Supplemental Table 1, related to Fig. 1

Estimated average cell volumes per genotype and region at St16+200 min

	B1-B2 average cell volume (μm^3)	**SD**	**T2-T3 average cell volume (μm^3)**	**SD**	**A8-A10 average cell volume (μm^3)**	**SD**
Control	**70.30**	**5.41**	**72.83**	**12.76**	**68.82**	**16.55**
ED225	**48.99 ** **	**9.19**	**48.99 ** **	**5.55**	**54.32 * **	**6.96**

The values for average cell volume in B1-B2, T2-T3 and A8-A10 were used to estimate total cell numbers in these three regions in control and *ED225* mutant embryos (Fig. 1h). The average cell volume is calculated as the ratio of the volume of restricted DAPI signal to the number of cells, manually counted in that region. Independent measurements were done for control and *ED225* mutant embryos in the specified regions of the brain, thorax and abdomen. No significant differences were observed between average cell volumes in the three regions for either genotypes (one-way ANOVA; control; $p \geq 0.9$, *ED225*; $p \geq 0.59$, $n \geq 5$ embryos per genotype and region). In all three regions, the average cell volumes for *ED225* mutants were significantly lower than those of control embryos (two-tailed Student's T-test, * $p \leq 0.05$, ** $p \leq 0.01$; SD= standard deviation; $n \geq 5$ embryos per genotype and region).

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Supplemental Table 2, related to Fig. 3

Estimated average cell volumes per genotype in B1-B2 at St16+200 min

	B1-B2 average cell volume (μm^3)	**SD**
prosG4,ED225	**71.10**	**12.82**
pros>BX-C,ED225	**82.69**	**14.16**

The average cell volume of each genotype was used to estimate total cell numbers in B1-B2 (Fig. 3m). The average cell volume is calculated as the ratio of the volume of restricted DAPI signal to the number of cells, manually counted in that region. Independent measurements were done for *prosG4,ED225* and *pros>BX-C,ED225* embryos in B1-B2 hemi-segments of the brain. No significant difference was observed between average cell volumes of the two genotypes (two-tailed Student's T-test, $p \geq 0.07$; SD= standard deviation; $n \geq 5$ embryos per genotype).

Supplemental Table 3, related to Fig. 4

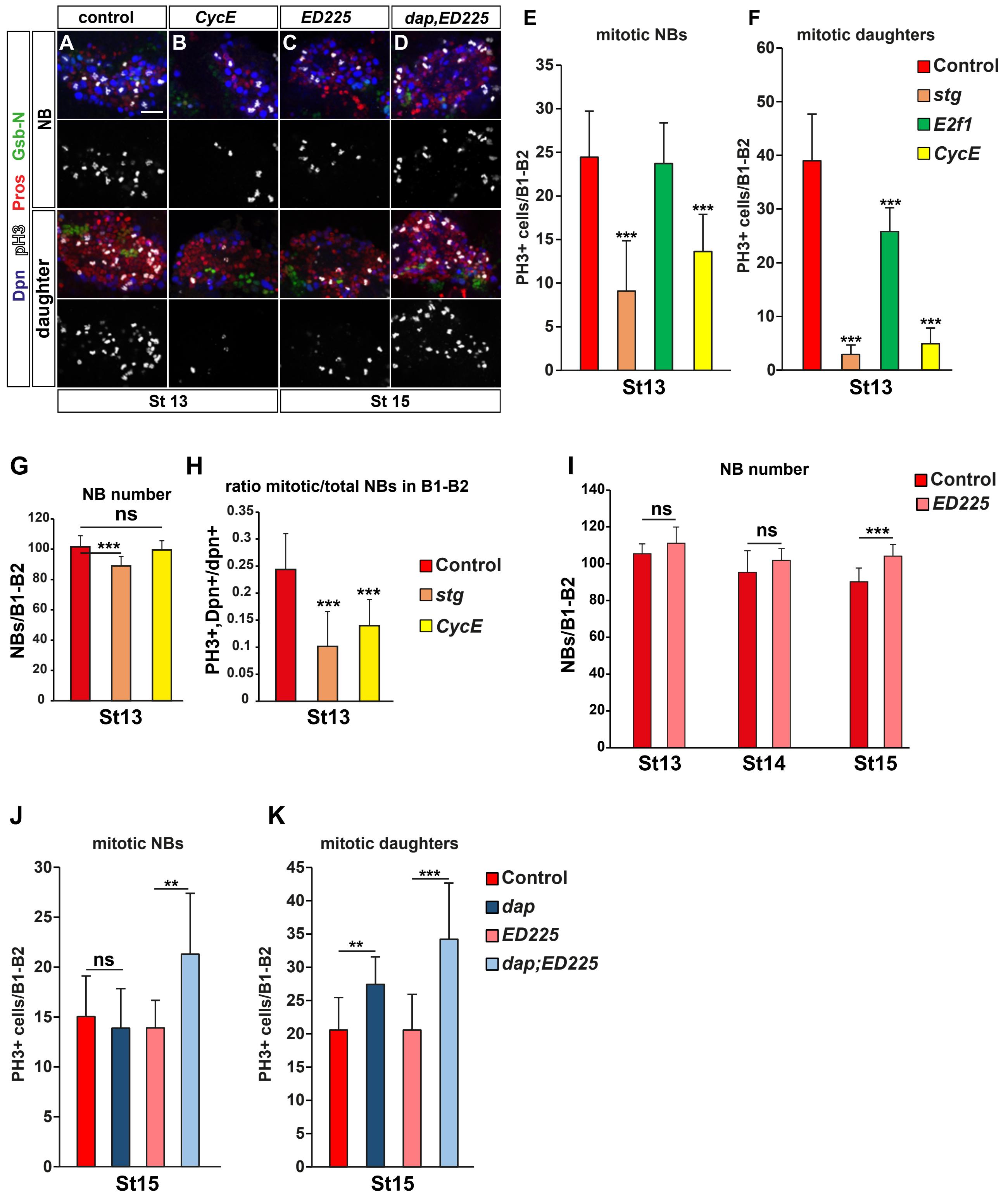
Estimated average cell volumes per genotype and region at St16+200 min

	B1-B2 average cell volume (µm^3)	**SD**	**A8-A10 average cell volume (µm^3)**	**SD**
ED225	**48.99**	**9.19**	**54.32**	**6.96**
esc;ED225	**89.65 *****	**17.48**	**66.49 ***	**15.56**

The values for average cell volume in B1-B2 and A8-A10 were used to estimate total cell numbers in these regions in *ED225* and *esc;ED225* mutant embryos (Fig. 4v). The average cell volume is calculated as the ratio of the volume of restricted DAPI signal to the number of cells, manually counted in that region. Independent measurements were done for *ED225* and *esc;ED225* mutant embryos in the specified regions of the brain and abdomen. In both regions, the average cell volumes for *ED225* mutants were significantly lower than those of *esc;ED225* embryos (two-tailed Student's T-test, * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$; SD= standard deviation; $n \geq 5$ embryos per genotype and region).

Supplemental Figures

The brain depends upon *stg, CycE, E2f1* and *dap*



Development • Supplementary information

Figure S1.

The brain depends on the same four key cell cycle genes as the nerve cord.

(A-D) z-Projections of brain lobes showing mitotic NBs (upper panels) and daughters (lower panels), at St13 in control, *CycE*, *ED225* and *dap;ED225* mutants, at St15 (scale bar, 20µm). (E-F) Quantification of mitotic NBs and daughters in B1-B2, at St13 in control, *stg*, *E2f1* and *CycE* mutants. (G) Quantification of total NB numbers in B1-B2 at St13 in control, *stg* and *CycE* mutants. (H) Ratio of mitotic to total NBs in control, *stg* and *CycE* mutants in B1-B2 at St13. (I) Quantification of total NB numbers in B1-B2 at stages 13,14 and 15 in control and *ED225*. (J-K) Quantification of mitotic NBs and daughters in B1-B2, at St15 in control, *dap*, *ED225* and *dap;ED225* (Student's t test; SD; n≥10 embryos per genotype).

