analysis was performed on 9 brains from 4 separate experiments per time point.

Slice Culture and Time-Lapse Imaging

Cux2$^{+/cre}$*;Ai9*$^{+/fl}$ embryos were dissected and brains processed for organotypic slice culture as described (*29*). Time-lapse imaging was performed using a 40x long-working distance objective on a Nikon A1R confocal laser microscope system with a temperature-controlled culture chamber containing 40% O_2 and 5% CO_2. An image z-stack ranging 10 μm in 2.5 μm steps was projected into a maximum intensity composite. Stacks were captured every 15 minutes. Image analysis was performed using Nikon NIS-Elements and Adobe Photoshop.

Statistics

Statistical analysis was performed using unpaired homoscedastic two-sample t-test, except for notch pathway experiments in Fig. S4. For notch pathway experiments, independence of the two variables, being electroporated (GFP$^+$) and being Cux2$^+$ (tdTomato$^+$), was determined for each construct. Each cell in a standardized field was counted as either red or not red (tdTomato±) and green or not green (GFP±) to calculate the expected and observed probabilities that a cell would be red given that it is green ([P(R|G)exp] and [P(R|G)obs], respectively). Averaged values from 3 separate experiments for each of the 4 red/green states were input into 2x2 contingency tables and Fisher's exact test was used to calculate the statistical significance (1-tailed p-value) of the contingency between GFP expression and tdTomato expression for each construct.

