APPENDIX

Mitotic WNT signalling orchestrates neurogenesis in the developing neocortex

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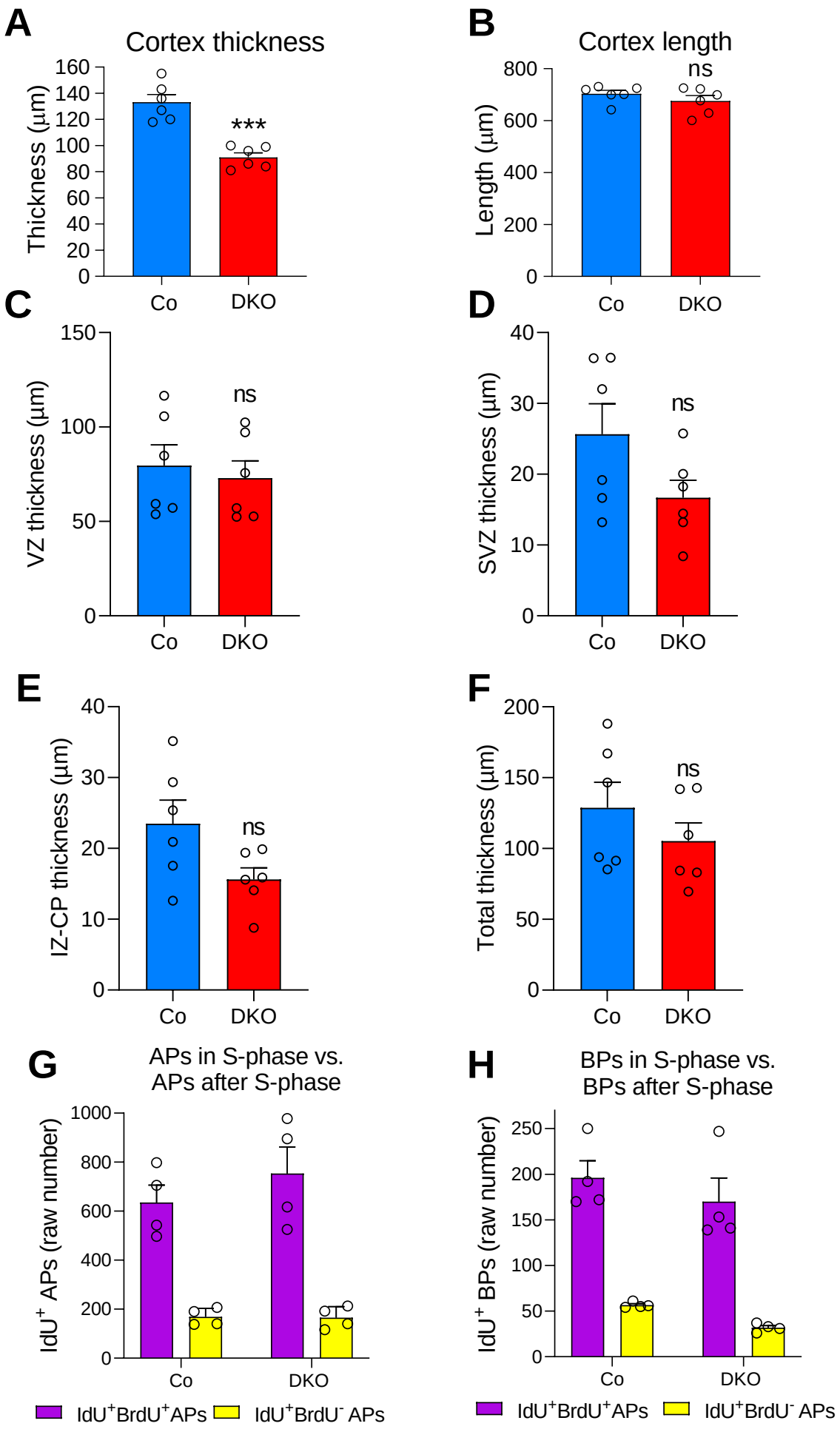

A
Cortex thickness
Thickness (µm)
160
140
120
100
80
60
40
20
0
***
Co
DKO
B
Cortex length
Length (µm)
800
600
400
200
0
ns
Co
DKO
C
VZ thickness (µm)
150
100
50
0
ns
Co
DKO
D
SVZ thickness (µm)
40
30
20
10
0
ns
Co
DKO
E
IZ-CP thickness (µm)
40
30
20
10
0
ns
Co
DKO
F
Total thickness (µm)
200
150
100
50
0
ns
Co
DKO
G
APs in S-phase vs.
APs after S-phase
IdU+ APs (raw number)
1000
800
600
400
200
0
Co
DKO
IdU+BrdU+APs
IdU+BrdU- APs
H
BPs in S-phase vs.
BPs after S-phase
IdU+ BPs (raw number)
250
200
150
100
50
0
Co
DKO
IdU+BrdU+APs
IdU+BrdU- APs

Appendix Figure S1. Data related to Figures 1 and 2.