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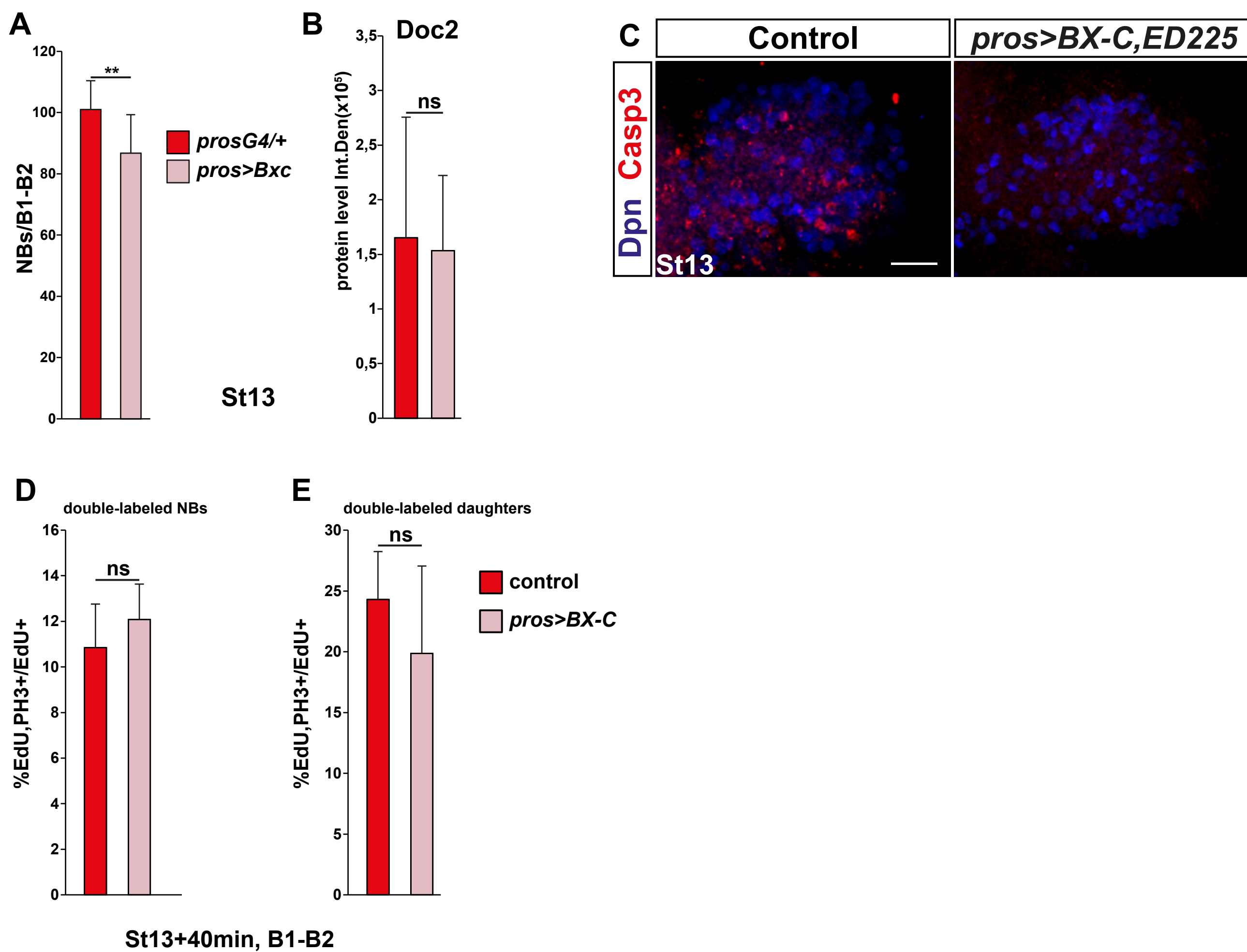
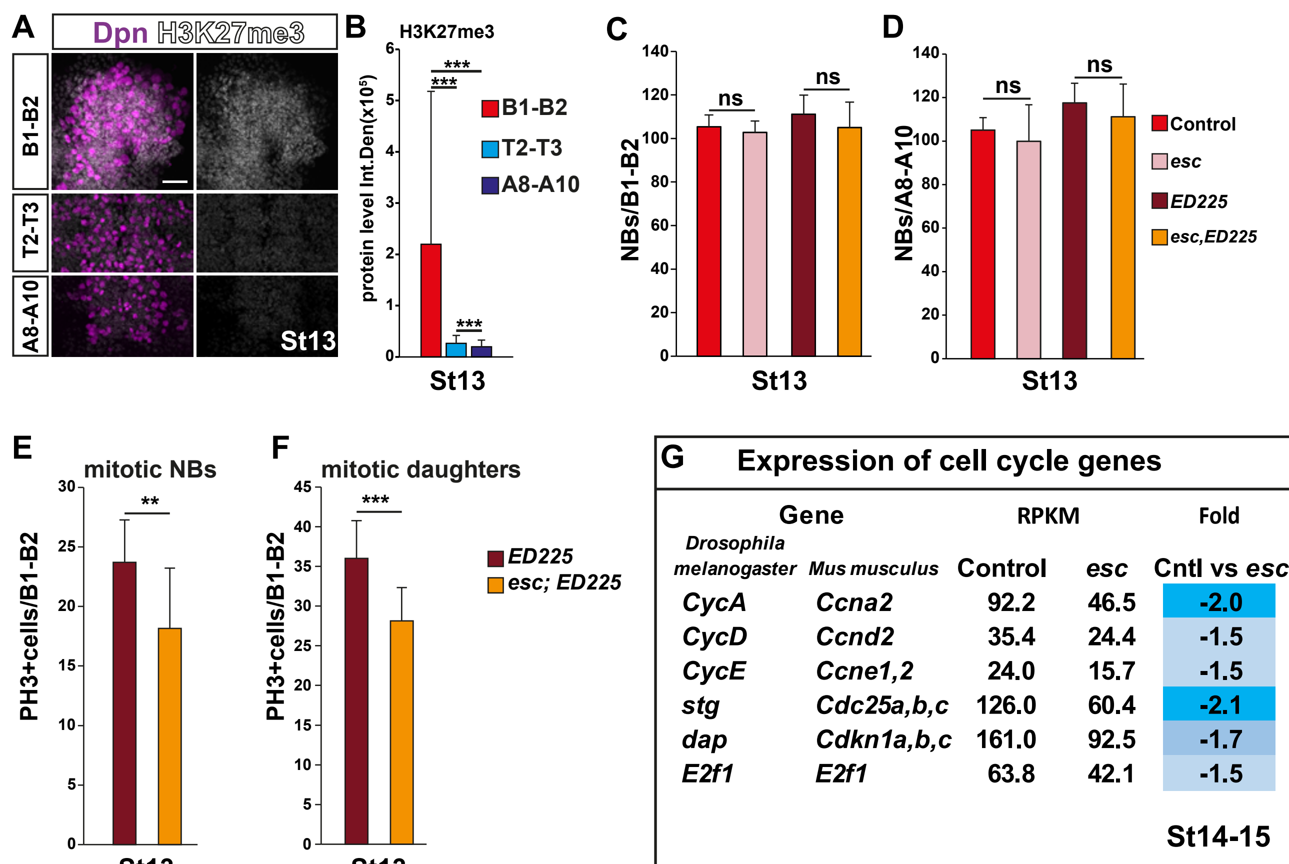


Figure S2.

