SUPPLEMENTAL INFORMATION

Inventory of Supplemental Information

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Cux2-Cre (Franco et al., 2012; 2011), *Cux2-CreERT2* (Franco et al., 2012), *Neurod6-Cre* (Goebbels et al., 2006), *Emx1-Cre* (*B6.129S2-Emx1tm1(cre)Krj*) (Gorski et al., 2002), *Rosa26-LacZ (Gt(ROSA)26Sor)* (Friedrich and Soriano, 1991), *Z/EG (Tg(ACTB-Bgeo/GFP)21Lbe)* (Novak et al., 2000), *Ai9* (Madisen et al., 2010) and *Rosa26-NZG* (*Gt(ROSA)26Sortm1(CAG-lacZ,-EGFP)Glh/J*) (Yamamoto et al., 2009) mice have been previously described. Induction of Cre-activity in *Cux2-CreERT2* mice was achieved by intraperitoneal injection of pregnant dams with 4-OHT (Sigma) (1 mg/20 g of body weight, dissolved as described (Guenthner et al., 2013), or with tamoxifen exactly as described (Franco et al., 2012)For postnatal analysis of induced animals, pups were delivered by cesarean section at E19.5 and provided with a foster mother until analysis.

Mouse genetic background and breeding

The *Cre* and *CreERT2* transgenes were knocked into the endogenous start site in exon 4 of the *Cux2* gene by homologous recombination in *C57BL/6*-derived ES cells. Positive clones were injected into *C57BL/6J-Tyr c-2J* blastocysts and the resulting chimeras were then mated to *C57BL/6J-Tyr c-2J* females to obtain germline transmission. Heterozygous F1 mice were mated with *B6.Cg-Tg(ACTFLPe)* mice (Rodríguez et al., 2000) to remove the *PGK-Neo* selection cassette and the resulting F2 offspring were

subsequently mated to *C57BL/6J* mice to remove the *FLPe* transgene.

All animals used for analyses in Figs. 2-3 and S2-S3 were heterozygous for the Cre allele (*Cux2*$^{+/Cre}$, *Cux2*$^{+/CreERT2}$ or *Neurod6*$^{+/Cre}$) and heterozygous/hemizygous for the Reporter allele (*Ai9*$^{+/fl}$, *Rosa26-LacZ*$^{+/fl}$ or *Z/EG*$^{+/-}$). For Fig. 1A-B and E, "Original" and "Sparse" data were generated by crossing *Cux2*$^{Cre/Cre}$ x *Ai9*$^{fl/fl}$ animals, both on congenic *C57BL/6J* backgrounds. The "Recovered" data in Fig. 1B were generated by first outcrossing *Cux2*$^{Cre/Cre}$ mice to ICR mice for 3 generations, then crossing these *Cux2*$^{+/Cre}$ mice to *Ai9*$^{fl/fl}$ animals on the congenic *C57BL/6J* background. For Fig. 1C-D and F, "Original" and "Expanded" data were generated by crossing *B6.Cg-Cux2*$^{Cre/Cre}$ x *Rosa26*$^{NZG/NZG}$ animals that were on a congenic *FVB/NJ* background. The "Recovered" data in Fig. 1D and F were generated by first outcrossing *Cux2*$^{Cre/Cre}$ mice to *FVB/NJ* mice for 5 generations, then crossing these *Cux2*$^{+/Cre}$ mice to *Rosa26*$^{NZG/NZG}$ animals on the congenic *FVB/NJ* background.