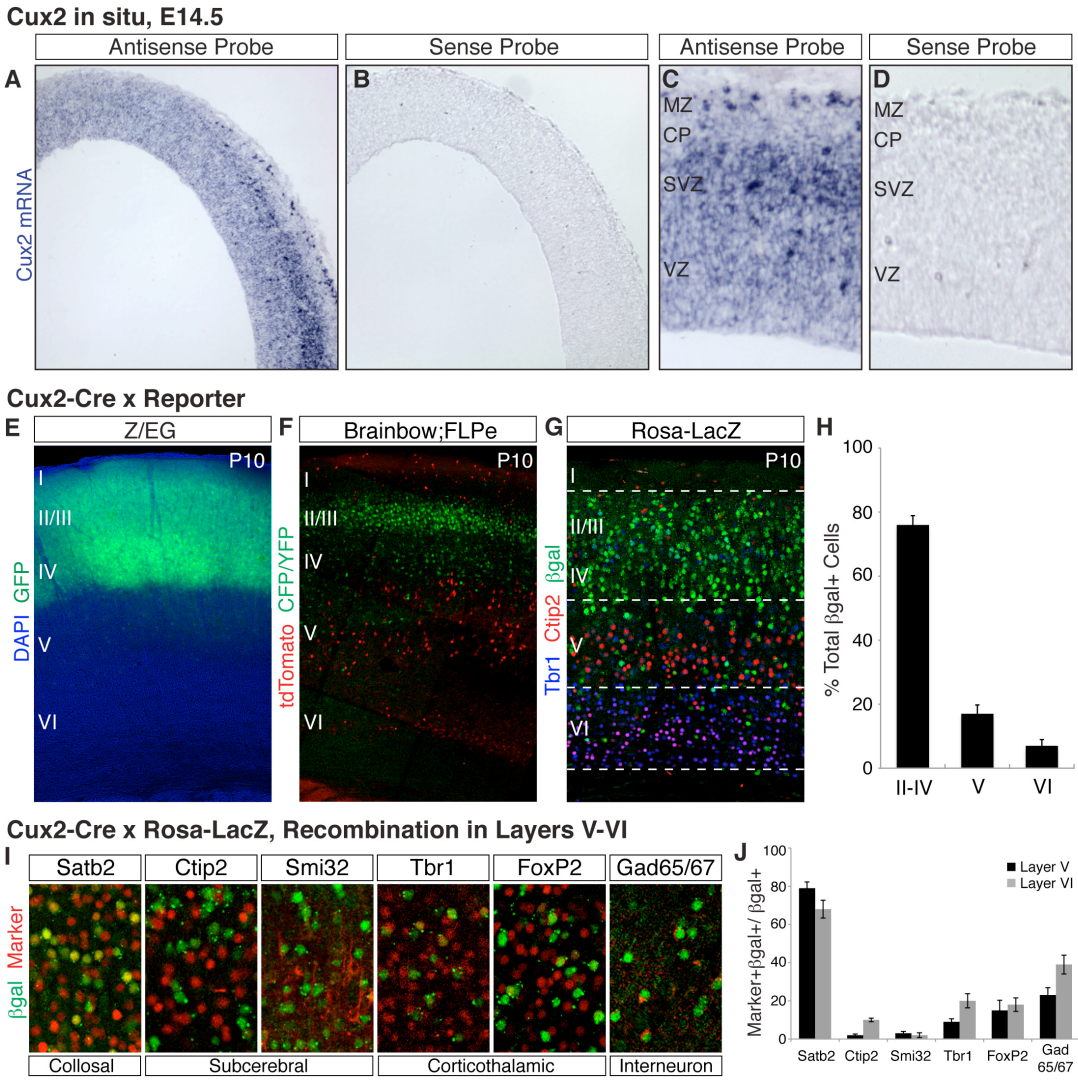

Fig. S1.

Related to Figure 1. (**A** and **B**) In situ hybridization of Cux2mRNA at E14.5 using antisense and sense probes. Note high expression in the marginal and subventricular zones and weaker expression in the cortical plate and ventricular zone. (**C** and **D**) Higher magnification images from examples in (A) and (B), respectively. (**E**-**H**) *Cux2-Cre* drives recombination of multiple reporter lines primarily in upper layer neurons at P10. (**E**) *Cux2-Cre* x *Z/EG* reporter. Recombined cells express GFP. (**F**) *Cux2-Cre* x *Brainbow;FLPe*[4]. Recombined cells express CFP or YFP, non-recombined cells express tdTomato. (**G**) *Cux2-Cre* x *Rosa-LacZ* reporter. Stained for markers of recombined cells (βgal), layer V neurons (Ctip2) and layer VI neurons (Tbr1). (**H**) Percentage of total βgal positive cells in each layer, ± SEM. (**I**) Characterization of recombined cells in lower layers V-VI in *Cux2-Cre*;*Rosa-LacZ* mice at P10. Stained for βgal and markers of callosal projection neurons (Satb2), subcerebral projection neurons (Ctip2, Smi32), corticothalamic projection neurons (Tbr1, FoxP2) and interneurons (Gad65/67). (**J**) Percentage of βgal^{+} cells in layer V or VI that express each marker, ± SEM.