Misexpression of *BX-C* reduces proliferation in brain daughters and NBs.

(A) Quantification of total NB numbers in B1-B2 in *prosG4/+* and *pros>BX-C*, at St13 (Student's t test; SD; n≥10 embryos per genotype). (B) Quantification of expression levels of Doc2 in B1-B2 NBs, at St13 in *prosG4/+* and *pros>BX-C* embryos (Integrated Density = area x mean gray value; Mann-Whitney U test; SD; n≥3 embryos, n≥248 NBs per genotype). (C) z-Projection images showing expression of the cell death marker, cleaved-Caspase3, in St13 brain lobes of control and *pros>BX-C,ED225* (scale bar; 20µm). (D-E) Ratios of double-labeled (EdU+, PH3+) to only EdU+, NBs and daughters in B1-B2, at St13 after a 40-min EdU pulse in control and *pros>BX-C* embryos (Student's t test; SD; n≥10 embryos per genotype).



Gene: *Drosophila melanogaster*	Gene: *Mus musculus*	RPKM: Control	RPKM: *esc*	Fold: Cntl vs *esc*
CycA	*Ccna2*	92.2	46.5	-2.0
CycD	*Ccnd2*	35.4	24.4	-1.5
CycE	*Ccne1,2*	24.0	15.7	-1.5
stg	*Cdc25a,b,c*	126.0	60.4	-2.1
dap	*Cdkn1a,b,c*	161.0	92.5	-1.7
E2f1	*E2f1*	63.8	42.1	-1.5

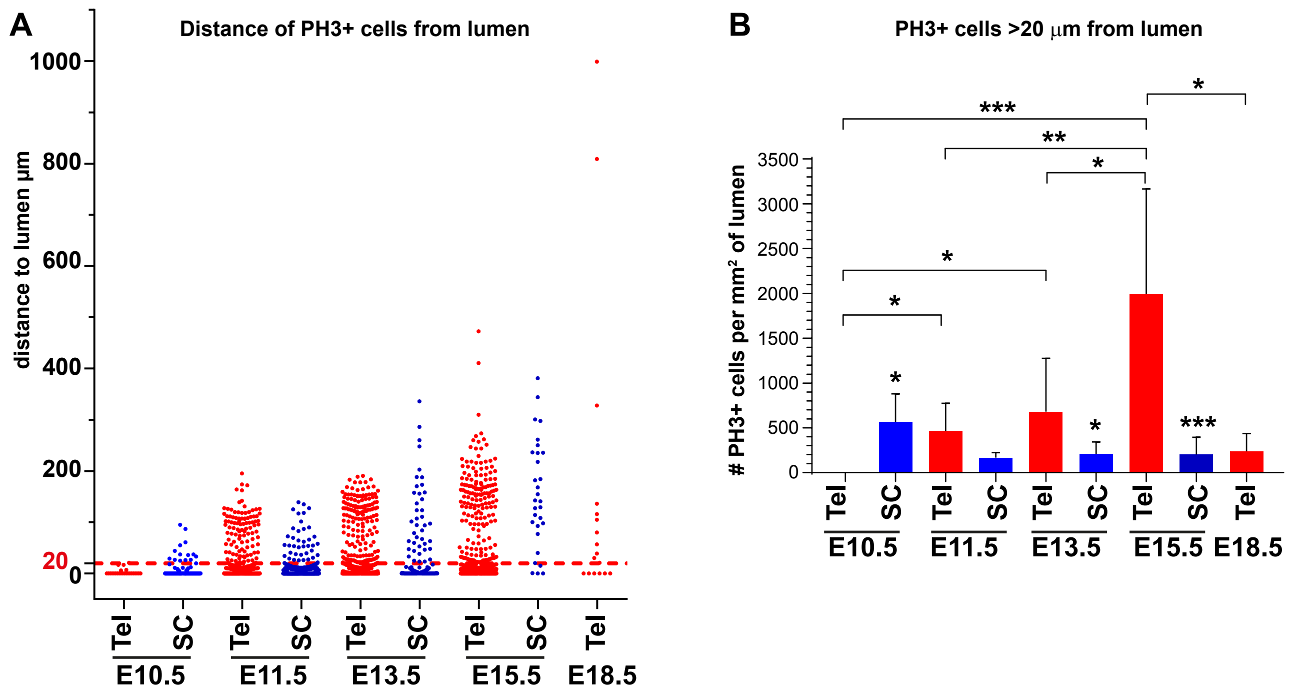
Figure S3.

Elevated H3K27me3 in brain, but no effect on NB numbers in *esc* mutants.

(A) z-Projections of H3K27me3 expression in B1-B2, T2-T3 and A8-A10, in St13 control embryos (scale bar; 20µm). (B) Quantification of H3K27me3 expression in NBs of St13 control embryos in B1-B2, T2-T3 and A8-A10 (Integrated Density = area x mean gray value; independent samples Kruskal-Wallis test, Mann-Whitney U test; SD; n≥3 embryos, n≥148 NBs per region). (C-D) Quantification of total NB numbers in B1-B2 and A8-A10, in control, *esc*, *ED225* and *esc;ED225* embryos, at St13 (Student's t test; SD; n≥6 embryos per genotype and region). (E-F) Quantification of mitotic NBs and daughters in B1-B2 in *ED225* and *esc;ED225* St13 embryos (Student's t test; SD; n≥8 embryos per genotype). (G) Whole embryo RNA-seq analysis of candidate cell-cycle genes in control and *esc* mutants. Columns from left to right; homologous genes in *Drosophila* and mouse, RPKM values in control and *esc* mutants, comparative *esc* to control fold changes (n=2 embryos per genotype, n=4 technical replicates per genotype, RPKM; reads per kilobases per million, FC; fold of change; FC>2;red, FC=1;white, FC<2;blue).

Development • Supplementary information

More dividing daughters in Telencephalon



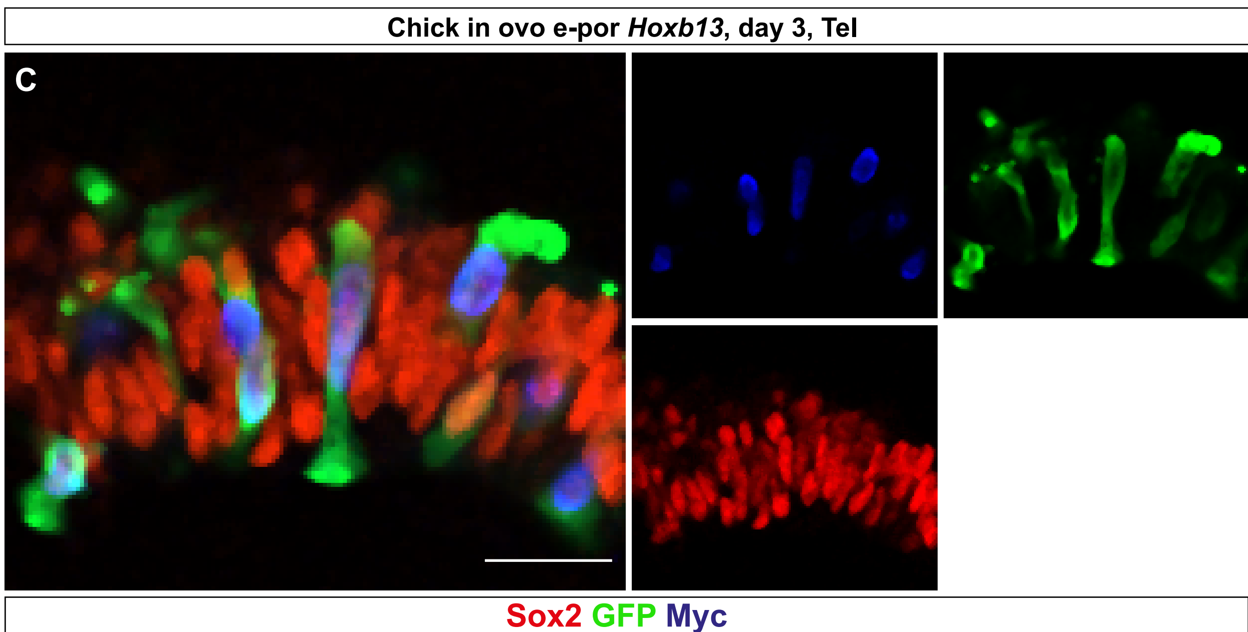


Figure S4.

