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Supplemental Information

Slit/Robo Signaling Modulates the Proliferation of Central Nervous System Progenitors

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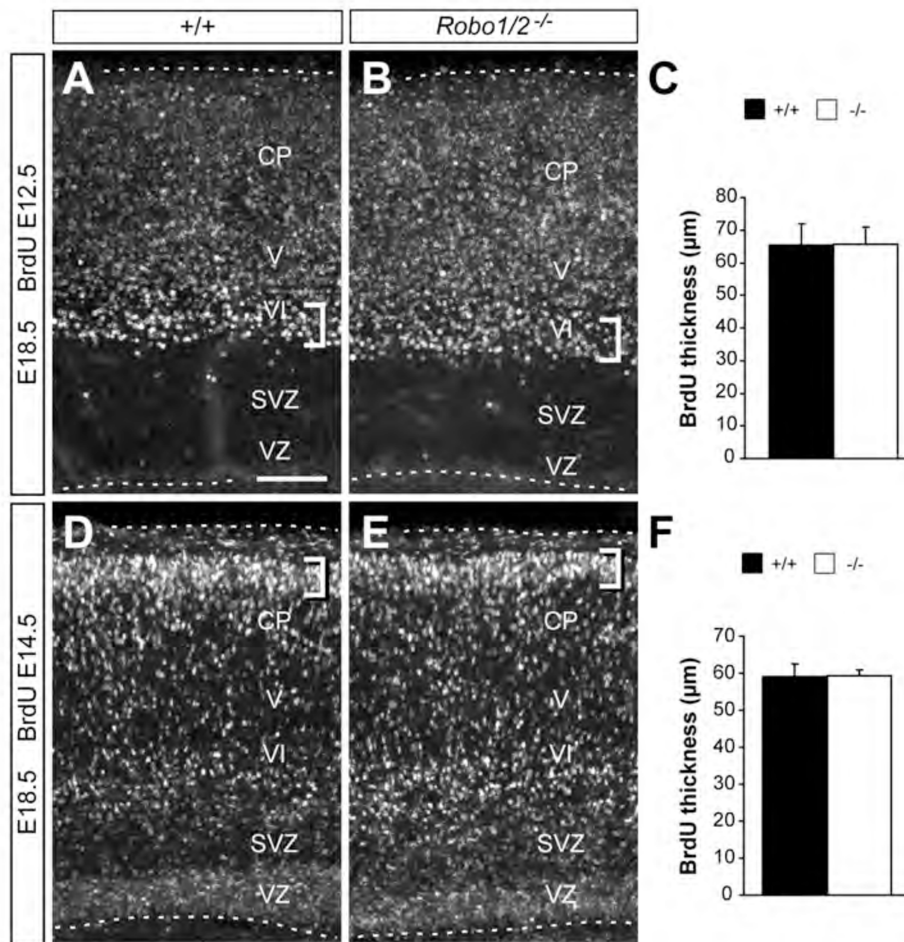


Figure S1. Neuronal production is normal in *Robo1/2* mutants.
(A, B, D and E) Coronal sections of the telencephalon showing the distribution of BrdU labeled cells in E18.5 control (A and D) and Robo1/2 mutants after BrdU injection at E12.5 (A and B) and E14.5 (D and E).
(C and F) Quantification of the thickness of the region containing cells with strong BrdU label in the two experimental conditions (brackets). Mean ± SEM. t-test, $P > 0.05$ for both comparisons.
Scale bar equals 100 μm.