Data in Fig. S1A, C are from *Cux2*$^{+/Cre}$*;Ai14*$^{+/fl}$ animals generated by crossing *Cux2*$^{+/Cre}$ mice to *Ai14*$^{fl/fl}$ animals, both on congenic *C57BL/6J*. Data in Fig. S1D are from *Cux2*$^{+/Cre}$*;Ai14*$^{+/fl}$ animals generated by crossing *Cux2*$^{+/Cre}$*;Ai14*$^{+/fl}$ animals to wild-type *C57BL/6J* mice. Data in Fig. S1E-F are from offspring resulting from crossing *B6.Cg-Cux2*$^{+/Cre}$*;Ai9*$^{+/fl}$ (generated as described above) to wild-type *C57BL/6J* mice Data in the left panels of Fig. S1G are from *Cux2*$^{+/Cre}$;*Rosa26*$^{+/NZG}$ generated by crossing *Cux2*$^{Cre/Cre}$ mice to *FVB/NJ* mice for 3 generations, then crossing these *Cux2*$^{+/Cre}$ mice to *Rosa26*$^{+/NZG}$ animals. Data in the center and right panels of Fig. S1G are from *Cux2*$^{+/Cre}$;*Rosa26*$^{+/ZG}$ and *Cux2*$^{+/+}$;*Rosa26*$^{+/ZG}$ mice, respectively, which were generated by crossing *Cux2*$^{+/Cre}$;*Rosa26*$^{+/NZG}$ mice (generated as described above) to wild-type *FVB/NJ* mice.

Immunohistochemistry and *in utero* electroporation

Embryonic brains were fixed in 4 % paraformaldehyde (PFA) overnight at 4°C. Postnatal mice were transcardially perfused with 4% PFA and brains postfixed in 4% PFA for 2 hours at 4°C. Brains were sectioned coronally at 50, 75 or 100 μm with a vibrating microtome (VT1200S; Leica). Immunostaining was performed as described and using the same antibodies (Franco et al., 2012; Gil-Sanz et al., 2013), with the addition of anti-Aldh1L1 (Antibodies Inc.), anti-Cre (Covance), anti-Parvalbunin (Swant), anti-RFP (LifeSpan Biosciences), anti-Somatostatin (Abcam), anti-Tle4 rabbit polyclonal (Abcam). In utero electroporations using the Cre responsive plasmid CβA-Flex (Franco et al., 2012)were carried out as described (Franco et al., 2012; Gil-Sanz et al., 2013). Confocal images were captured using a Nikon C2 or a Nikon-A1 laser-scanning confocal microscope system and widefield images were captured on a Nikon Eclipse 80i microscope.

Quantification of recombination and molecular markers

For the Sabt2 and Ctip2 double immunostainings in the *Cux2-Cre;Ai9* mice, at least 3 histological sections from 3 different animals at 3 distinct rostro-caudal levels were analyzed in the medio-lateral part of the neocortex comprising primarily the somatosensory cortex. Confocal optical sections were used for quantification. Cells were first categorized by molecular marker expression (single-positive for either marker or double-positive) and then classified as recombined or not based on tdTomato expression. 2902 cells were analyzed in contiguous columns spanning the region between layer I and the white matter. Values are mean ± SEM. For analysis of interneurons in the *Cux2-Cre;Rosa26-LacZ* mice, 3 sagittal sections from 2 different animals at distinct rostro-caudal levels of the neocortex were analyzed. The proportion of

recombined interneurons, the proportion Ctip2$^+$ cells that were interneurons and the proportion of the recombined Ctip2$^+$ cells that were interneurons were calculated. More than 1200 cells were quantified in contiguous columns spanning the region between layer I and the white matter. Values are mean ± SEM. For Satb2 and Ctip2 double immunostaings in the *Cux2-CreERT2;Ai9* mice 4-6 serial sections from each of 9 mice injected with 4-OHT were analyzed. Confocal optical sections were used for quantification. The total number of recombined tdTomato$^+$ cells present in the cortical plate were analyzed and classified according to the expression of one marker, both markers or neither marker. A total of 1745 cells were analyzed. Quantifications are presented as percentage of the total number of recombined cells expressing the different combinations of markers. For analysis of interneurons in *Cux2-CreERT2;Ai9* mice, 6-9 serial sections from 2 mice injected with 4-OHT were analyzed. Confocal optical sections were used for quantification. The total number of recombined tdTomato$^+$ cells present in the cortical plate were analyzed and classified according to the expression of one marker or both markers. A total of 265 cells were analyzed. Values are mean ± SEM. For quantifications of layer distribution in sparsely recombined *Cux2-Cre;Ai9* brains, 502 cells were quantified from 3 animals. For *Cux2-Cre;Rosa-NZG* mice, 3 sections from each of at least 2 animals were used to quantify >2100 cells for each condition. Layer positions of recombined cells were quantified separately for somatosensory cortex and motor cortex, but did not show significant differences between areas and were therefore combined in the final counts. Values are mean ± SEM.