Elevated daughter proliferation in telencephalon, and Hoxb13 misexpression.

(A) Position of mitotic cells in relation to the lumen, of Tel and SC tissues in control, at E11.5, E13.5 and E15.5 (red dashed line denotes 20 µm). (B) Number of mitotic cells in Tel and SC more than 20 µm distant from the lumen, normalized for luminal area (comparison at each stage using Mann-Whitney U-test; comparison between stages using Kruskal-Wallis with Bonferroni correction: SD; n≥3 embryos and 6-9 sections, 20-40 µm, per genotype and region). (C) Sox2, GFP and Myc staining of telencephalon to show co-electroporation of GFP and Hoxb13-myc plasmids in the chick embryo (scale bar = 20 µm).

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Loss of H3K27me3 in *Eed-cKO* CNS

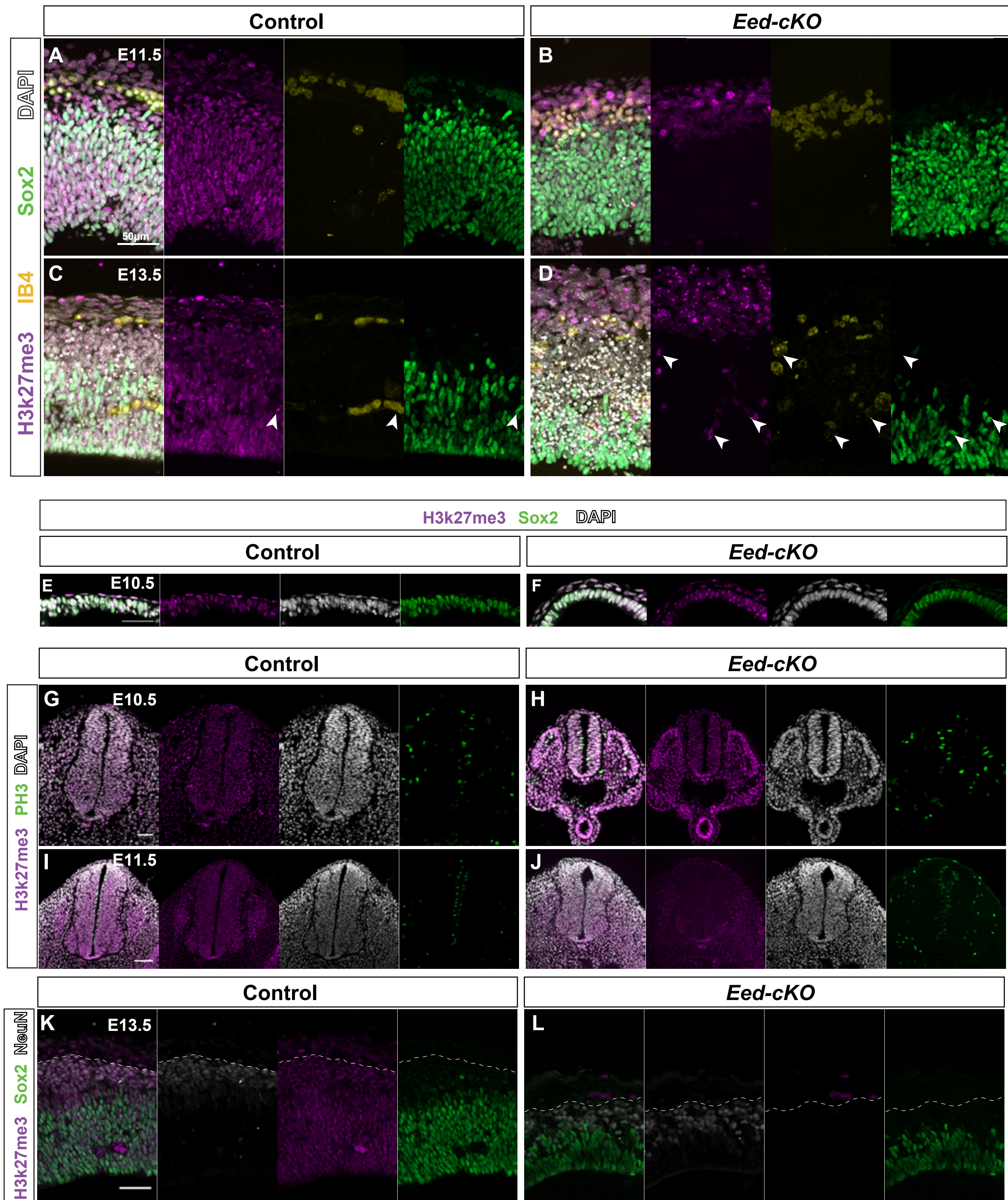

Figure S5.

H3K27me3 is lost in the CNS of *Eed-cKO* embryos, but still present in vascular tissue.
(A-D) 12 to 18 µm confocal image projections of horizontal sections of Tel of control and *Eed-cKO* embryos stained for H3K27me3 and the vasculature marker IB4, at E11.5 (A-B) and E13.5 (C-D). *Eed-cKO* shows loss of H3K27me3 in the CNS proper, but staining is evident in vasculature (white arrows; scale bar = 50µm). (E-F) Staining for H3K27me3 in horizontal sections of Tel, of control and *Eed-cKO* embryos, at E10.5. *Eed-cKO* shows staining for H3K27me3 in the CNS proper (Sox2 cells; scale bar = 50µm). (G-J) Staining for H3K27me3 in horizontal sections of SC, of control and *Eed-cKO* embryos, at E10.5 (top) and E11.5 (bottom). The SC of *Eed-cKO* embryos shows reduced expression of H3K27me3 at E10.5 (H) and the expression is lost at E11.5 (J) when compared to control at the respective age (G, I) (scale bar = 50µm E-F, 100µm G-H). (K-L) Staining for the neuron-specific nuclear marker NeuN, which marks the outer-most layer of the CNS-proper/differentiated cells of CNS and H3K27me3 in horizontal sections of Tel, of control and *Eed-cKO* embryos, at E13.5. *Eed-cKO* shows no H3K27me3 in the CNS-proper when compared to control, but staining is evident outside the NeuN marker limit (15 µm projection confocal images, white dashed line; scale bar = 50µm).