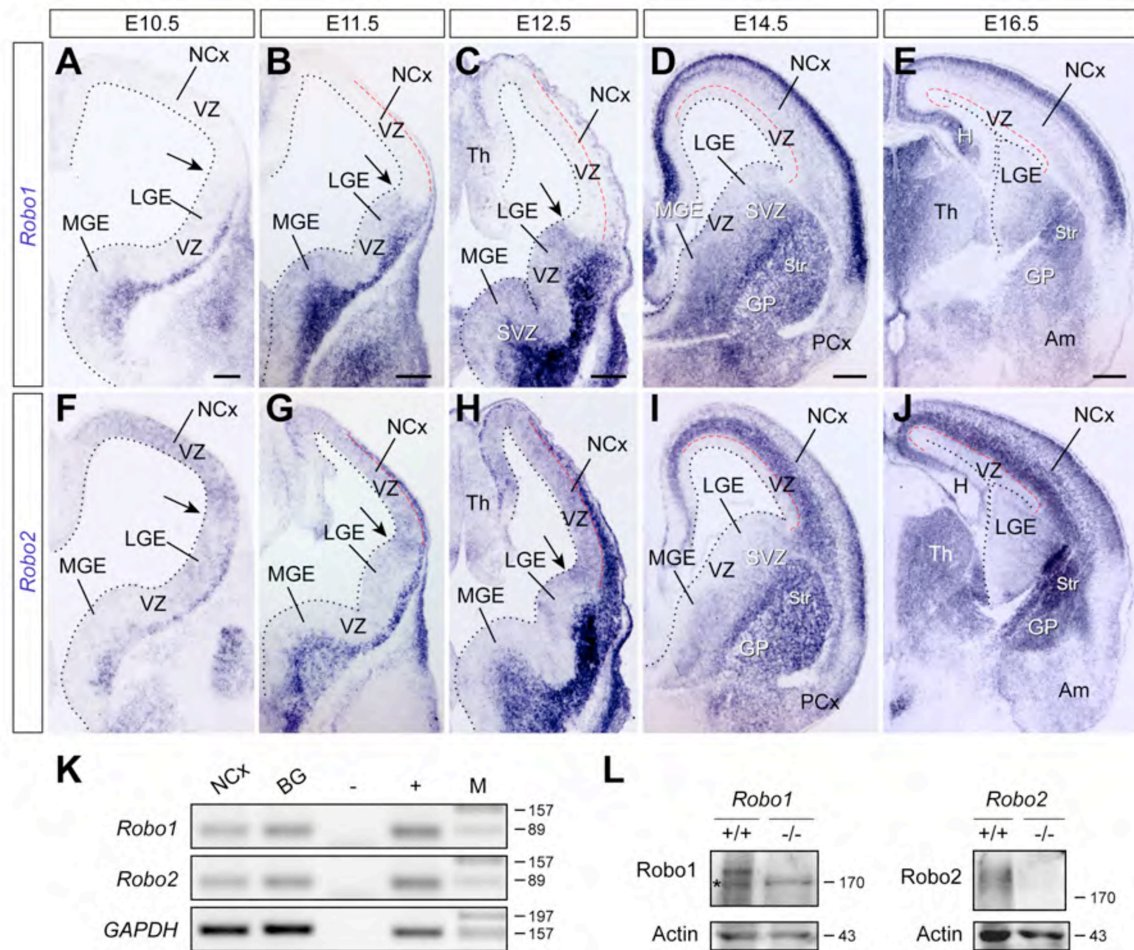


Figure S2. Robo1 and Robo2 are expressed in telencephalic progenitors during embryogenesis.
(A–J) Coronal sections of the telencephalon showing the expression patterns of *Robo1* and *Robo2* mRNA at the indicated ages. Arrows point at the pallial-subpallial boundary. Dotted and dashed lines indicate the apical and basal border of the ventricular zone (VZ), respectively.
(K) Semi-quantitative RT-PCR analysis comparing Robo1 and Robo2 expression in neocortex and basal ganglia of E12.5 embryos. Negative (-, all reagents except cDNA) and positive (+, E14.5 MGE cDNA) controls were included in each run. Amplicon and molecular marker (M) base pairs (bp) are shown at the left and right sides of the panels, respectively. GAPDH was used as loading control.
(L) Western blot analysis of the expression of Robo1 and Robo2 in the neocortex of E10.5 control and Robo1/2 mutant embryos.
Am, amygdala; BG, basal ganglia; GP, globus pallidus; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, pyriform cortex; Str, striatum; Th, thalamus.
Scale bar equals 150 μm (A and F), 250 μm (B and G), 300 μm (C and H) and 500 μm (D, E, I and J).

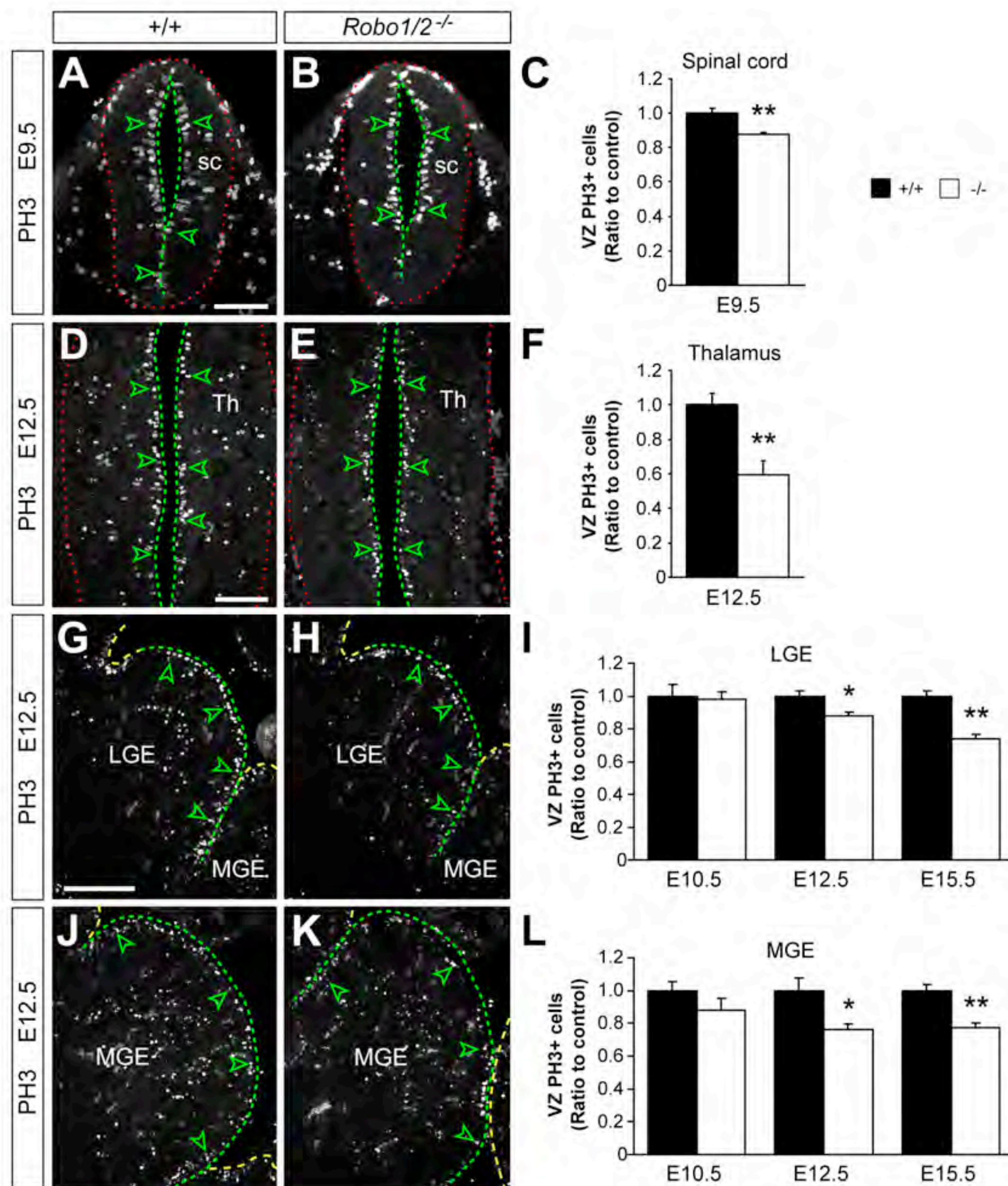


Figure S3. Robo1 and Robo2 are required to sustain ventricular mitosis throughout the CNS.

(A, B, D, E, G, H, J and K) PH3 stains in the spinal cord [(A and B); sc], thalamus [(D and E); Th], lateral ganglionic eminence [(G and H); LGE] and medial ganglionic eminence [(J and K); MGE] in control and mutant embryos at the ages indicated.

(C, F, I and L) Quantification of the linear density of PH3+ nuclei in the ventricular zone (VZ) (arrowheads). Values are expressed as relative to measurements in control embryos. Mean ± SEM. *t*-test; **, $P < 0.01$ (n = 5–6 embryos per group). Dashed green lines indicate VZ included in quantifications and red dotted lines indicate basal border of the tissue. Scale bars equal 50 μm (A and B) and 250 μm (D, E, G, H, J and K).