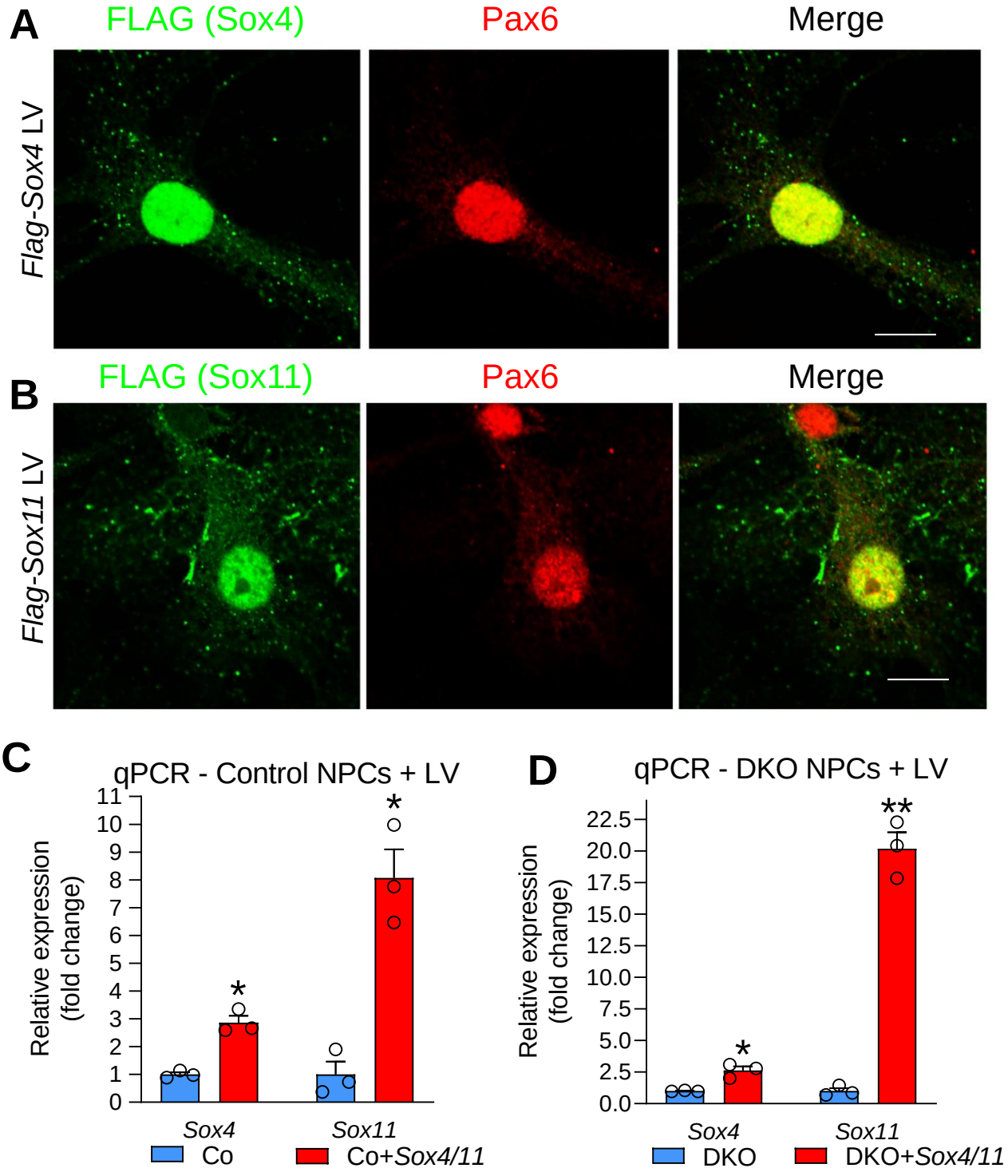
(A) Measurement of neocortex thickness on H & E stained sections of control and DKO E13.5 embryos from Fig 1A. Data are means ± SEM (n=6, 4 litters).