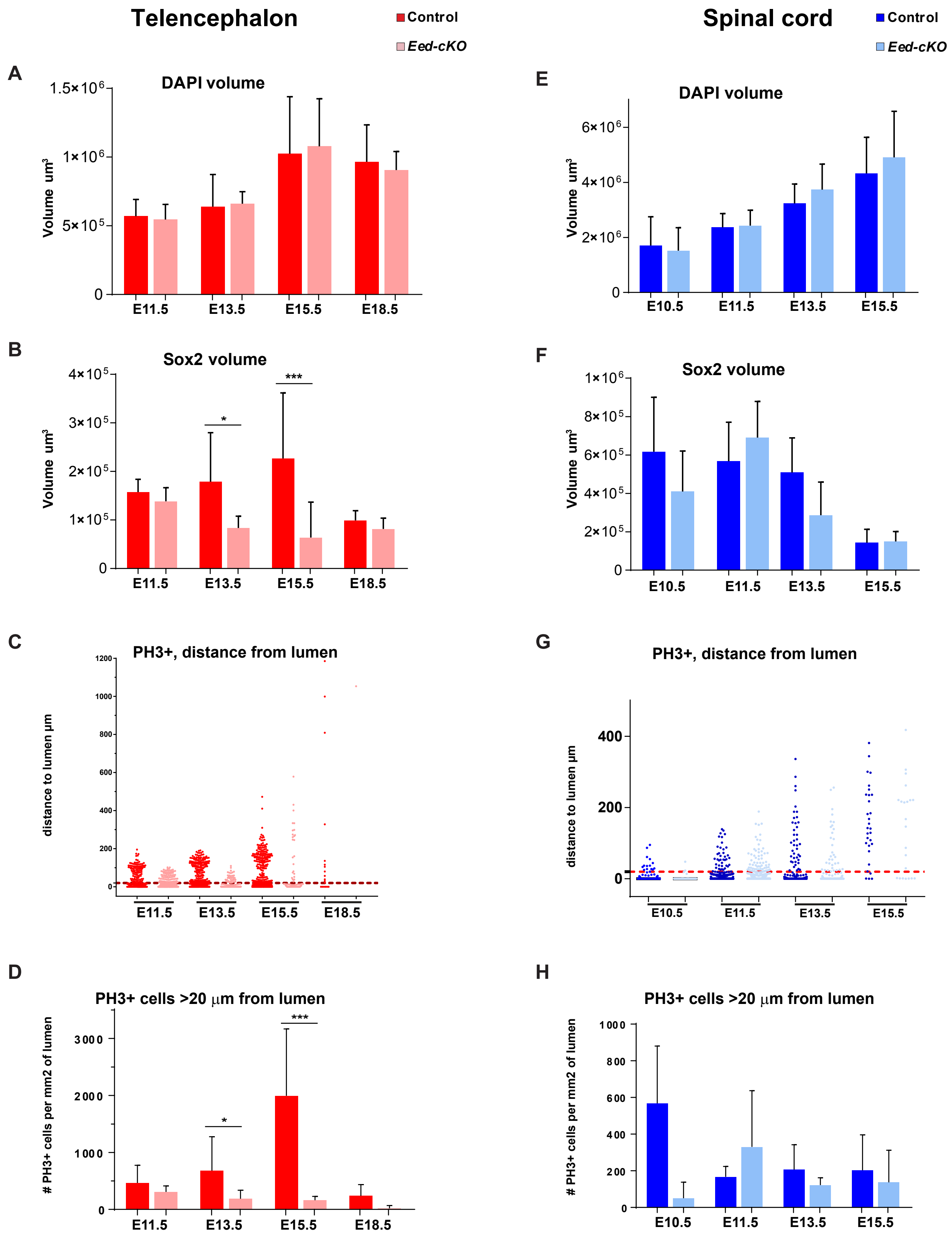

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Figure S6.

Proliferation reduction in *Eed-cKO* embryos.

(A-B, E-F) Quantification of volume of DAPI and Sox2 signal in 200 µm bins along the lateral ventricles of horizontal sections, of Tel (A-B) and SC (E-F) tissues in control and *Eed-cKO* embryos at E10.5 (only SC), E11.5, 13.5, 15.5 and E18.5 (only Tel). (C, G) Position of mitotic cells in relation to the lumen, of Tel and SC tissues in control and *Eed-cKO* embryos, at E10.5 (only SC), E11.5, 13.5, 15.5 and E18.5 (only Tel; red dashed line denotes 20 µm). (D, H) Number of mitotic cells in Tel and SC more than 20 µm distant from the lumen, normalized for luminal area (comparison at each stage using Mann-Whitney U-test; SD; n≥3 embryos and 6-9 sections, 20-40 µm, per genotype and region).

p27 and PCD in telencephalon

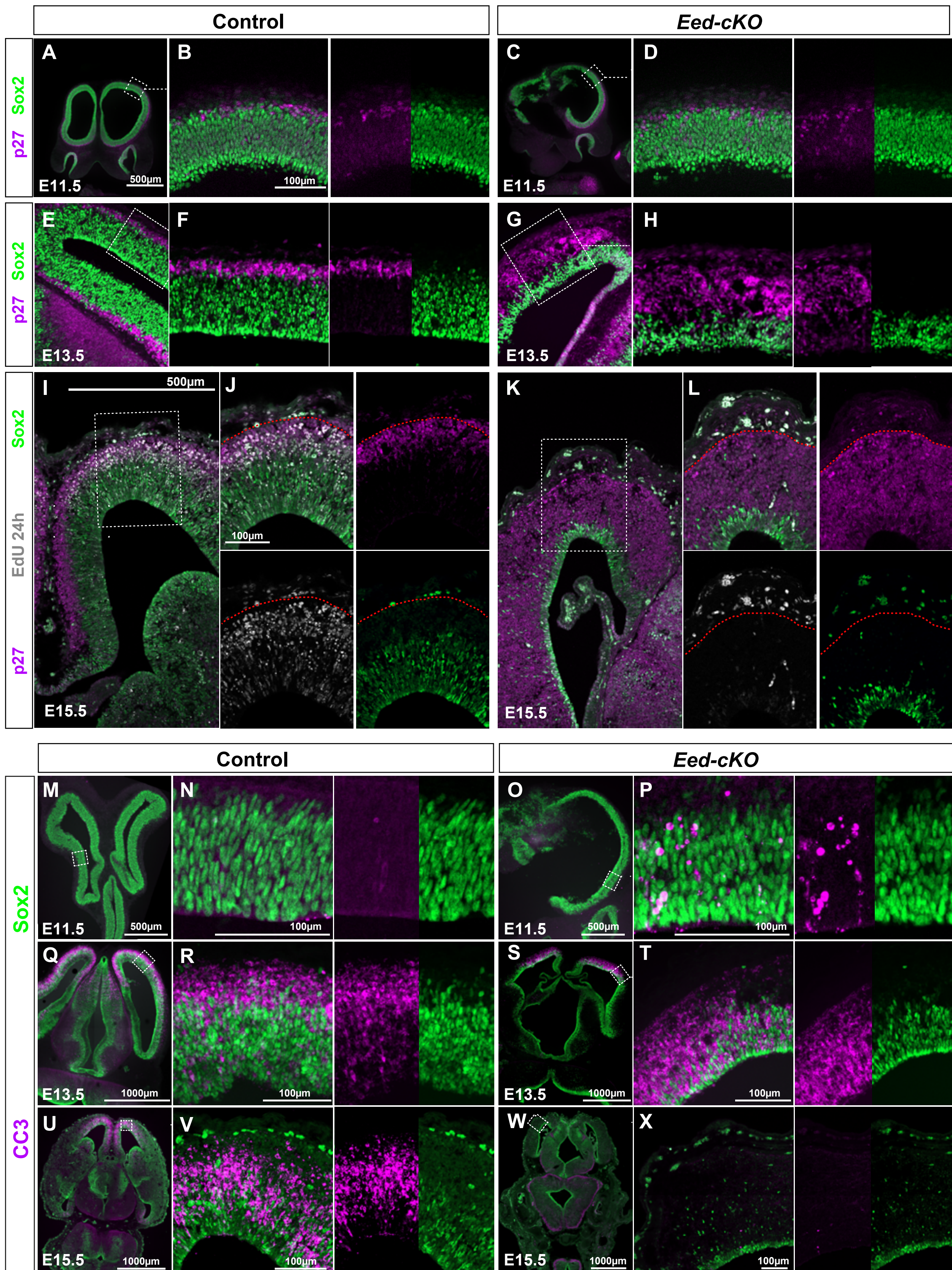


Figure S7.