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SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1 (Gil-Sanz, et al.)

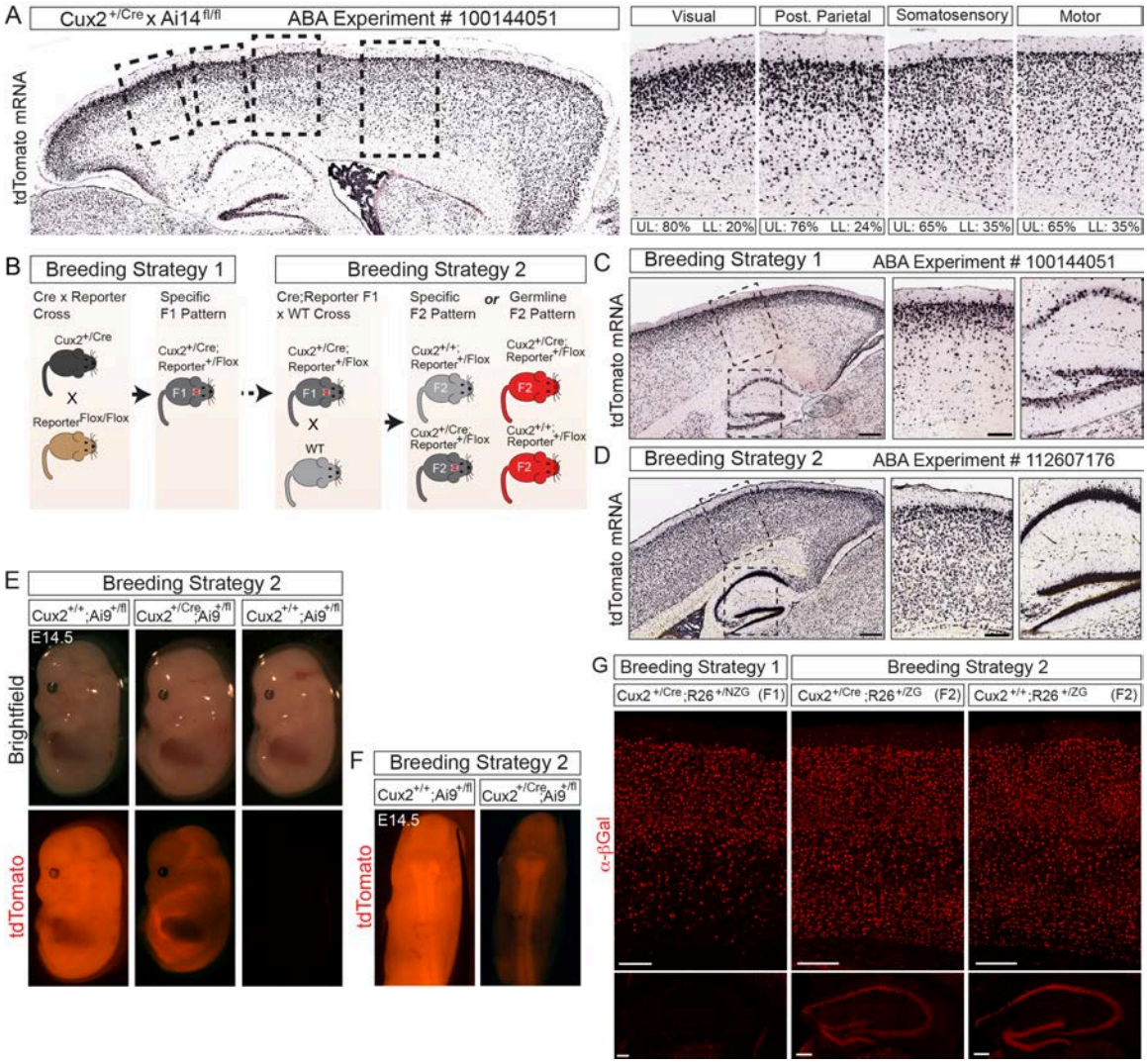

Figure S1. **Related to Figure 1**. ***Cux2*** **genetic locus is active in the developing germline.** (**A**) Sagittal sections displaying *tdTomato* in situ hybridizations in the brains of a *Cux2-Cre;Ai14* mouse generated by Cre x Reporter breeding as in Breeding Strategy 1 shown in (B). Images at right are higher magnifications of insets in overview at left. Quantification is percentage of total recombined cells that are found in upper layers 2-4 (UL) or in lower layers 5-6 (LL). (**B**) Different breeding strategies used to generate mice for analysis. Breeding Strategy 1 involves breeding a *Cux2-Cre* heterozygous animal to

a homozygous Reporter animal (i.e., *Ai14*$^{fl/fl}$, *Ai9*$^{fl/fl}$ or *Rosa26-NZG*$^{fl/fl}$) to produce double heterozygous F1 animals that exhibit the layer-specific recombination pattern. For Breeding Strategy 2, these double heterozygous animals were then crossed to WT mice to produce F2 animals. Some F2 mice showed the expected specific recombination patterns, whereas others exhibited ubiquitous reporter expression indicating germline transmission of the recombined reporter allele. (**C**) Sagittal sections displaying *tdTomato* in situ hybridizations in the brains of a *Cux2-Cre;Ai14* mouse generated by Breeding Strategy 1 as described in (B). Note the restricted recombination patterns in the neocortex (left, middle panels) and the hippocampus (right panel). (**D**) Sagittal sections displaying *tdTomato* in situ hybridizations in the brains of a *Cux2-Cre;Ai14* mouse generated by Breeding Strategy 2 as described in (B). Note the equal distribution of *tdTomato*$^{+}$ cells throughout all layers of the neocortex and the extensive recombination throughout the hippocampus. (**E**) Wide-field images (transmitted light and fluorescence) showing the F2 offspring from the cross shown in (K). Note that some embryos without Cre have no recombination (right panels) and some with Cre exhibit the normal recombination pattern (center panels), whereas others display widespread recombination even without the Cre allele (left panels), indicating germline recombination. (**F**) Fluorescence images of one germline recombined embryo (left) showing widespread recombination and a normal recombined embryo (right) displaying fluorescence in the brain and spinal cord. (**G**) Confocal images displaying the neocortex (top) and the hippocampus (bottom) of animals with normal recombination (Breeding Strategy 1) and animals showing germline recombination (Breeding Strategy 2). Note that essentially all cells present in these animals appear to be recombined, even in the absence of Cre. Scale bars: (C-D) 400 μm; (G) 200 μm.