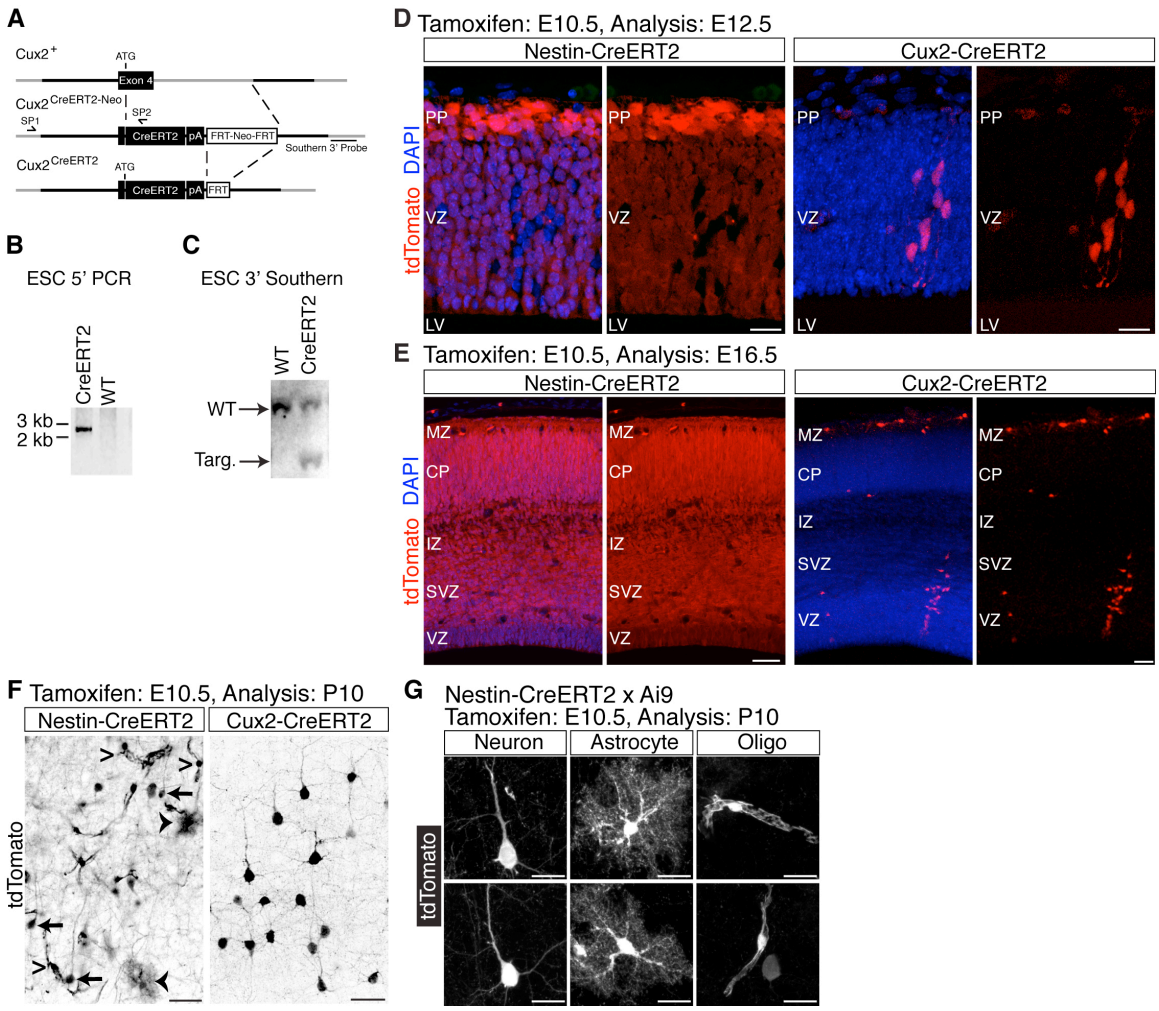

Fig. S2.

Related to Figure 2. (**A**) Targeting strategy for *Cux2-CreERT2* mice. SP1 and SP2, screening primers. (**B**) PCR analysis of 5' arm targeting using SP1 and SP2 in embryonic stem cells (ESC). (**C**) Southern blot for 3' arm targeting in ESCs. (**D** and **E**) CreERT2 mice were crossed to *Ai9* reporter mice. Pregnant females were injected with tamoxifen (2 mg) at E10.5. (**D**) *Nestin-CreERT2* mice drive recombination in nearly all RGCs at E12.5, including progenitors of preplate neurons. *Cux2-CreERT2* mice recombine in only a subset of RGCs, which are often found in clonal columns. (**E**) By E16.5, recombined cells in *Nestin-CreERT2;Ai9* mice include nearly all RGCs, intermediate progenitors, and their neuronal offspring in the cortical plate. Recombined cells in *Cux2-CreERT2* animals are mostly found in sparse clonal columns in the ventricular and subventricular zones, with very few recombined neurons. Scale bars, 10 μm (D) and 50 μm (E). (**F** and **G**) Tamoxifen induction at E10.5, analysis at P10. *Nestin-CreERT2* mice received 100-fold less tamoxifen than *Cux2-CreERT2* mice to facilitate visualization of single cells. (**F**) Recombination in *Nestin-CreERT2* brains is found in neurons (arrows), astrocytes (solid arrowheads) and oligodendrocytes (open arrowheads), whereas only neurons are labeled in *Cux2-CreERT2* brains. Scale bars, 50 μm. (**G**) Higher magnification of the various cell types labeled in *Nestin-CreERT2* mice. Scale bars, 10 μm.

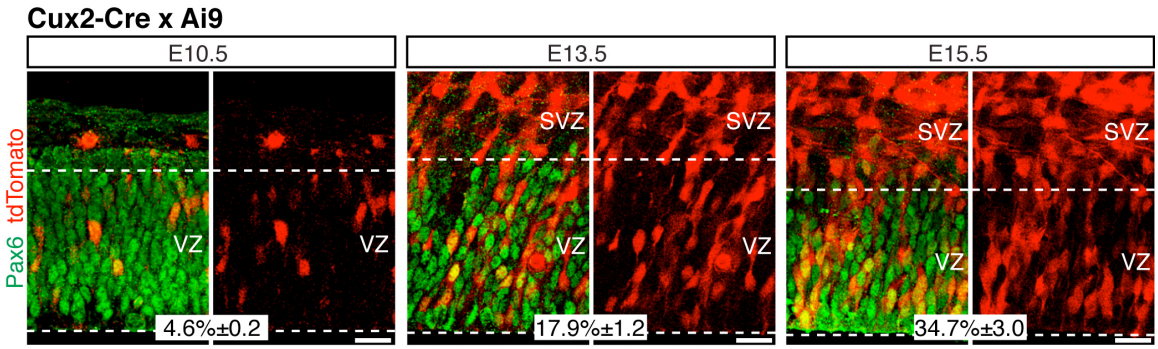

Fig. S3.