(B) Measurement of neocortex length on H & E stained sections of control and DKO E13.5 embryos from Fig 1A. Data are means ± SEM (n=6, 4 litters).

(**C-F**) Measurement of ventricular zone (VZ) (A), subventricular zone (SVZ) (B), intermediate zone-cortical plate (IZ-CP) (C), and total thickness (D) in neocortex sections from E13.5 control and DKO embryos. Data shown are specific values (in µm) from Fig 1C. Data are means ± SEM (n=6, 4 litters).

(**E-F**) Raw numbers of $Brdu^{+}IdU^{+}$ vs. $BrdU^{-}IdU^{+}$APs (E) and BPs (F) in neocortex sections from E13.5 control and DKO embryos. Data are means ± SEM (n=4, 3 litters). Data shown are specific values from Fig 2H.

Unpaired two-tailed *t*-test for all statistical analyses: ns, not significant; *** $p < 0.001$. $p = 0.00040$ (A); $p = 0.30$ (B); $p = 0.65$ (C); $p = 0.11$ (D); $p = 0.061$ (E); $p = 0.32$ (F).


A
FLAG (Sox4)
Pax6
Merge
Flag-Sox4 LV
B
FLAG (Sox11)
Pax6
Merge
Flag-Sox11 LV
C
qPCR - Control NPCs + LV
Relative expression (fold change)
Sox4
Sox11
Co
Co+Sox4/11
D
qPCR - DKO NPCs + LV
Relative expression (fold change)
Sox4
Sox11
DKO
DKO+Sox4/11
E
GFP DAPI
CAG-IRES-EGFP LV
F
FLAG (Sox4/11) DAPI
Flag-Sox4 +
Flag-Sox11 LVs

Appendix Figure S2. Data related to Figure 6.

Validation of *Sox4/11* lentiviral overexpression in NPC cultures

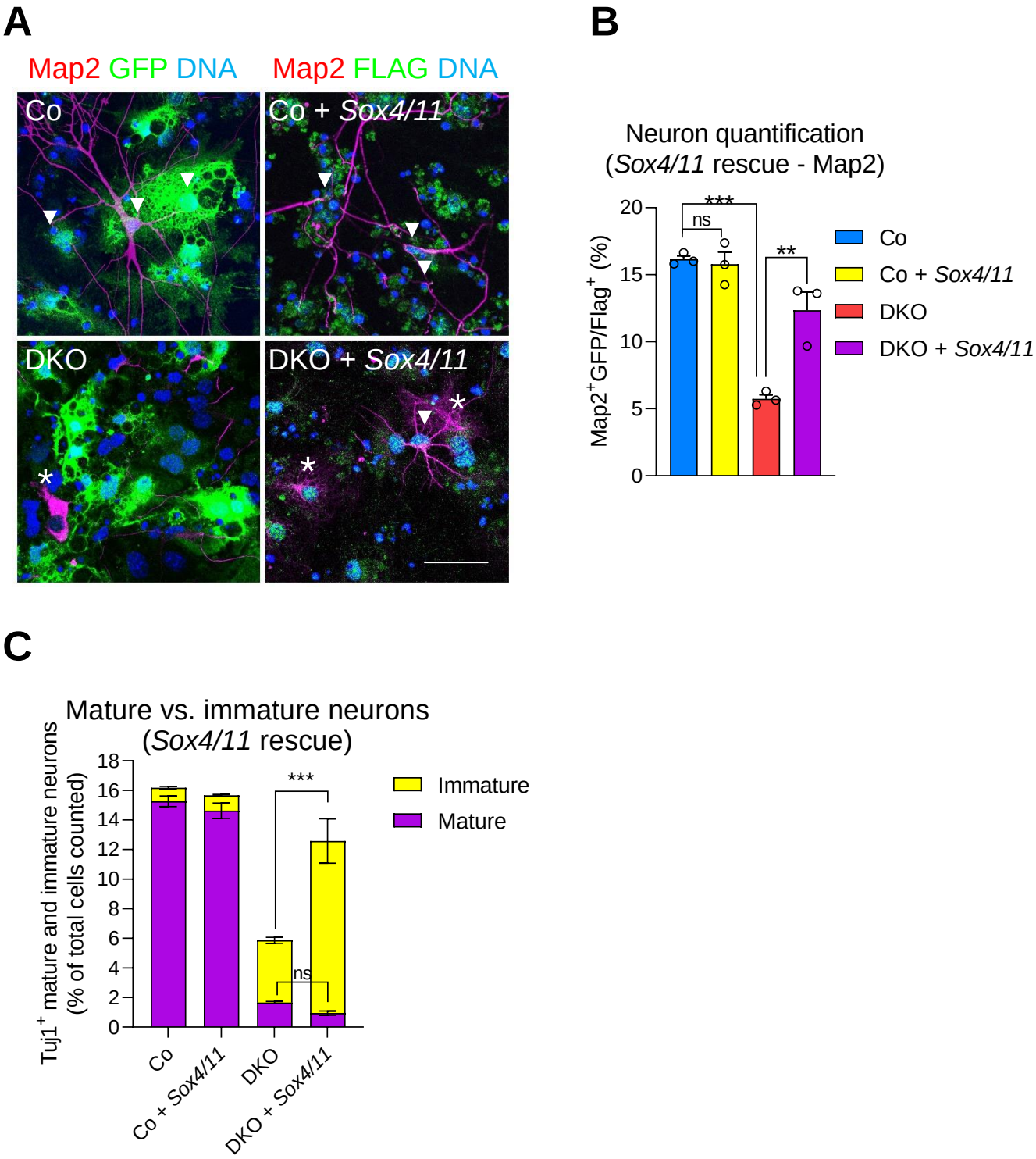
(**A-B**) Co-IF for FLAG and Pax6 in E13.5 NPCs transduced with (A) *Flag-Sox4* or (B) *Flag-Sox11*-overexpressing lentiviruses (LV). Specific nuclear co-staining is detected for both *Sox4* and *Sox11*. Scale bar 5 μm.