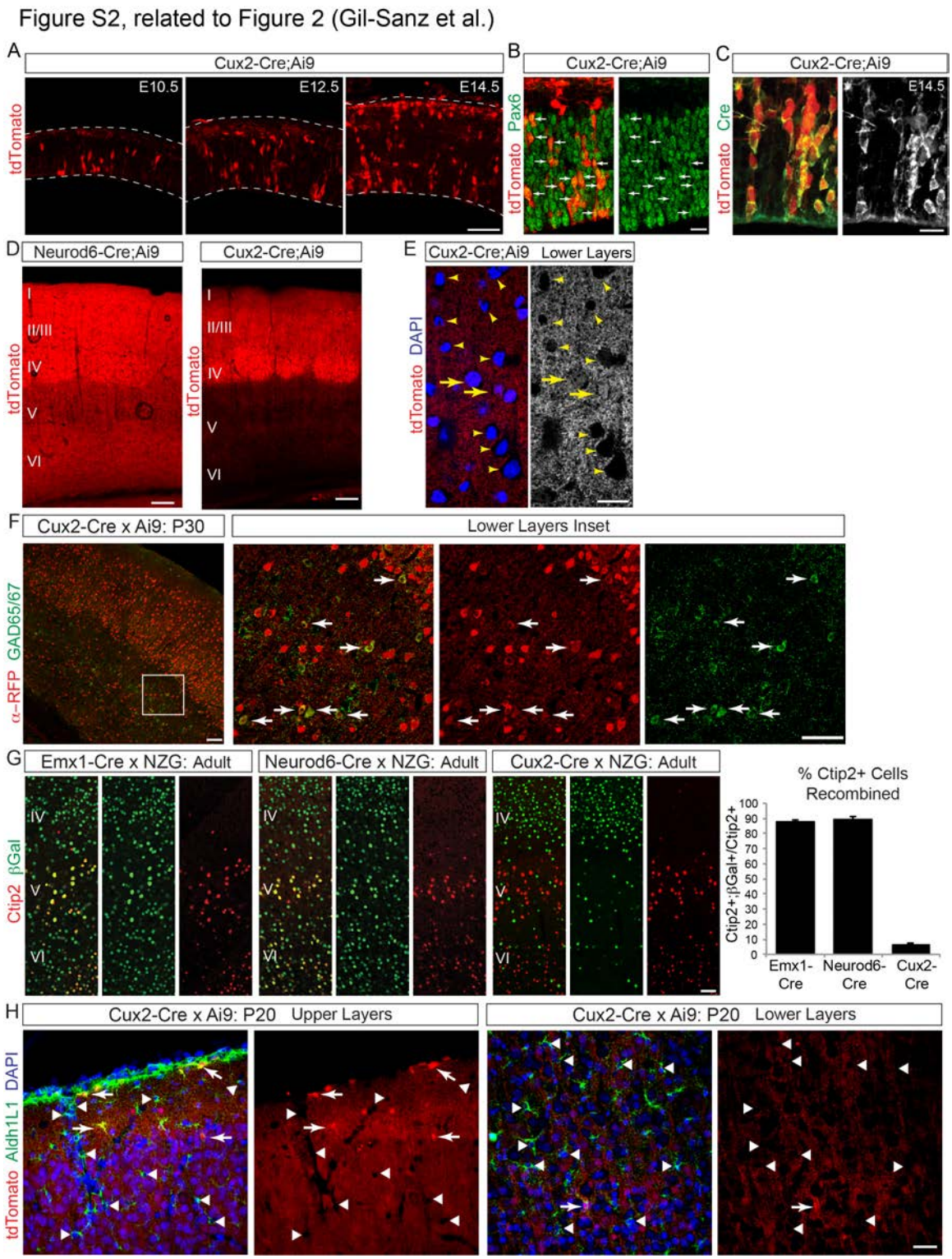

Figure S2. Related to Figure 2. *Cux2-Cre* is expressed by a subpopulation of interneurons and astrocytes. (**A**) Recombination patterns in the neocortex at E10.5, E12.5 and E14.5 in *Cux2-Cre*;*Ai9* mice. Note the presence of recombined cells with