Related to Figure 3. Pax6 immunostaining in *Cux2-Cre;Ai9* embryos at E10.5, E13.5 and E15.5 demonstrates that the percentage of $Cux2^{+}$ RGCs increases over time. Percentage of $Pax6^{+}$ cells that are $tdTomato^{+}$ for each timepoint is indicated at the bottom of each set of images.

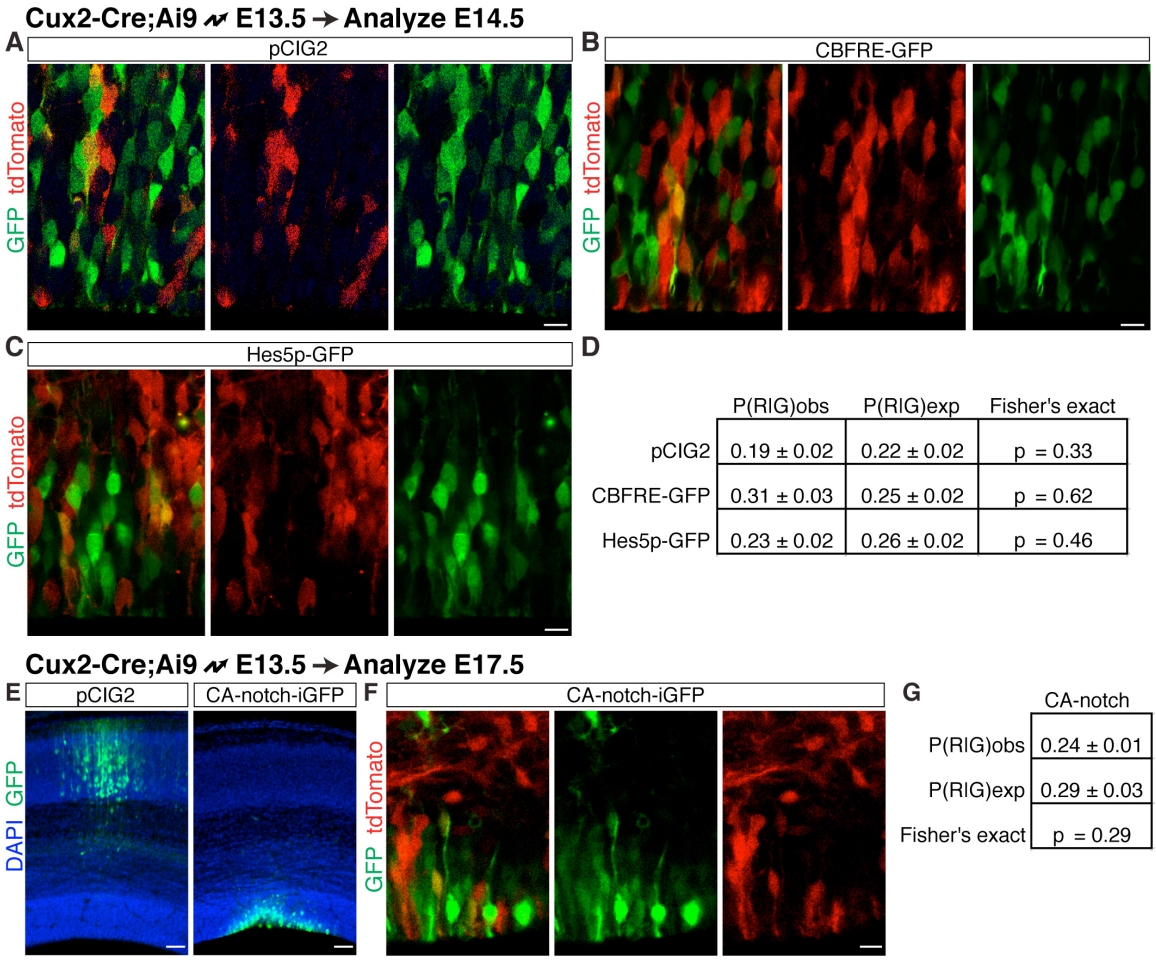

Fig. S4.