(**C-D**) *Sox4* and *Sox11* qPCR analysis of control (C) and DKO (D) E13.5 NPCs transduced with *Sox4/11*-overexpression LVs. Data are expressed as fold change vs. controls and are means ± SEM (n=3).

(**E**) IF for GFP in control NPCs transduced with empty CAG-IRES-EGFP lentivirus. Note the high transduction efficiency. Scale bar 40 μm.

(**F**) IF for FLAG in control NPCs transduced with *Flag-Sox4* and *Flag-Sox11* overexpressing lentiviruses. Note the high transduction efficiency. Scale bar 40 μm.

Unpaired two-tailed *t*-test for all statistical analyses: ns, not significant; * $p < 0.05$, ** $p < 0.01$. $p = 0.010$ (C); $p = 0.0036$ (D).


A
Map2 GFP DNA
Map2 FLAG DNA
Co
Co + Sox4/11
DKO
DKO + Sox4/11
B
Neuron quantification
(Sox4/11 rescue - Map2)
Map2+GFP/Flag+ (%)
20
15
10
5
0
***
ns
**
Co
Co + Sox4/11
DKO
DKO + Sox4/11
C
Mature vs. immature neurons
(Sox4/11 rescue)
Tuj1+ mature and immature neurons
(% of total cells counted)
18
16
14
12
10
8
6
4
2
0
***
ns
Immature
Mature
Co
Co + Sox4/11
DKO
DKO + Sox4/11

Appendix Figure S3. Data related to Figure 6.

Validation of *Sox4/11* rescue in DKO NPCs

(**A**) IF for Map2 and Tuj1 (not shown) to identify newly formed neurons in NPCs for Sox4/11 rescue (same experiment as Fig 6B-C). NPCs transduced with empty CAG-IRES-EGFP (Co and DKO) or *Flag-Sox4/11* lentiviruses as indicated. Transduced cells identified by IF for GFP (Co and DKO) or FLAG (Co + *Sox4/11* and DKO + *Sox4/11*). Arrowheads indicate mature neurons and asterisks denote immature neurons lacking neurite extension. Scale bar 20 μm.

(**B**) Quantification of transduced $Map2^+$ cells in (A). Data are means ± SEM (experiment performed twice in triplicates, representative experiment shown). One way ANOVA test for statistical analyses.

(**C**) Quantification of transduced mature and immature $Tuj1^+$ neurons in control, control + *Sox4/11*, DKO, and DKO + *Sox4/11* NPCs from Fig 6B-C. Data are means ± SEM (experiment performed twice in triplicates, representative experiment shown). Two way ANOVA test for statistical analyses.