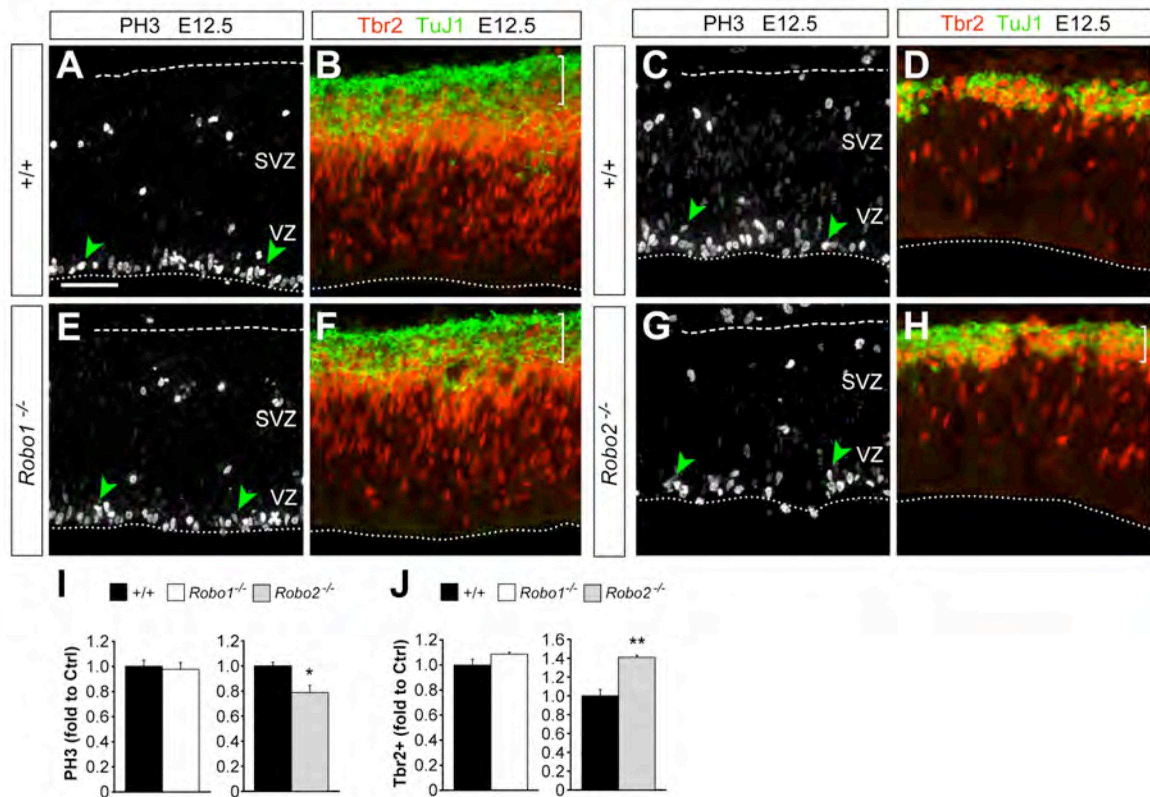


Figure S4. Genetic analysis of *Robo1* and *Robo2* in cortical neurogenesis.
(A–H) PH3, Tbr2 and Tuj1 stains in the neocortex of control (A–D), *Robo1* (E and F) and *Robo2* (G and H) mutant embryos at E12.5. Green arrowheads point to PH3+ nuclei in the ventricular zone (VZ).
(I and J) Quantification of the linear densities of PH3+ nuclei in the VZ and Tbr2+ cells. Mean ± SEM; t-test, *, $P < 0.05$; **, $P < 0.01$ (n = 3 embryos per group).
SVZ, subventricular zone.
Scale bars equal 150 μm (A and F), 250 μm (B, G, K–P), 300 μm (C and H) and 500 μm (D, E, I and J).