RGC morphology. (**B**) Confocal images of the VZ of a *Cux2-Cre;Ai9* embryo at E12.5. Arrows highlight recombined tdTomato$^+$ cells (red) that express Pax6 (green). (**C**) Sections of *Cux2-Cre;Ai9* VZ at E14.5 stained with α-Cre (green) to reveal expression in recombined tdTomato$^+$ cells (red). (**D**) Recombination patterns in the neocortex at P30 from *Neurod6-Cre* and *Cux2-Cre* crossed to Ai9 reporters. (**E**) Coronal section of a *Cux2-Cre*;*Ai9* brain showing that the majority of fluorescence signal in lower layers is in the neuropil from upper layer neurons. Nuclei are stained with DAPI (blue). (**F**) Coronal sections from a *Cux2-Cre;Ai9* neocortex immunostained with the interneuron marker Gad65/67 (green) and an antibody against RFP (red) to enhance the soma staining in the recombined cells. Boxed inset is shown enlarged at right to demonstrate recombined interneurons (arrows). (**G**) Coronal neocortical sections from different Cre mice crossed to the *Rosa26-NZG* reporter, immunostained for Ctip2 (red) and βGal (green). Note that the majority of Ctip2$^+$ cells are recombined (βGal$^+$) in *Emx1-Cre;NZG* and *Neurod6-Cre;NZG* mice, whereas very few Ctip2$^+$ cells are recombined in *Cux2-Cre;NZG* brains. Graph shows the percentage of Ctip2$^+$ cells that are recombined in each strain, ± SEM. (**H**) Coronal section from a *Cux2-Cre;Ai9* neocortex immunostained with the general astrocyte marker Aldlh1L1 (green). tdTomato$^+$ recombined cells are shown in red and DAPI-stained nuclei in blue. Note that some astrocytes are recombined (arrows), but the vast majority of astrocytes are not recombined, as revealed by the absence of tdTomato signal (arrowheads). Abbrev. I-VI: layers I-VI. Scale bars: (A, D) 100 μm; (B, C) 25 μm; (E) 20 μm; (F) 100 μm, insets 50 μm; (G, H) 50 μm.

Figure S3, related to Figure 3 (Gil-Sanz et al.)