Cleaved-Caspase3 and p27 expression in telencephalon.

(A-L) Staining for p27 and Sox2 in horizontal sections of Tel of control and *Eed-cKO* embryos at E11.5 (A-D), E13.5 (E-H) and E15.5 (I-L). Cell cycle progression is shown in white by EdU detection (24 hours pulse) at E15.5 (I-L). Red dashed lines indicate proper CNS/Tel tissue. Marked dashed square in A, C, E, G, I, K, indicate magnification area in B, D, F, H, J, L, respectively. (M-X), Staining for CC3 and Sox2 in horizontal sections of Tel of control and *Eed-KO* embryos at E11.5 (M-P), E13.5 (Q-T) and E15.5 (U-X). Marked dashed square in M, O, Q, S, U, W, indicate magnification area in N, P, R, T, V, X, respectively (overview panel scale bar; 500µm, insert panel scale bar; 100µm).

Expression of Cdc25C and E2F3 in Telencephalon

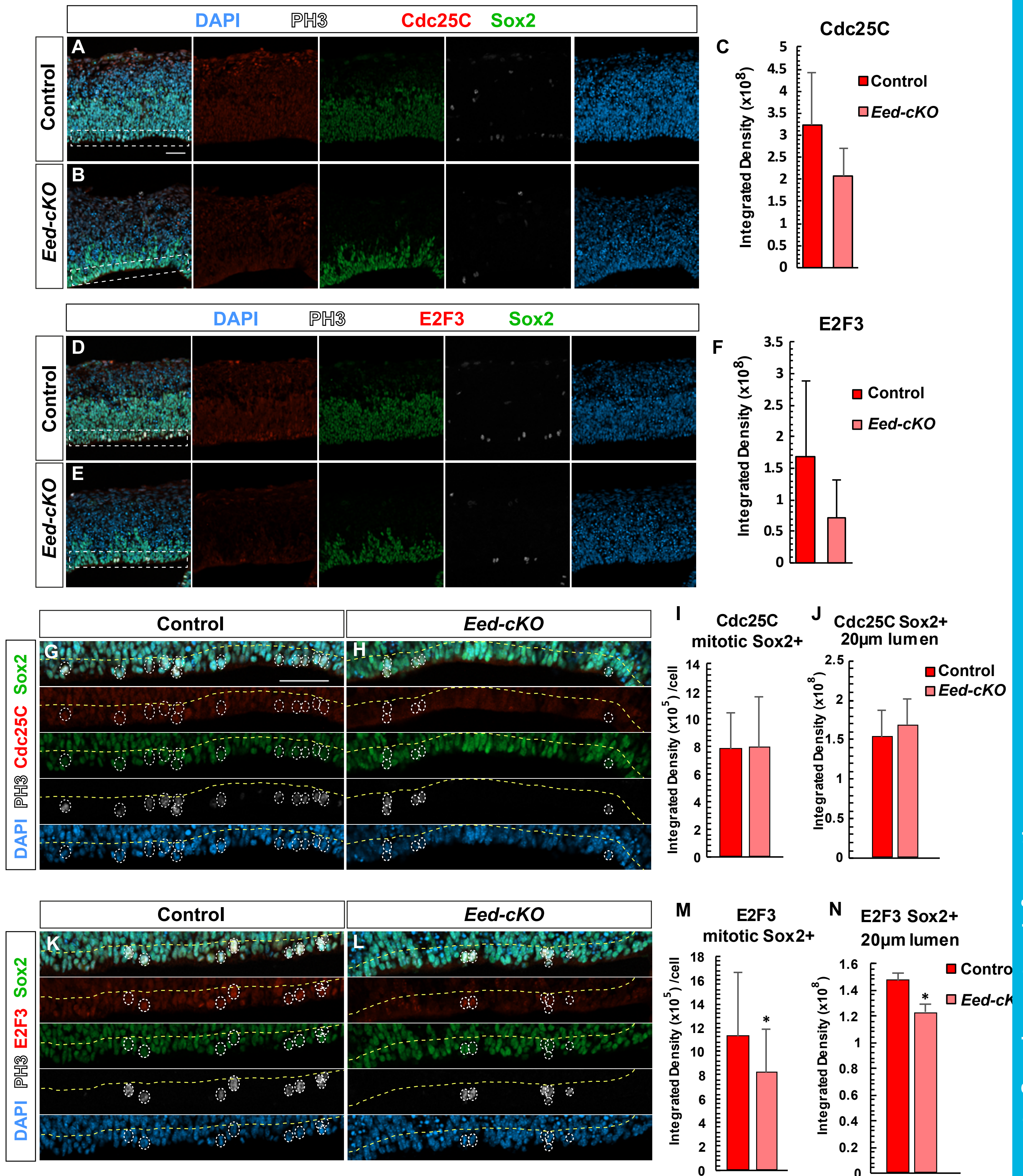
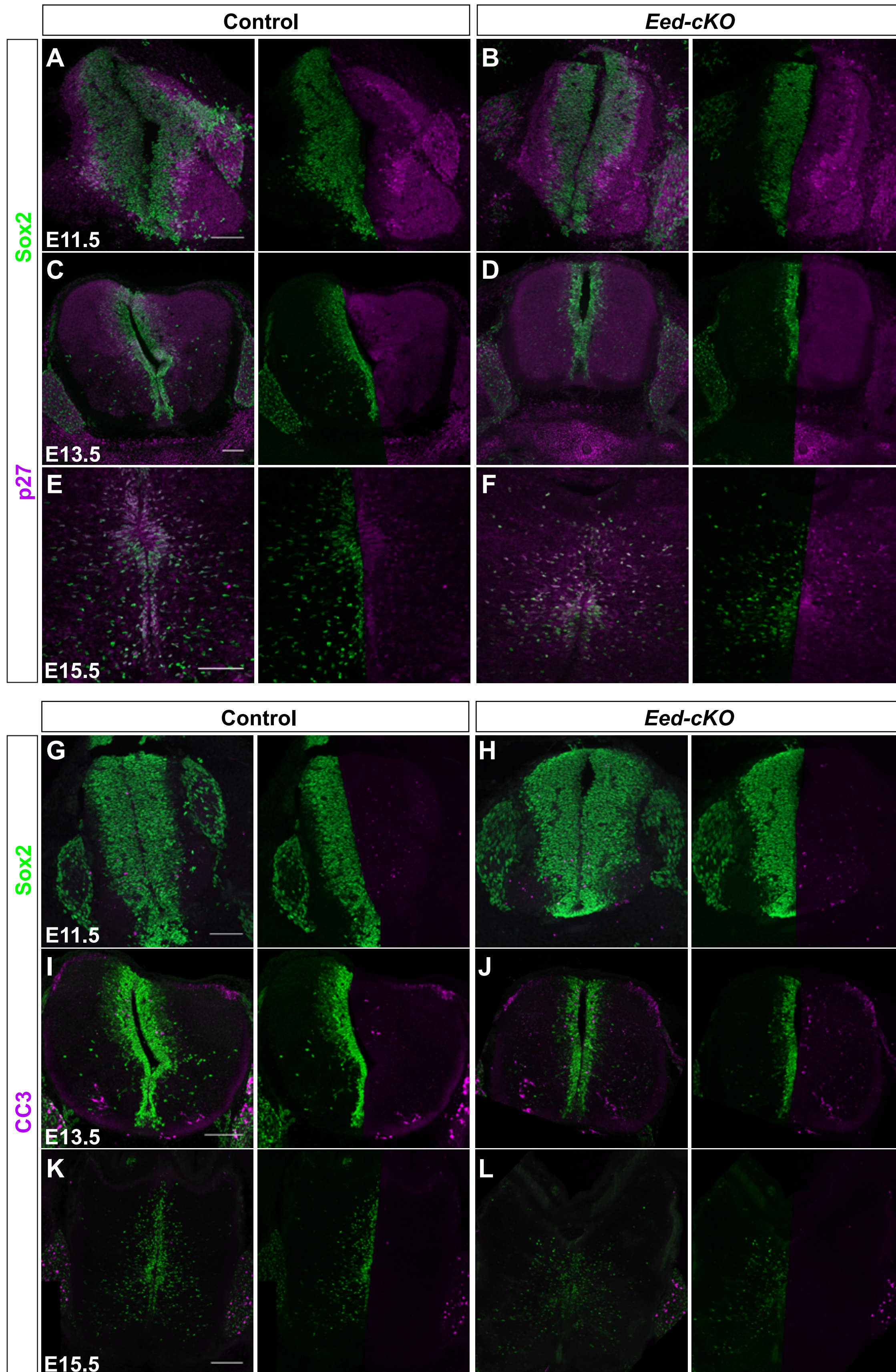