For all statistical analyses: ns, not significant; ** $p < 0.01$, *** $p < 0.001$. All scale bars 20 μm. $p = 0.99$ (B, yellow bar); $p = 0.00010$ (B, red bar); $p = 0.0022$ (B, purple bar); $p = 0.0000030$ (C, yellow bars); $p = 0.99$ (C, purple bars).

Appendix Methods

Image acquisition and analysis

HE-stained sections were imaged with a Leica DMIL LED microscope mounted with a Canon EOS 600D camera. IF samples were imaged using a Zeiss LSM 700 microscope. The pinhole was set to 1 Airy unit at maximal optical resolution and gain and offset were calibrated so that fluorescent signal intensities were within the liner range of detection.

Neocortex thickness and length

Average cortical wall thickness was measured on HE stained sections of the cortical forebrain in 5 different areas per section. Neocortex length was measured from the junction between the lateral ganglionic eminence and lateral neocortex to the most medial point of the neocortex (see Fig 1A). At least 6 matched (similar regions of the neocortex) sections were measured per embryo for both analyses.

Measurement of VZ, SVZ and IZ+CP thickness

VZ, SVZ and IZ+CP thickness was measured using Fiji. VZ vs. SVZ vs. IZ+CP were defined based on the orientation and density of the cell nuclei as revealed by DNA staining and the immunofluorescence pattern of the basal progenitor marker Tbr2. Two pictures (one picture near the lateral ganglionic eminence and the other in a more medial position) were taken per section and at least 7 matched sections were measured per embryo.

Quantification of specific cell types in developing neocortex

IF using anti-Pax6 + Tbr2, Tbr2 only, and Tbr1 antibodies was performed to quantify the number of APs, BPs and post-mitotic neurons, respectively. Data were expressed as percentages of $Pax6^{+}Tbr2^{-}$ (APs), $Tbr2^{+}$ (BPs), or $Tbr1^{+}$ (neurons) nuclei per total nuclei (DNA^{+}) in at least 5 matched sections per embryo for each analysis. Two pictures (see above) were taken per section. Average number of cells (DNA^{+}) counted for AP quantification per embryo: 5158 (Co) and 4733 (DKO). Average number of cells counted for BP quantification per embryo: 5771 (Co) and 4613 (DKO). Average number of cells counted for post mitotic neuron quantification per embryo: 2770 (Co) and 2321 (DKO).

Quantification of Ccny/l1 and pLRP6 immunoreactivity in non-mitotic and mitotic BPs, and in mitotic APs

Triple IF for Tbr2, pHH3 and Ccny or Ccnyl1 was performed on E13.5 control embryos and the number of $Ccny/l1^{+}$ non-mitotic and mitotic BPs, as well as mitotic APs were counted on tile scans from 2 sections of the lateral cortex. Cells counted for Ccny: 1356 non-mitotic BPs, 35 mitotic BPs, and 86 mitotic APs. Cells counted for Ccnyl1: 1432 non-mitotic BPs, 22 mitotic BPs, 92 mitotic APs. For pLRP6, co-IF for pHH3 and pLRP6 (T1479 or S1490) was performed on E13.5 control embryos and the number of pLRP6+ mitotic APs and BPs were counted on tile scans from 2 sections of the lateral neocortex. Cells counted for pLRP6 T1479: 27 mitotic BPs and 105 mitotic APs. Cells counted for pLRP6 S1490: 34 mitotic BPs and 110 mitotic APs.

Quantificaion of pLRP6 T1479 staining intensity in mitotic APs and BPs

Pixel intensity was calculated for an average of 417 mitotic APs and 114 mitotic BPs per embryo from 10 matched sections and values were normalized to background fluorescence. Regions of interest (ROIs) were drawn around mitotic cells using the square tool and the integrated density function from Fiji was used to calculate pixel intensity. The area of the cells in the ROIs was measured with the freehand tool and subtracted from the pixel intensities for final values.

RNAScope

Axin2 expression levels in the cortex as revealed by RNAScope analysis was calculated by scoring the *Axin2*-positive area divided by the total cortex area in 6 matched sections per embryo. Areas were calculated with Fiji software by selecting the threshold value and then using the analyse→measure function. Areas were calculated for the entire image (as shown in Fig 1O) and not for individual cells. No AP or BP-specific markers were used for this analysis.