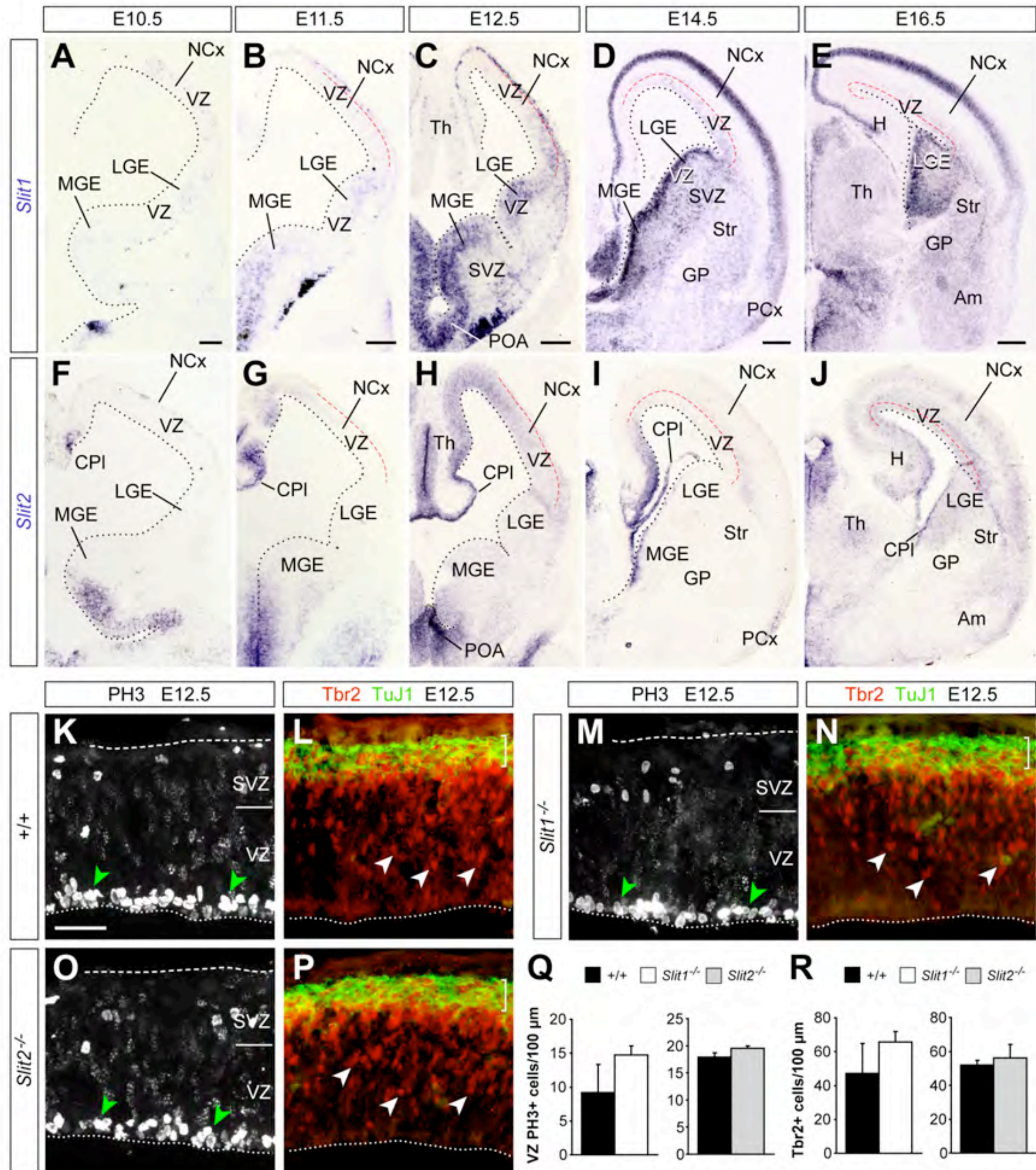
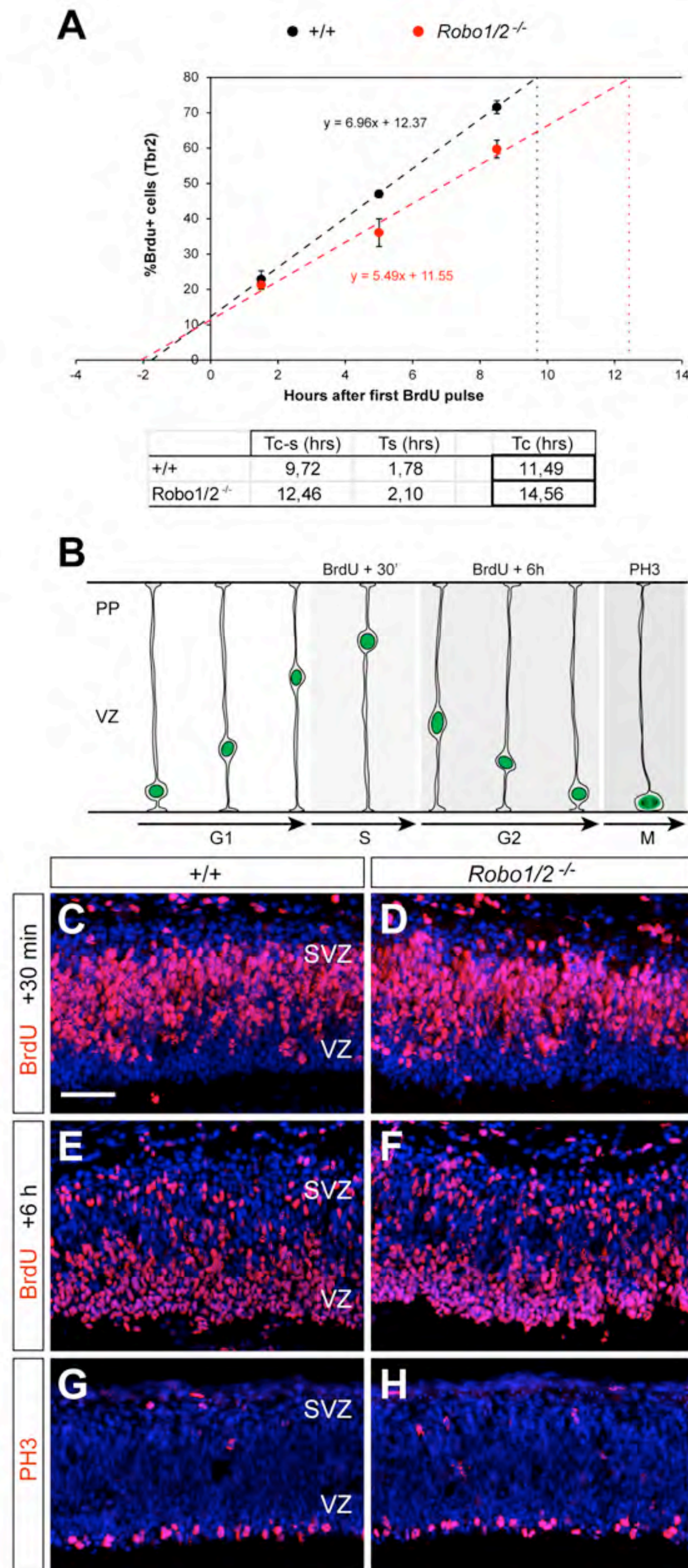


Figure S5. Slit1 and Slit2 cooperate during cortical neurogenesis.
(A–J) Coronal sections of the telencephalon showing the expression patterns of *Slit1* and *Slit2* mRNA at the indicated ages. Dotted and dashed lines indicate the apical and basal border of the cortex, respectively.
(K–P) PH3, Tbr2 and Tuj1 stains in the neocortex of control (K and L), Slit1 (M and N) and Slit2 (O and P) mutant embryos at E12.5. Green arrowheads point to PH3+ nuclei in the ventricular zone (VZ), while white arrowheads point to Tbr2+ cells.
(Q and R) Quantification of the linear densities of PH3+ nuclei in the ventricular zone (VZ) and Tbr2+ cells. Mean ± SEM; t-test, $P > 0.05$ for all comparisons, n = 3–5 embryos per group.
Am, amygdala; CPl, Choroid plexus; GP, globus pallidus; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, pyriform cortex; Str, striatum; SVZ, subventricular zone; Th, thalamus.
Scale bars equal 150 μm (A and F), 250 μm (B, G, K–P), 300 μm (C and H) and 500 μm (D, E, I and J).



	Tc-s (hrs)	Ts (hrs)	Tc (hrs)
+/+	9,72	1,78	11,49
Robo1/2 -/-	12,46	2,10	14,56

Figure S6. Cell cycle length and interkinetic nuclear migration in *Robo1/2* mutants.
(A) Cumulative BrdU labeling in cortical Tbr2+ cells of E12.5 control (+/+) and *Robo1/2* mutant embryos to calculate the duration of the cell cycle without S phase (Tc-s), S phase (Ts), and of the entire cell cycle (Tc). Because only 80% of Tbr2+ cells are progenitors in control and mutant embryos, we estimated that saturation of BrdU labeling is reached at 80%. The table indicates the duration of Tc-s, Ts and Tc (in hours, hrs) and the fold- change for these values, between control and mutant embryos. Data between experimental groups were statistically different, as assessed by using the generalized linear model of a binary logistic regression associated to a χ^2-test, 2 d.f., P < 0.001.
(B) Schematic drawing summarizing cell cycle phases and the strategy used for their identification.
(C–F) Coronal sections through the neocortex of E12.5 control and *Robo1/2* mutants showing the distribution of cells labeled by a BrdU injection 30 min (D and E) or 6 hours before sacrifice.
(G and H) Coronal sections through the neocortex of E12.5 control and Robo1/2 mutants showing PH3 stains.
SVZ, subventricular zone; VZ, ventricular zone.
Scale bar equals 100 μm.