Figure S8.

Expression of cell cycle factors Cdc25C and E2F3 in telencephalon and spinal cord in *Eed-cKO*.

(A-B, D-E, G-H, K-L) Staining for Cdc25C and E2F3 in horizontal sections of Tel of control and *Eed-cKO* embryos, at E13.5, on the same slide for comparison. (C, F) Quantification of the Integrated density of Cdc25C (C) and E2F3 (F), showed reduced staining level, albeit not significant, in *Eed-cKO* embryos when compared to control. Square dashed lines indicate magnification areas showed in G-L respectively (scale bar; 50µm). (G-H, K-L) Magnification of the lumen area showed in (A-B, D-E) horizontal sections of Tel stained for Cdc25C and E2F3 in control and *Eed-cKO* embryos, at E13.5, on the same slide for comparison. Yellow dashed line show 20 µm from the lumen border area, white dashed line highlight mitotic progenitors. (I-J, M-N) Quantification of integrated intensity of Cdc25C and E2F3 was performed in progenitors (Sox2 positive) within 20 µm from the lumen border and in mitotic progenitors (Sox2 and PH3 positive) in control and *Eed-cKO* embryos. Significant reduction of E2F3 levels were revealed both for progenitors close to the lumen area (M) and for mitotic progenitors (N) in *Eed-cKO*, when compared to control.



Development • Supplementary information

Figure S9.

Cleaved-Caspase3 and p27 expression in mouse spinal cord.

(A-F) Staining for p27 and Sox2 in horizontal sections of spinal cord of control and *Eed-cKO* embryos at E11.5 (A-B), E13.5 (C-D) and E15.5 (E-F) (scale bar; 100µm). (G-L) Staining for CC3 and Sox2 in horizontal sections of spinal cord of control and *Eed-cKO* embryos at E11.5 (G-H), E13.5 (I-J) and E15.5 (K-L) (scale bar; 100 µm G-H, 500 µm I-L).

Tbr2/Eomes and Tbr1 expression in Telencephalon

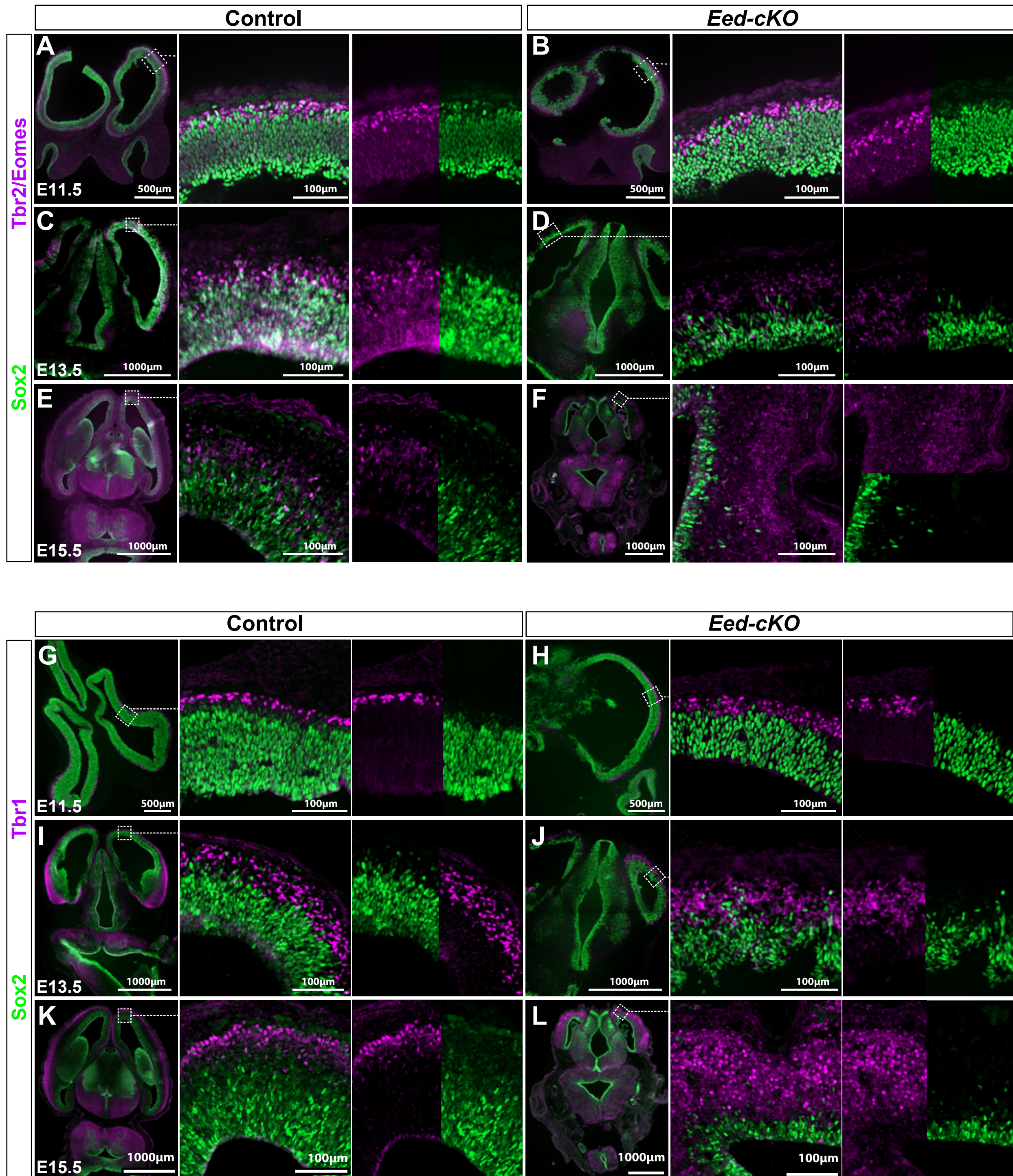


Figure S10.

Expression of Tbr1 and Tbr2 (Eomes) in the mouse telecenphalon.