Analysis of BrdU-labeled NPCs

Co-IF using anti-BrdU plus either anti-Pax6 + Tbr2 and anti-Tbr2 only antibodies was performed to quantify the number of APs and BPs in S-phase, respectively. Data were expressed as percentages of $BrdU^{+}Pax6^{+}Tbr2^{-}$ or $BrdU^{+}Tbr2^{+}$ cells per the total number of $Pax6^{+}Tbr2^{-}$ or $Tbr2^{+}$ cells, respectively. Two pictures (see VZ, SVZ and CP measurement) from at least 6 matched sections were counted per embryo.

Calculation of the length of S-phase, total cell cycle, and mitosis

S-PHASE LENGTH: E13.5 neocortex sections were triple-stained for IdU, BrdU and Tbr2, and the numbers of $IdU^+/BrdU^-$ cells (i.e. NPCs having left S-phase 1.5 hours after the IdU labeling) and $IdU^+/BrdU^+$ cells (i.e. NPCs still remaining in S-phase 1.5 hours after the IdU labeling) were counted for APs ($Tbr2^-$) and BPs ($Tbr2^+$) on at least 10 images from matched sections of control and DKO embryos. For each of the four NPC populations, the numbers obtained were expressed as percentages of the total number of IdU^+ cells (see Fig 2H). S-phase length (T_S) was calculated by dividing 1.5 hours (the interval between IdU and BrdU injection) by the percentage values for the NPCs having left S-phase after this time period ($IdU^+/BrdU^-$ cells, see Fig 2H, yellow) and multiplying the resulting values times 100, which yielded the times until all IdU-labeled NPCs would have left S-phase, that is, the length of S-phase (Fig 2I).

The formula used was: $$T_S = 1.5 \text{ hours} / \frac{IdU^+BrdU^- \text{ NPCs}}{IdU^+ \text{ NPCs}}$$

TOTAL CELL CYCLE LENGTH: Total cell cycle (T_C) length was calculated by dividing, for each type of NPC, the S-phase length value by the percentage value for this type of NPC in S-phase (as determined after a 1-hour BrdU pulse, see above) and multiplying the resulting value times 100 (Fig 2J).

The formula used was: $$T_C = T_S / \frac{BrdU^+ \text{ NPCs}}{\text{total NPCs}}$$

LENGTH OF MITOSIS: Different sections from the same embryos were stained for pHH3, Ki67 and Tbr2, and the percentage of NPCs in mitosis was determined for APs ($pHH3^+Ki67^+Tbr2^-/Ki67^+Tbr2^-$) and BPs ($pHH3^+Ki67^+Tbr2^+/Ki67^+Tbr2^+$) on at least 10 matched sections per embryo. The length of mitosis (T_M) was calculated for each type of NPC multiplying the total cell cycle length value (T_C) times the percentage value for the respective type of NPC in mitosis, and dividing the resulting value by 100 (Figure 2M).

The formula used was: $$T_M = T_C \text{ x } \frac{pHH3^+ \, Ki67^+ \text{ NPCs}}{Ki67^+ \text{ NPCs}}$$

The strategies for the above cell cycle analyses were adapted from Arai *et al*, 2011 and Turrero Garcia *et al*, 2016. It is worth noting that the total cell cycle length values for E13.5 wt APs (18.1 hours) and E13.5 wt BPs (22.1) determined in the present study (see Fig 2) fit quite well to those determined previously at E14.5 by cumulative EdU labeling (Arai *et al*, 2011; 19.1 hours for APs, 26.5 hours for BPs; cell cycle length is known to increase at later developmental stages, i.e. E14.5 vs. E13.5).

Apoptosis analysis

Quantification of apoptotic cells was performed by IF with an anti-active caspase3 antibody or by fluorescent TUNEL staining. Results were obtained after scoring the percentage of active caspase 3 or TUNEL cells/5000 cells on at least 5 matched sections per embryo.

Asymmetric vs. symmetric AP division

Asymmetric vs. symmetric AP division was determined as described previously (Kosodo *et al*, 2004). Briefly, to identify asymmetrically vs. symmetrically dividing APs at the ventricular surface of the neocortex, sections were immunostained with an anti-pan-cadherin antibody to visualize the apical-most lateral plasma membrane and to thereby identify the apical plasma membrane (pan-cadherin-negative region, referred to as "cadherin hole"). Anaphase and early telophase APs were identified by DNA staining using Hoechst 33258 dye. The orientation of the cleavage plane was deduced from the position of the Hoechst-stained sister chromatids. APs whose cleavage plane was predicted to result in an unequal (bypassing the cadherin hole) or equal (bisecting the cadherin hole) distribution of apical membrane were considered as asymmetrically and symmetrically dividing cells, respectively. At least 25 mitotic APs were analyzed per embryo.