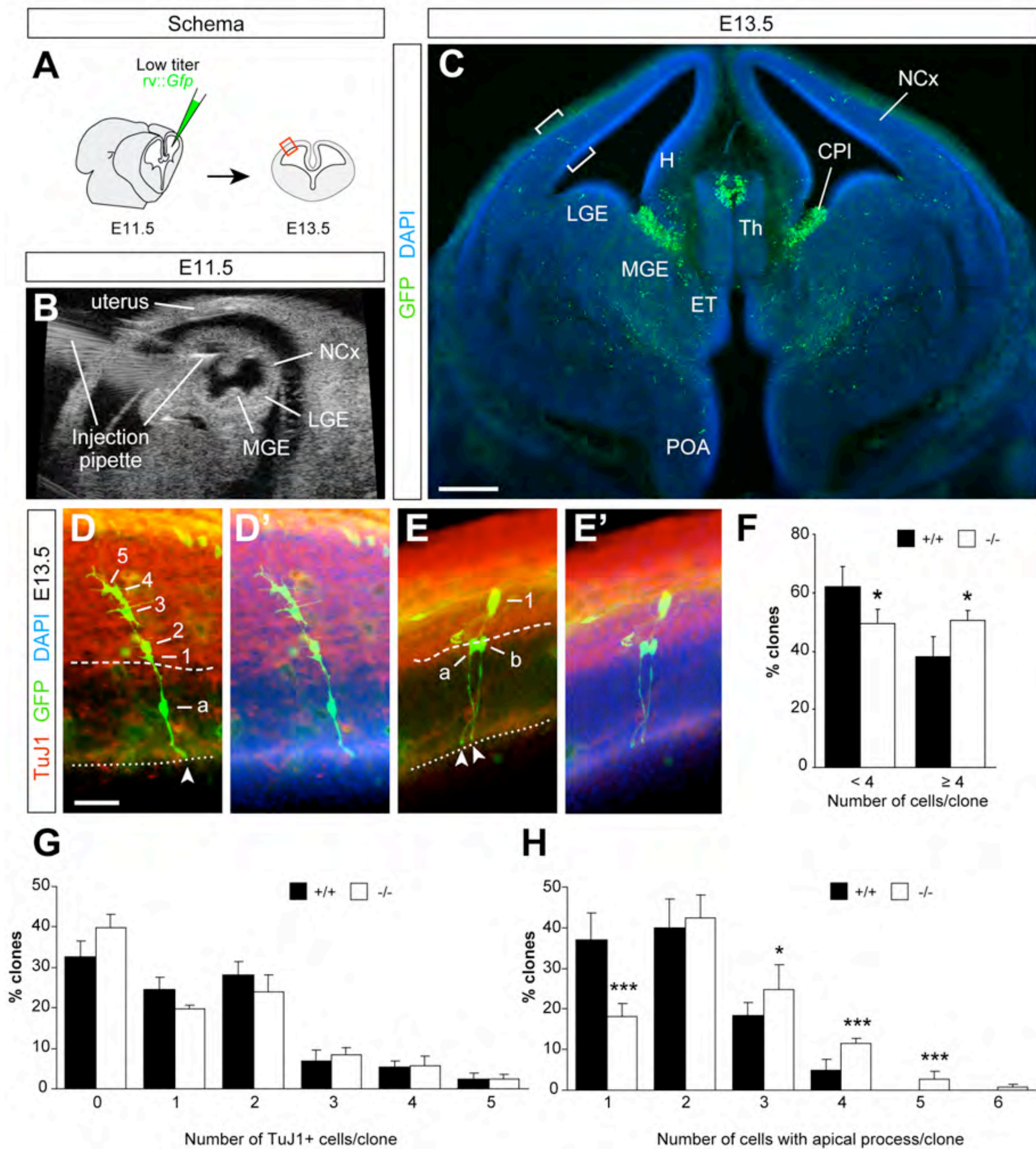


Figure S7. Clonal analysis of progenitors in the embryonic neocortex.
(A–C) Experimental paradigm. Low titer of *Gfp*-encoding retroviruses were injected in utero in the lumen of the telencephalic vesicles of E11.5 embryos, while being monitored with real time ultrasound imaging (B). Embryos were analyzed at E13.5 (C), when isolated clones could be clearly identified. Brackets indicate area shown in (D).
(D–E') Examples of individual clones in control embryos. The clone shown in (D and D') is composed of one TuJ1- cell (a) with an apical process (arrowhead), and five multipolar TuJ1+ cells (1 to 5). The clone shown in (E and E') is composed of two TuJ1- cells (a and b) with apical process (arrowheads), and one TuJ1+ cell (1). Dotted lines indicate apical border of VZ, and dashed lines indicate border between VZ and TuJ1+ layers.
(F) Quantification of the proportion of clones with less than 4 cells per clone, and equal or more than 4 cells per clone. Mean ± SEM; χ^2-test. $\chi^2 = 6.34$, 1 d.f.; *, $P < 0.05$. Control, n = 206 clones from 5 embryos. *Robo1/2* mutants, n = 186 clones from 4 embryos. (G)

Quantification of the proportion of clones containing 0-5 TuJ1+ cells. Mean ± SEM; χ^2-test. $\chi^2 = 2.86$, 5 d.f., $P > 0.7$ for the entire distribution.
(H) Quantification of the proportion of clones containing 1-6 cells with an apical process. $\chi^2 = 62.66$, 5 d.f., $P < 0.001$ for the entire distribution. Mean ± SEM; χ^2-test for pair comparisons, *, $P < 0.05$; ***, $P < 0.001$.
CPl, choroid plexus; ET, eminentia thalami; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; POA, preoptic area; Th, thalamus.
Scale is 500 μm in (C), 50 μm in (D to E').