(A-F) Staining for Sox2 and Tbr2/Eomes in the developing telencephalon, at E11.5, E13.5 and E15.5, in control and *Eed-cKO*. At E11.5, Tbr2/Eomes expression is not strikingly different, while at E13.5 and E15.5 there is a marked decrease in strongly expressing cells in *Eed-cKO*. (G-L) Staining for Sox2 and Tbr1 in the developing telencephalon, at E11.5, E13.5 and E15.5, in control and *Eed-cKO*. At E11.5, Tbr1 expression is not strikingly different, while at E13.5 and E15.5 there is a marked increase in strongly expressing cells in *Eed-cKO*.

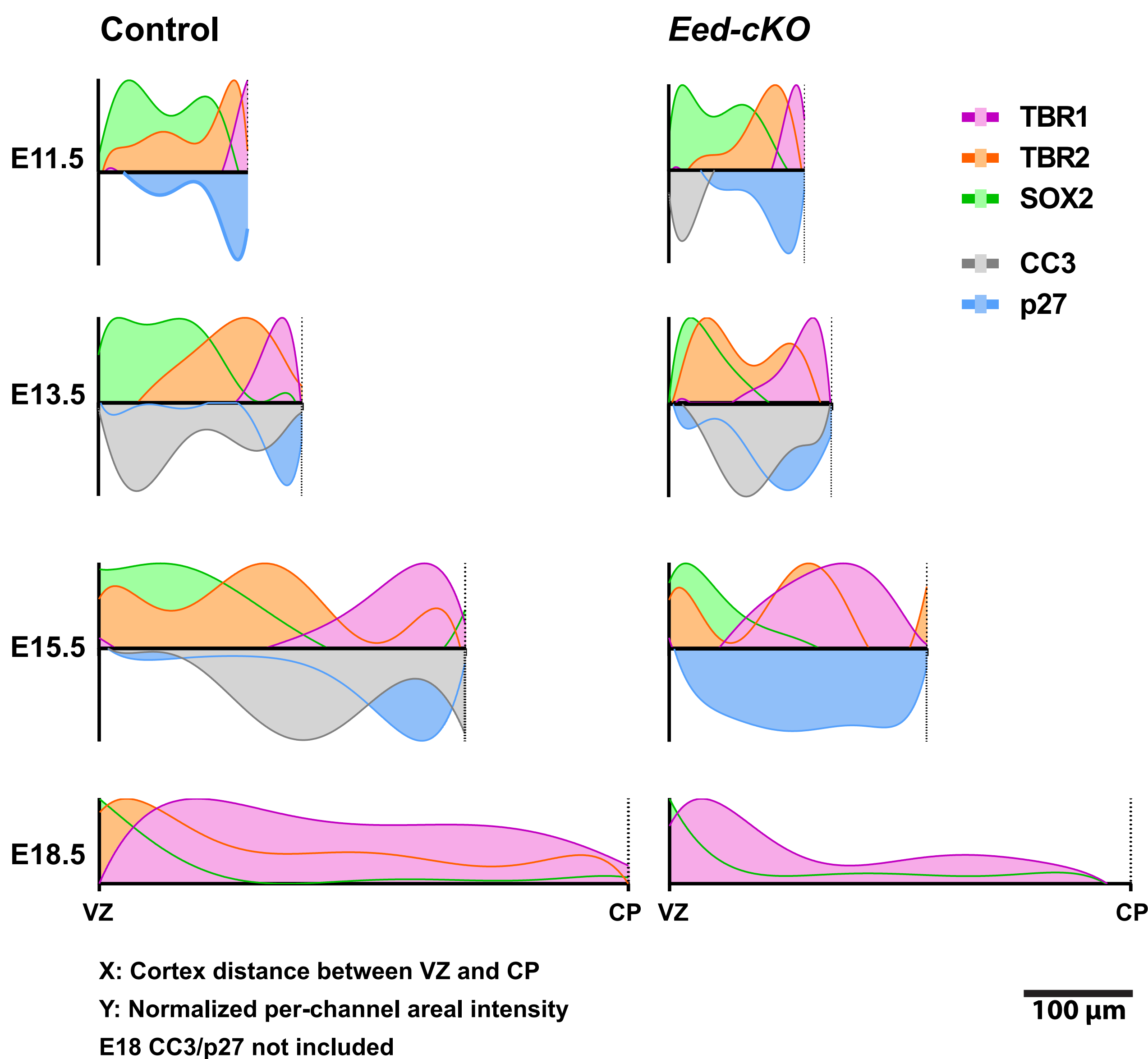


Figure S11.

Staining profiles of mouse telecenphalon.

Comparative telencephalon thickness during development, and zones of highest signal intensity of Sox2 (green), Tbr2/Eomes (orange), Tbr1 (lavender), Cleaved-caspase 3 (CC3; grey) and p27 (blue). Y-axes: intensity is per-channel ratio-normalized and range-normalized across all three channels, in arbitrary units (0 to 1, and inverted 0 to 1); X-axes: average tissue length between ventricular zone (VZ) and cortical plate (CP) of 7-26 measurements per age and genotype, to scale (µm).

Supplementary Materials and Methods

Supplemental DNA sequences

DNA sequences for optimized Hoxb9 and Hoxb13. Start ATGs underlined and bold.

Hoxb9:

gaattcgccgccacc**ATG**GCCGAGCAGAAGCTGATCAGCGAGGAGGACCTGGGACCACCTGGAATGTCCA
TCTCTGGCACCCTGAGCAGCTACTACGTGGACTCCATCATCTCTCACGAGAGCGAGGATGCCCCACCCGC
TAAGTTCCCTTCCGGACAGTACGCTAACCCAAGGCAGCCTGGACACGCTGAGCACCTGGATTTCCCAAGC
TGCTCCTTTCAGCCAAAGGCTCCCGTGTTTGGAGCTAGCTGGGCTCCTCTGTCCCCACACGCTTCTGGAA
GCCTGCCATCCGTGTACCACCCATACCTCCAGCCTCAGGGCGCCCCAGCCGCTGAGAGCAGATACCTGAG
AACATGGCTGGAGCCTGCTCCAAGAGCTGAGGCTGCTCCAGGACAGGGACAGGCCGCTGTGAAGGCTGAG
CCACTGCTGGGCGCTCCTGGAGAGCTGCTGAAGCAGGGCACCCCTGAGTACTCCCTGGAGACATCTGCCG
GACGCGAGGCTGTGCTGTCTAACCAGAGGGCTGGCTACGGAGACAACAAGATTTGCGAGGGATCTGAGGA
CAAGGAGAGACCAGATCAGACCAACCCAAGCGCCAACTGGCTGCACGCTCGGTCTAGCCGCAAGAAGAGG
TGTCCCTACACCAAGTACCAGACACTGGAGCTGGAGAAGGAGTTCCTGTTTAACATGTACCTGACACGGG
ATAGGAGACACGAGGTGGCCAGACTGCTGAACCTGAGCGAGCGGCAGGTGAAGATTTGGTTCCAGAACCG
GCGCATGAAGATGAAGAAGATGAACAAGGAGCAGGGCAAGGAGtaatgatagtctagagaattc

Hoxb13:

gaattcgccgccacc**ATG**GCCGAGCAGAAGCTGATCTCCGAGGAGGACCTGGGACCACCTGGAATGGAGC
CAGGCAACTACGCTACCCTGGACGGAGCCAAGGATATCGAGGGACTGCTGGGAGCTGGAGGAGGCCGGAA
CCTGGTGAGCCACAGCTCCCCTCTGGCTTCCCACCCCGCCGCTCCTACCCTGATGCCAACAGTGAACTAC
GCTCCCCTGGATCTGCCTGGCTCCGCCGAGCCACCAAAGCAGTGCCACCCATGTCCTGGCGTGCCTCAGG
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GCACCACACCTtaatgatagtctagagaattc

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