Astral microtubule quantification

IF for α-tubulin was performed on sections of E13.5 control and DKO embryos containing neocortex. Z-stacks (8-10 μm thick) were acquired at 1-μm intervals and the number of apical, basal and central astral microtubules was counted for at least 10 metaphase cells per embryo, as described previously (Mora-Bermúdez *et al*, 2014) using the *Cell Counter* plugin in Fiji.

IUE quantification

For all analyses, indicated co-immunostaining quantified on at least 3 bins per embryo for each treatment.

Neurite length quantification

Neurite length measurement was performed with the Fiji NeuronJ plugin according to Pemberton *et al*, 2018. Briefly, the IF images of Tuj1 labeled neurons were opened with the NeuronJ macro, and each neurite path was traced with the cursor. After being traced, the length of each neurite was converted to microns. In each group the neurites of 60 neurons were counted.

Sox4/Sox11 in vivo quantification

IF for Sox4/Sox11 plus Tbr2 and pHH3 was performed on sections of E13.5 control and DKO embryos. At least 30 mitotic BPs were analyzed per embryo. Cells were scored as high or low based on fluorescence intensity compared to non-mitotic cells. Ratio of high vs. low intensity cells was plotted as the final result. For quantification of Sox4 staining in non-mitotic BPs, pixel intensity was calculated for at least 20 non-mitotic BPs per embryo and values were normalized to background fluorescence. ROIs were drawn around mitotic cells manually using the square tool and the integrated density function from Fiji was used to calculate pixel intensity. The area of the cells in the ROIs was measured with the freehand tool and subtracted from the pixel intensity for final values.

For quantification of Sox11 in BPs, the number of $Tbr2^+/Sox11^+$ cells divided by the total number of $Tbr2^+$ cells was counted on at least 6 matched sections per embryo (2 pictures per section). Average number of BPs counted per embryo was 1108 for controls and 697 for DKOs.

For all analyses Fiji software was used to calculate the various parameters such as cortex thickness and length, cell number, pixel area etc. For *in vivo* analyses, 1-2 pictures per section were taken at 20x or 40x magnification. At least 3-5 independent embryos per genotype from different litters were quantified. *In vitro* experiments were performed in duplicates, triplicates or quadruplicates, and were repeated once or twice as indicated in the figure legends. Each well was considered an independent sample for all *in vitro* quantifications. For western blots representative samples shown and numbers represent quantifications from all samples analysed.

Table S1: List of primers used in this study

Primer name and sequence
qRT-PCR primers for mouse *Axin2* (UPL probe #50): FW:CCATGACGGACAGTAGCGTA RV: GCCATTGGCCTTCACACT
qRT-PCR primers for mouse *N-myc* (UPL probe #12): FW:CCTCCGGAGAGGATACCTTG RV:TCTCTACGGTGACCACATCG
qRT-PCR primers for mouse *Gapdh* (UPL probe #9): FW**:**AGCTTGTCATCAACGGGAAG RV: TTTGATGTTAGTGGGTCTCG
qRT-PCR primers for mouse *βIII-tubulin* (UPL probe #104): FW: GCGCATCAGCGTATACTACAA RV:TTCCAAGTCCACCAGAATGG
qRT-PCR primers for mouse *Doublecortin* (UPL probe #18): FW:AGCTGACTCAGGTAACGACCA RV:GCTTTGACTTAGGTGTTGAGAGC
qRT-PCR primers for mouse *Sox2* (UPL probe #67): FW:GGCAGAGAAGAGAGTGTTTGC RV: TCTTCTTTCTCCCAGCCCTA
qRT-PCR primers for mouse *Sox4* (UPL probe #79): FW: ACAGCGACAAGATTCCGTTC RV: GTCAGCCATGTGCTTGAGG
qRT-PCR primers for mouse *Sox11* (UPL probe #20): FW: GAGCTGAGCGAGATGATCG RV: GAACACCAGGTCGGAGAAGT
Cloning primers for *Sox4*-Flag (*PCS2*$^{+}$*): FW:TAAGCAGGATCCATGGATTACAAGGATGACGACGATAAGGTACAACAGACCAACAA RV: TGCTTACTCGAGTCAGTAGGTGAAGACCAGGT
Cloning primers for *Sox11*-Flag (*PCS2*$^{+}$*): FW:TAAGCAGGATCCATGGATTACAAGGATGACGACGATAAGGTGCAGCAGGCCGAGAGC RV:TGCTTACTCGAGTCAATACGTGAACACCAGGTC