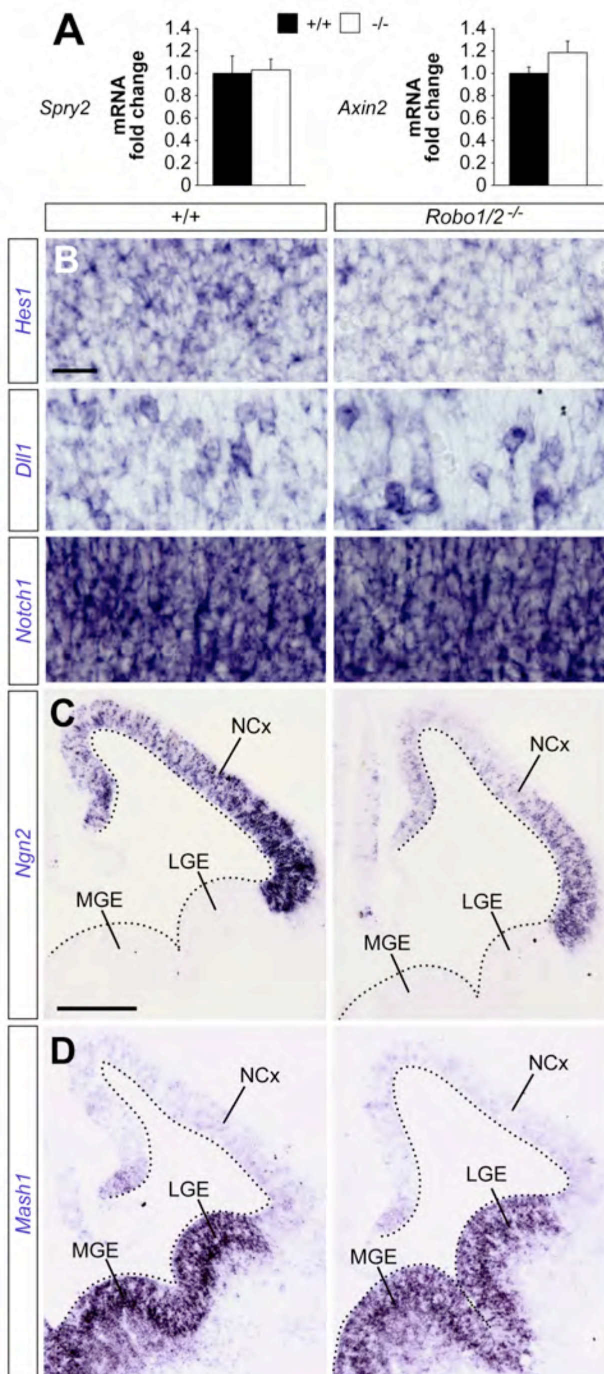


Figure S8. Gene expression changes in the cortex of *Robo1/2* mutants.
(A) Quantitative PCR (qPCR) measurements of Spry2 and Axin2 mRNA expressed as values relative to control embryos (n = 3–5 embryos per group). *t*-test, $P > 0.05$.
(B) High magnification images of the neocortex of E12.5 control and Robo1/2 mutant embryos showing expression of *Hes1*, *Dll1* and *Notch1* mRNA in individually-identified cells.
(C–D) Coronal sections through the telencephalon of control and *Robo1/2* mutant embryos at E12.5 showing *Ngn2* and *Mash1* mRNA expression.
LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; PP, preplate; VZ, ventricular zone.
Scale bar equals 20 μm (B) and 300 μm (C and D).

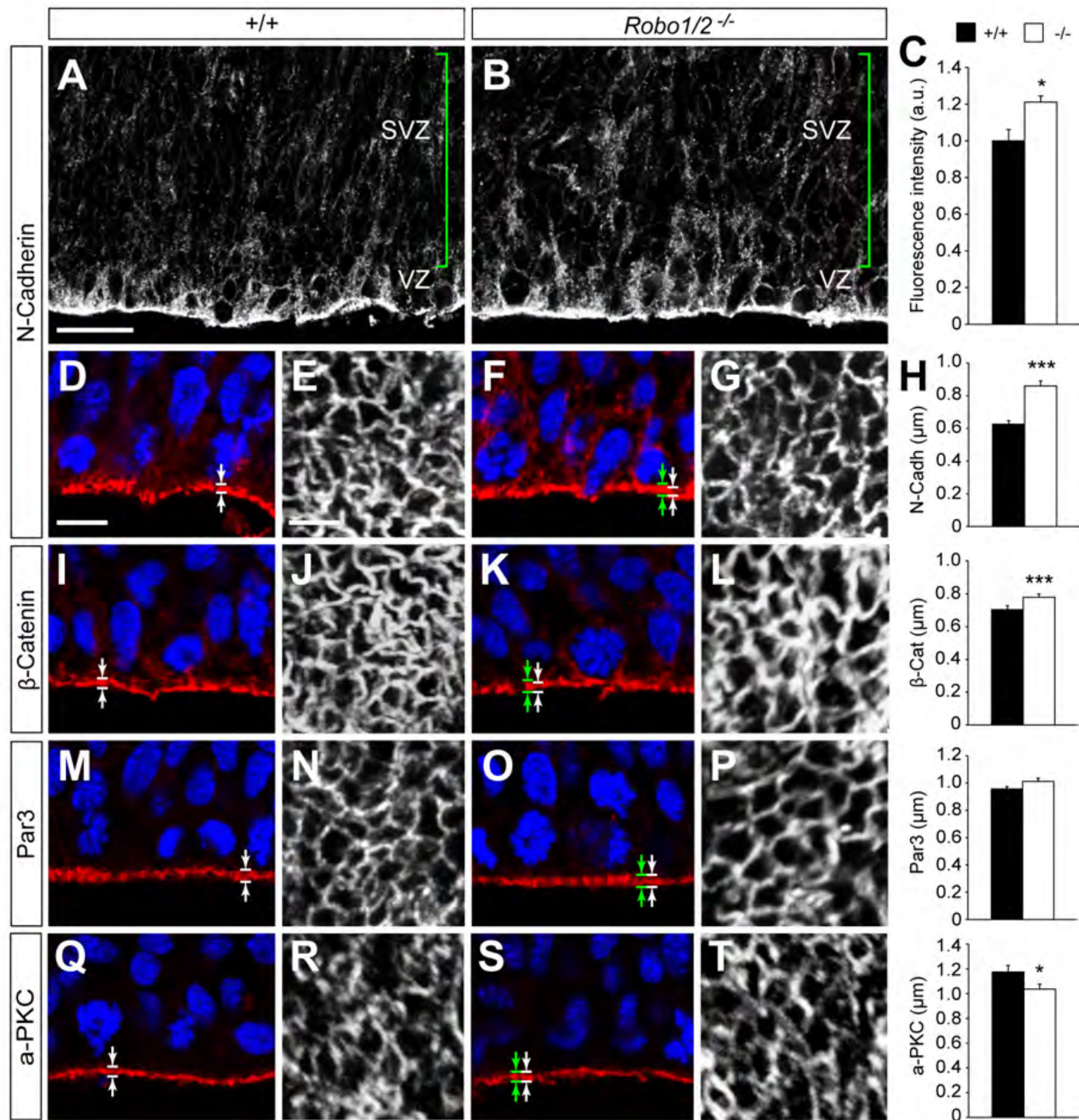


Figure 9. Analysis of adherens junction proteins in *Robo1/2* mutants.
(A, B) Coronal sections through the neocortex of E12.5 control and *Robo1/2* mutant embryos showing immunohistochemistry against N-Cadherin.
(C) Quantification of relative fluorescence intensity of N-Cadherin through the SVZ of control and *Robo1/2* mutant embryos.
(D–G, I–L, M–P and Q–T) Details of the apical band of immunoreactivity against N-Cadherin (D-G), ß-Catenin (I–L), Par3 (A-D) and a-PKC (F–I) in coronal (D, F, I, K, M, O, Q and S) or apical en-face (E, G, J, L, N, P, R and T) views.
(H) Quantification of the thickness of the apical band in N-Cadherin and ß-Catenin stains. *t*-test. *, $P < 0.05$; ***, $P < 0.001$.
Scale bars equal 25 μm (A and B), 10 μm (D, F, I, K, M, O, Q and S), and 10 μm (E, G, J, L, N, P, R and T).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

Wild type mice maintained in a CD1 background were used for cell culture experiments and some expression analyses. Mice carrying loss-of-function alleles for Robo1 and both Robo1 and Robo2 were maintained in an ICR background, while Robo2 mice were maintained in a C57b6 background. The day of vaginal plug was considered as embryonic day (E) 0.5. Mice were kept at the Instituto de Neurociencias de Alicante in accordance with Spanish and EU regulations.