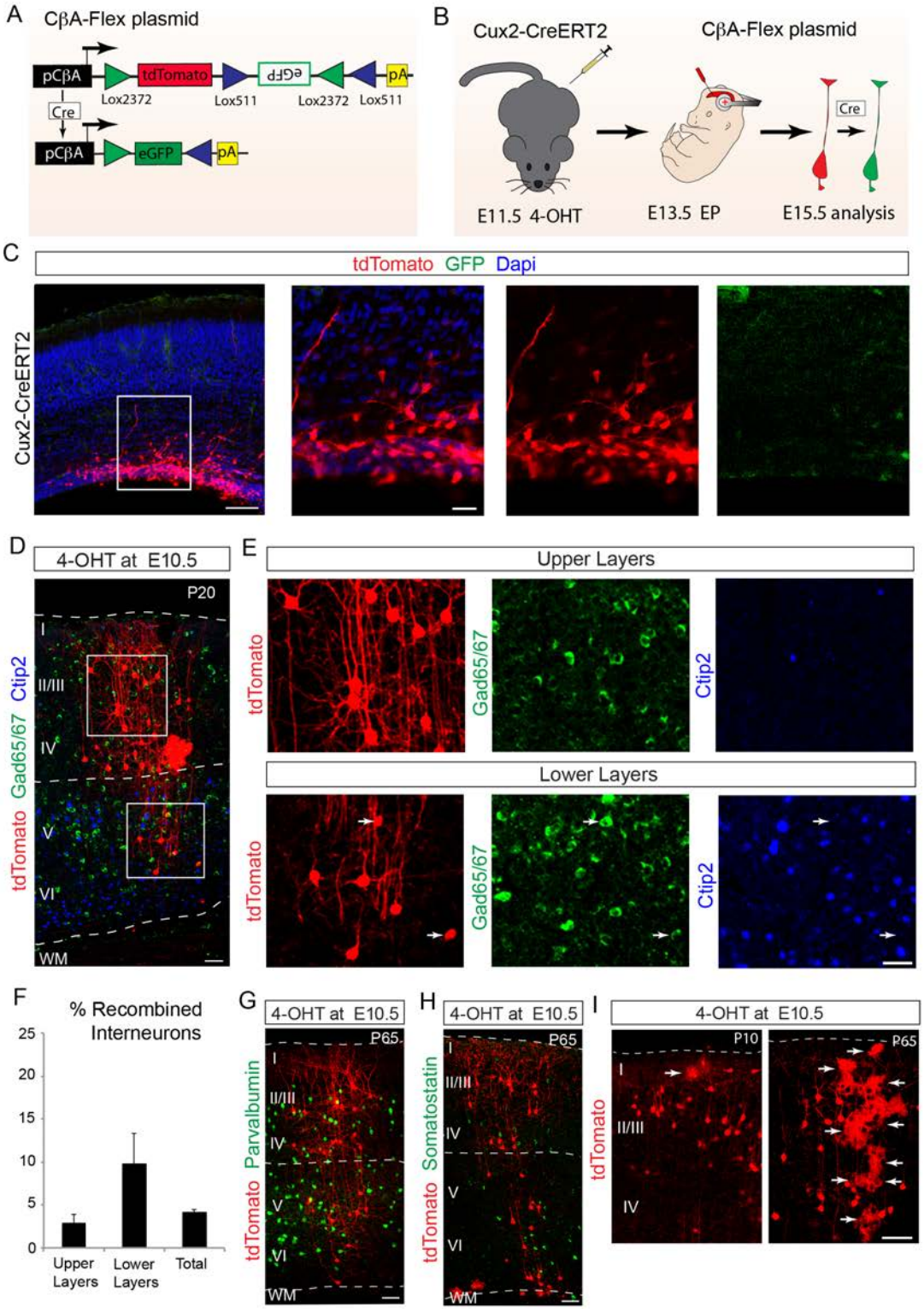

Figure S3. Related to Figure 3. *Cux2-CreERT2*$^{+}$ progenitors generate a small subpopulation of cortical interneurons and astrocytes. (**A**) Schematic of the CβA-Flex plasmid. This plasmid drives differential expression of tdTomato or EGFP upon Cre recombination. (**B**) Schematic of the strategy used to evaluate the duration of the activity of tamoxifen to mediate Cre recombination. *Cux2-CreERT2* pregnant dams were injected with 4-OHT at E11.5 and 2 days later the embryos were electroporated with the Cre responsive plasmid. Brains were analyzed 2 days after electroporation. (**C**) Confocal images of embryonic brains showing the neocortex after performing the experiment described in (B). Non-recombined cells express tdTomato (red) and recombined cells express GFP (green). DAPI-stained nuclei are in blue. Note the absence of EGFP expression in the ventricular zone of the electroporated brains. (**D**) Overview of the adult neocortex from a *Cux2-CreERT2;Ai9* mouse injected with 4-OHT at E10.5, immunostained with the interneuron marker Gad65/67 (green) and Ctip2 (blue). tdTomato^{+} recombined cells are in red. (**E**) Higher magnification of the boxed areas in (D). (**F**) Quantification (mean ± SEM) of the percentage of recombined interneurons present in the *Cux2-CreERT2;Ai9* neocortex after 4-OHT injections at E10.5. (**G**-**H**) Confocal images of the *Cux2-CreERT2;Ai9* neocortex after 4-OHT injection at E10.5, immunostained with interneuron markers parvalbumin (G) and somatostatin (H) (green), tdTomato recombined cells are red. (**I**) Confocal images of the *Cux2-CreERT2;Ai9* neocortex after injection with 4-OHT at E10.5, analyzed at P10 or P65. Note the presence of a small number of astrocytes (arrow) at P10 and larger numbers of astrocytes present in groups at P65 (arrows). Scale bars: (D, G-H) 100 μm; (E) 50 μm.