Table S2: List of Antibodies used in this study

Antibodies	Manufacturer	Identifier
Mouse monoclonal anti-active β-catenin	Millipore	Cat#05-665
Mouse monoclonal anti-β-catenin	BD Bioscience	Cat#610154
Mouse monoclonal anti-BrdU	BD Bioscience	Cat#555627
Rabbit polyclonal anti-active caspase 3	R & D	Cat#AF835
Rabbit polyclonal anti-CCNY	Homemade	Davidson *et al*, 2009
Rabbit polyclonal anti-CCNYL1	Homemade	Koch *et al*, 2015
Guinea pig polyclonal anti-SOX4	Homemade	Hoser *et al*, 2008
Guinea pig polyclonal anti-SOX11	Homemade	Hoser *et al*, 2008
Rabbit polyclonal anti-SOX4	Diagenode	Cat#C15310129
Mouse monoclonal anti-SOX2	R & D	Cat# MAB2018
Rabbit polyclonal anti-MAP2	Millipore	Cat#AB5622
Rat monoclonal anti-CTIP2	Abcam	Cat#Ab18465
Rabbit polyclonal anti-CDK14	Abnova	Cat#PAB2949
Mouse monoclonal anti-FLAG M2	Sigma Aldrich	Cat#F3165
Goat polyclonal anti-GFP	genomics	Cat#ABIN100085
Rat monoclonal anti-TBR2	Invitrogen	Cat#14-4875-80
Rabbit polyclonal anti-TBR2	Abcam	Cat# ab23345
Mouse monoclonal anti-GSK3β	BD bioscience	Cat#610201
Mouse monoclonal anti-tubulin	Sigma Aldrich	Cat#T5168
Rat monoclonal anti-Ki67	ebioscience	Cat#14-5698-82
Rat monoclonal anti-IdU	Abcam	Cat#ab187742
Rabbit monoclonal anti-LRP6	CST	Cat#2560S
Rabbit polyclonal anti-Phospho-LRP6 (S1490)	CST	Cat#2568S
Rabbit polyclonal anti-Phospho-LRP6 (T1479)	Homemade	Davidson *et al*, 2005
Rabbit polyclonal anti-PAX6	Biolegend	Cat#901301
Mouse monoclonal anti-nestin	EMD Millipore	Cat#MAB353
Mouse monoclonal anti-pan-cadherin	Santa Cruz	Cat#sc-59876
Mouse monoclonal anti-phospho-Histone H3	CST	Cat#9706S
Rat monoclonal anti-HA	Roche	Cat# ROAHAHA
Goat anti-mouse IgG (H+L) HRP	Jackson ImmunoResearch	Cat#115-035-146
Goat anti-rabbit igG (H+L) HRP	Jackson ImmunoResearch	Cat#111-035-144
Goat anti-Guinea pig (H+L) HRP	Jackson ImmunoResearch	Cat#106-035-003
Goat anti-Rat IgG Light chain specific HRP	Jackson ImmunoResearch	Cat#112-035-175
Donkey polyclonal Anti-Mouse IgG C^{TM}y3	Jackson ImmunoResearch	Cat#715-165-150
Donkey polyclonal Anti-Mouse IgG, Alexa Fluor 647	Jackson ImmunoResearch	Cat#715-605-151
Donkey polyclonal Anti-Mouse IgG, Alexa Fluor 488	Jackson ImmunoResearch	Cat#715-545-150
Donkey polyclonal Anti-Rabbit IgG C^{TM}y3	Jackson ImmunoResearch	Cat#711-165-152
Donkey polyclonal Anti-Rabbit IgG, Alexa Fluor 647	Jackson ImmunoResearch	Cat#711-605-152
Goat polyclonal Anti-Rabbit IgG, Alexa Fluor 488	Invitrogen	Cat#A11008
Goat polyclonal Anti-Guinea Pig IgG, Alexa Fluor 546	Invitrogen	Cat#A11074
Donkey polyclonal Anti-Rat IgG, Alexa Fluor 647	Jackson ImmunoResearch	Cat#712-605-153