Immunohistochemistry and in situ hybridization

Twenty micrometer frozen brain sections were hybridized with digoxigenin-labeled probes as described before (Flames et al., 2007). For immunohistochemistry of frozen or vibratome brain sections, the tissue was incubated with primary antibodies overnight, followed by appropriate secondary antibodies. In non-fluorescent stainings, sections were further processed by the ABC histochemical method (Vector). Fluorescent stainings were counterstained with DAPI. Primary antibodies used were: anti-BrdU (1:100, Accurate); anti-N-Cadherin (1:1000, BD Transduction); anti-cleaved Caspase 3 (1:150, Cell signaling); anti-ß-Catenin (1:1000, Sigma); anti-GFP (1:1000, Aves Inc.); anti-Ki67 (1:500, Novocastra); anti-Par3 (1:500, Millipore); anti-phosphohistone H3 (1:500, Upstate); anti-a-PKC (1:200, BD Transduction); anti-Robo1 and anti-Robo2 (1:1000, generous gifts of F. Murakami) (Tamada et al., Neural Dev. 3:29, 2008); anti-Tbr1 and anti-Tbr2 (1:1000, generous gifts of R.F. Hevner); anti-ßIII tubulin (1:3000, Covance). Secondary antibodies used were: biotinylated anti-rabbit IgG (Vector); Alexa488 and Alexa546 anti-mouse, anti-rabbit and anti-chicken (Molecular Probes), all diluted 1:200.

Slit binding experiments

Slit2-AP fusion proteins were produced as described before (Fouquet et al., 2007). The brains of wild-type embryos aged E12.5 were dissected out, and the neocortex isolated. After incubation with concentrated conditioned medium containing Slit2-AP or control secretable AP at room temperature, cortices were rinsed, fixed and processed for alkaline phosphatase histochemistry. Cortices incubated with control or Slit2-AP solution were developed in parallel and photographed with identical settings.

Single progenitor clonal analysis and rescue experiments

Robo1/2$^{+/-}$ pregnant females carrying E11.5 embryos were deeply anesthetized with isoflurane and individual embryos were injected with 200 nl of Gfp-encoding retroviruses (5x106 cfu/ml) into the telencephalic ventricles using an ultrasound backscatter microscope, as previously described (Pla et al., 2006). For testing cell-autonomy, E11.5 wild type embryos were injected with retroviruses encoding a dominant negative form of *Robo2* along with *Gfp* (*DN-Robo2-IRES-Gfp*). Retroviral stocks were prepared and concentrated as described previously (Zhao et al., 2006). After 48 h of survival embryos were sacrificed, their heads fixed in 4% paraformaldehyde, and then vibratome-sectioned and processed for immunohistochemistry as described above. For the unequivocal assignment of GFP+ cells as belonging to a particular individual clone, the three-dimensional arrangement of labeled cells within the embryonic neocortex was assessed by scanning the entire thickness of brain sections under confocal microscopy. Once all cells within a single clone were identified, they were counted and classified according to their

expression of Tuj1, Tbr2, and for containing an apical process extended to the ventricular surface.

For gain of function experiments, E12.5 wild type embryos were electroporated in utero with a plasmid encoding a myristoylated form of the cytoplasmic domain of Robo2 (mR2), which acts as a constitutively active form of the receptor, along with a plasmid encoding Gfp. Plasmid DNA was then introduced into telencephalic progenitors by passing electric pulses (5 pulses of 30V, 50 ms On – 950 ms Off) through the whole head with a CUY21SC electroporator and 5 mm paddle electrodes (Nepa Gene). Electroporated embryos were fixed 48 h later, cryosectioned, and processed for immunohistochemistry.
For Hes1 rescue experiments, E12.5 embryos were obtained from Robo1/2+/- pregnant females and placed in ice-cold PBS. Each embryo was injected ex utero into the lateral telencephalic ventricle with 1 l of plasmids encoding Hes1 and Gfp, or Gfp alone. Immediately after electroporation, the brain of each embryo was extracted and vibratome sectioned at 300 microns. The resulting slices were maintained in organotypic culture for 24 hrs, then fixed, cryosectioned, and stained for immunohistochemistry.

For *Hes1* RNAi experiments, E12.5 wild type embryos were electroporated in utero with a cocktail of two siRNA that have been previously shown to produce significant knockdown of mouse *Hes1* (Noda et al., 2011; Ross et al., 2004) or with control siRNA, along with a plasmid encoding *Gfp.* siRNAs were produced by Qiagen against the following target sequences: 5’-GTATTAAGTGACTGACCAT-3’ and 5’-CGACACCGGACAAACCAAA-3’. Electroporated embryos were fixed 48 h later, cryosectioned, and processed for immunohistochemistry.

Primary dissociated cell cultures

The brains of wild-type embryos aged E12.5 were dissected out, and the neocortex isolated. For dissociation, the neocortical tissue was incubated in trypsin-EDTA and DNAse at 37oC for 6 min, followed by gentle trituration. Dissociated cells were plated on glass coverslips coated with poly-lysine and laminin at a density of 4,500 cells/mm^2, and were cultured in Neurobasal medium and incubated at 37^oC in 95% humidity, 5% CO_2.

Luciferase assays

Primary dissociated cell cultures prepared as described above were transfected after 48 hr in culture using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were collected and treated for the detection of luciferase and renilla activity. Briefly, cells were washed, whole cell extracts were obtained in PLB (Promega) and the signal detected with a Berthold luminometer using the Dual-Luciferase® Reporter Assay (Promega). On a different set of experiments, cells from the Neuro-2a cell line were transfected using Lipofectamine 2000 (Invitrogen), and luciferase and renilla activity were detected as described above 24 hours later. For co-culture assays Neuro-2a cells were transfected as above and after 6 hours they were replated in presence of Delta1 expressing or control cells followed by luciferase detection 24 hours later. Constructs used for transfection were: *Hes-Luc* (mouse *Hes1* promoter -192 to +129 driving *Luciferase*) or 2.6 *Hes-Luc* (mouse *Hes1* promoter -2600 to +74 driving *Luciferase*), *RL-CMV* (Promega), empty vector *pCI* (Promega), plus myristoylated-Cyan fluorescent protein (*mCFP*), myristoylated-Robo2 (*mR2*) and/or Notch intracellular domain (*NICD*), or truncated forms of the myristoylated-*Robo2* construct, with truncations spanning the intracellular domains CC3 (*mR2D1*), CC2 and CC3 (*mR2D2*), or CC1, CC2 and CC3 (*mR2D3*).