Related to Figure 3. (**A-D**) Notch-activity reporters are not preferentially active in Cux2^{+} or Cux2^{-} RGCs. *Cux2-Cre;Ai9* embryos electroporated at E13.5 with: (**A**) control pCIG2 vector, which expresses GFP ubiquitously from the chicken β-actin promoter; (**B**) notch activity reporter plasmid CBFRE-GFP, which expresses GFP from the CBF1 responsive element; (**C**) notch activity reporter plasmid Hes5p-GFP, which expresses GFP from the Hes5 promoter. Analysis at E14.5. Scale bars, 10 μm. (**D**) Quantification of tdTomato expression in electroporated GFP^{+} cells, as described in Materials and Methods. Shown for each construct are the expected and observed probabilities that a cell would be red given that it is green ([P(R|G)exp] and [P(R|G)obs], respectively), ± SEM, and the 1-tailed p-value (by Fisher's exact test) of the contingency between expression of GFP and tdTomato. (**E-G**) Overexpression of constitutively active notch maintains the RGC state, but does not alter subtype identity. *Cux2-Cre;Ai9* embryos electroporated at E13.5, analyzed at E17.5. (**E**) Representative images of electroporations with control pCIG2 or CA-notch-iGFP, illustrating that CA-notch prevents neurogenesis and maintains cells in the RGC state. Scale bar 50 μm. (**F**) Representative images of CA-notch-iGFP electroporations demonstrating both Cux2^{+} and Cux2^{-} RGCs amongst the electroporated cells, indicating that CA-notch did not alter RGC subtype fate. Scale bar 10 μm. (**G**) Quantification of tdTomato expression in CA-notch-iGFP cells, as in (D).

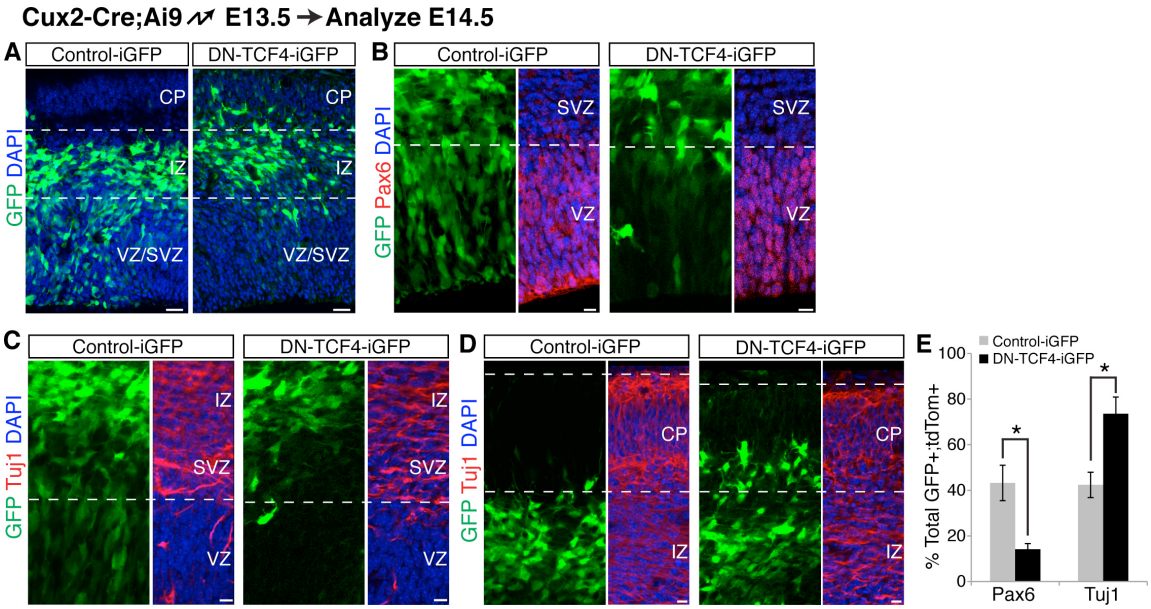

Fig. S5.

Related to Figure 4. (**A**-**E**) Interfering with β-catenin signaling forces premature differentiation of Cux2^{+} RGCs in vivo. *Cux2-Cre;Ai9* embryos were electroporated in utero at E13.5 with control IRES-GFP or dominant-negative TCF4-IRES-GFP to block β-catenin signaling. (**A**) At E14.5, control cells are located in the ventricular, subventricular and intermediate zones, whereas most cells expressing DN-TCF4 have left the ventricular and subventricular zones and are already found entering the cortical plate. (**B**) Fewer DN-TCF4 expressing cells are in the Pax6^{+} ventricular zone, compared to controls. (**C** and **D**) More DN-TCF4 expressing cells are in the Tuj1^{+} subventricular and intermediate zones (C) and cortical plate (D), compared to controls. Scale bars, 25 μm. (**E**) Quantification of Pax6 and Tuj1 expression in the electroporated Cux2^{+} (GFP^{+}, tdTomato^{+}) population. *, $P < 0.03$.