Semi-quantitative RT-PCR and qPCR

Total RNA from E12.5 cortex and basal ganglia was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A total of 500 ng RNA was treated with DNaseI RNase-free (Fermentas) for 30 min at 37°C prior to reverse transcription into single-stranded cDNA using SuperScriptII Reverse Transcriptase and Oligo(dT)12-18 primers (Invitrogen) for 1 hour at 42°C. The reaction was performed using 2 µl of cDNA, appropriate mouse primers (Robo1-forw, 5'-AGGGAAGCCTACGCAGATG-3'; Robo1-rev, 5'-TGGACAGTGGGCGATTTTAT-3', Robo2-forw, 5'-GAGAATCGGGTGGGAAAAGT-3'; Robo2-rev, 5'-CACAAACTGTGGAGGAGCAA-3'), recombinant Taq DNA polymerase (Invitrogen), and the following conditions: 2 min at 95°C followed by 30 or 35 cycles of 30 s at 95°C (for GAPDH and Robo1/2, respectively), 30 s at 60°C, 30 s at 72°C and final extension at 72°C for 5 min. PCR negative (all reagents except cDNA) and positive (E14.5 MGE cDNA) controls were included in each PCR run. PCR products were analyzed by electrophoresis on a 2% agarose gel. Each independent sample was assayed in duplicate and gene expression levels were normalized using GAPDH.

For quantitative (q) PCR, total RNA was extracted from E12.5 cortical slices at matching rostro-caudal and latero-medial levels between +/+ (n = 16) and Robo1/2-/- (n = 8) embryos. qPCR was carried out in an Applied Biosystems 7300 real-time PCR unit using the Platinum SYBR Green qPCR Supermix UDG with ROX (Invitrogen), 5 l of cDNA, and the appropriate mouse primers: Notch1-forw, 5'-CGCAAGCACCCAATCAAG -3'; Notch1-rev, 5'-TGTCGATCTCCAGGTAGACAATG -3'; Delta1-forw, 5'-CGGCTCTTCCCCTTGTTCTAA -3'; Delta1-rev, 5'-GGGGAGGAGGCACAGTCATC-3'; Hes1-forw, 5'-CGGCATTCCAAGCTAGAGA-3'; Hes1-rev, 5'-GCGGGTCACCTCGTTC-3'. For *Spry2* and *Axin2* qPCR, we used TaqMan® probes (Life Technologies) according to the manufacturer's specifications. Each independent sample was assayed in duplicate. Gene expression levels were normalized using *GAPDH*.

Western blot and dot blot

For detection of Robo2 in E10.5 mouse, the telencephalon of eight embryos was collected in 25 µl of lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.1% (v/v) glycerol and a mixture of protease inhibitors) and sonicated for 15 seconds. Dithiothreitol and Triton X-100 were added to the cell homogenates to final concentrations of 5 mM and 1% (v/v), respectively, followed by overhead rotation for 30 min at 4oC. After centrifugation at 10,000 g for 15 min the supernatant was analyzed by 7.5% SDS-PAGE and blotted onto nitrocellulose. Membranes were incubated at room temperature with 5% BSA in Tris-buffered saline containing 0.05% (v/v) Tween20 (TBST) for 1h, probed with anti-Robo1 (a kind gift of F. Murakami) and anti-Robo2 (R&D Systems) antibodies overnight at 4o C, followed by incubation with HRP-conjugated secondary antibodies and ECL. For the detection of Slit ligands in the CSF, 10 l of CSF from the lateral ventricles of E12.5 embryos, or from COS cell conditioned medium, were adsorbed onto nitrocellulose membranes in a single dot. Membranes were then blocked, followed by incubation with recombinant human ROBO2-Fc chimera (R&D Systems), HRP-conjugated secondary antibodies and ECL.

Quantification and statistics

Volume, thickness and length measurements. Cavalieri estimates of the volume of the whole telencephalon and of the thalamus were measured from DAPI –stained coronal brain

sections using StereoInvestigator software (Microbrightfield™). Total thickness of the cerebral cortex, or thickness of the TUJ1+ or BrdU+ layer, and length of the VZ, were measured from DAPI-stained or immunostained coronal sections using ImageJ software. Cell counts. Cells were counted from the entire medio-lateral extent of the dorso- parietal neocortex and at mid-rostro-caudal levels, identical between controls and mutants. For each section the total cell count was normalized to the length of the VZ. For PH3, nuclei positive for PH3 located at less than 5 cell diameters from the ventricular surface were considered as VZ nuclei, and the remaining were considered as SVZ nuclei. For cleaved Caspase-3, all positive nuclei were counted regardless of their apico-basal position. For Tbr2, all positive nuclei located outside of the TUJ1+ layer were counted. For studies of colocalization, single plane images were obtained using a Leica TCS SL confocal microscope and analyzed with Leica Confocal SoftwareTM.

N-Cadherin and apical surface measurements. Levels of N-Cadherin immunoreactivity (measured as mean grey value), and thickness of apical band for adherens junction proteins, were measured on single plane confocal images using ImageJ. The area for measuring levels of N-Cadherin staining did not include the first 20 microns from the apical surface of the cortex, where immunoreactivity levels are much higher than in the rest of the tissue.

Calculation of Ratio-to-Control, and criterion for phenotypic penetrance. Each parameter in our study was measured on individual embryos. Phenotypic penetrance was variable in different litters of mutant embryos, but roughly 60-70% of the mutant embryos analyzed displayed the phenotypes described in this study. For each litter independently, the mean value among control embryos was calculated. This was then used to calculate the Ratio-to-Control, defined as the ratio between the measurement on each embryo and the mean value among controls for that litter. By definition, the mean value of Ratio-to-Control in control embryos is equal to 1. Next we measured the Standard Deviation (SD) of this Ratio-to-Control among control embryos from all litters pooled. The Ratio-to-Control was then calculated for all mutant embryos, each referred to the mean control value of its own litter. Those mutant embryos with a Ratio-to-Control value closer than 1 SD to the control average were considered phenotypically non-penetrant, and discarded. For the remaining, phenotypically penetrant mutants, the mean and standard error of the mean (SEM) of Ratio-to-Control was calculated.

Data were statistically analyzed with SPSS software using χ^2-test, pair-wise t-test or independent samples t-test, where appropriate. Histograms represent mean ± SEM.