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Supplemental Information

Tissue-Specific Actions of Pax6 on Proliferation and Differentiation Balance in Developing Forebrain Are Foxg1 Dependent

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Supplementary Figures

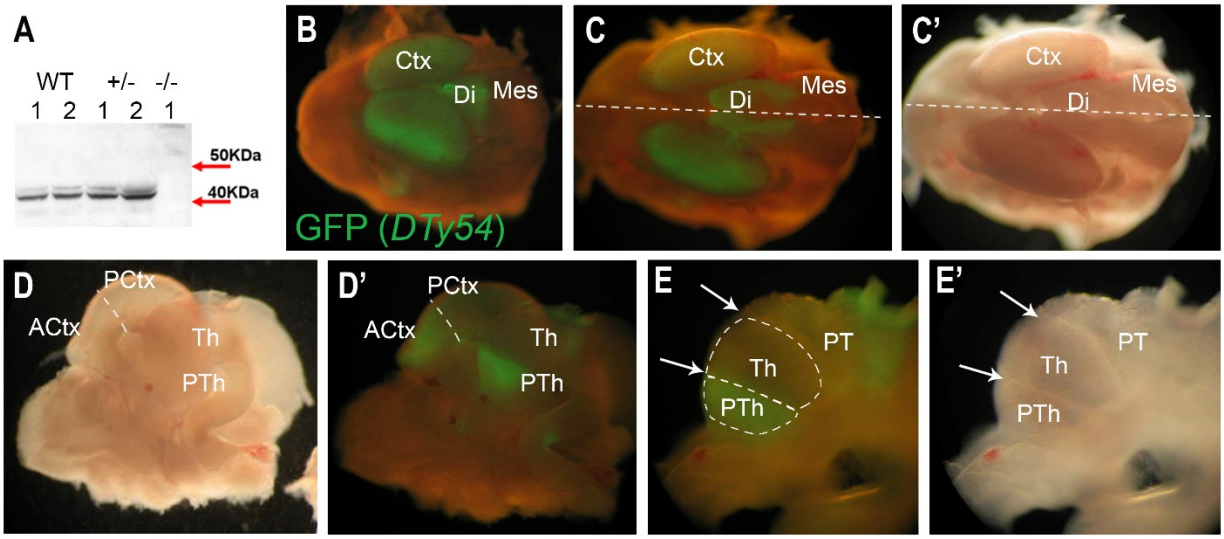

Figure S1 Preparation of samples for RNA-seq. Related to Figure 1 and STAR methods.

(A) Western blots showing Pax6 levels in two wild-type (WT), two *Pax6*$^{+/-}$ and a *Pax6*$^{-/-}$ E13.5 forebrain(s).

(B) GFP expression from the *DTy54* transgene in the E13.5 forebrain; Ctx, cortex; Di, diencephalon; Mes, mesencephalon.

(C,C') The two cortices were teased apart and the brain was cut along the midline (broken line).

(D,D') The anterior cortex (ACtx; high *Pax6*/GFP expression) was separated from posterior cortex (PCtx; broken line) in each hemi-brain. Th, thalamus; PTh, prethalamus.

(E,E') Thalamus (Th) and prethalamus (PTh) were dissected (broken lines). Arrows indicate boundaries of these structures visible in bright-field. PT, pretectum.

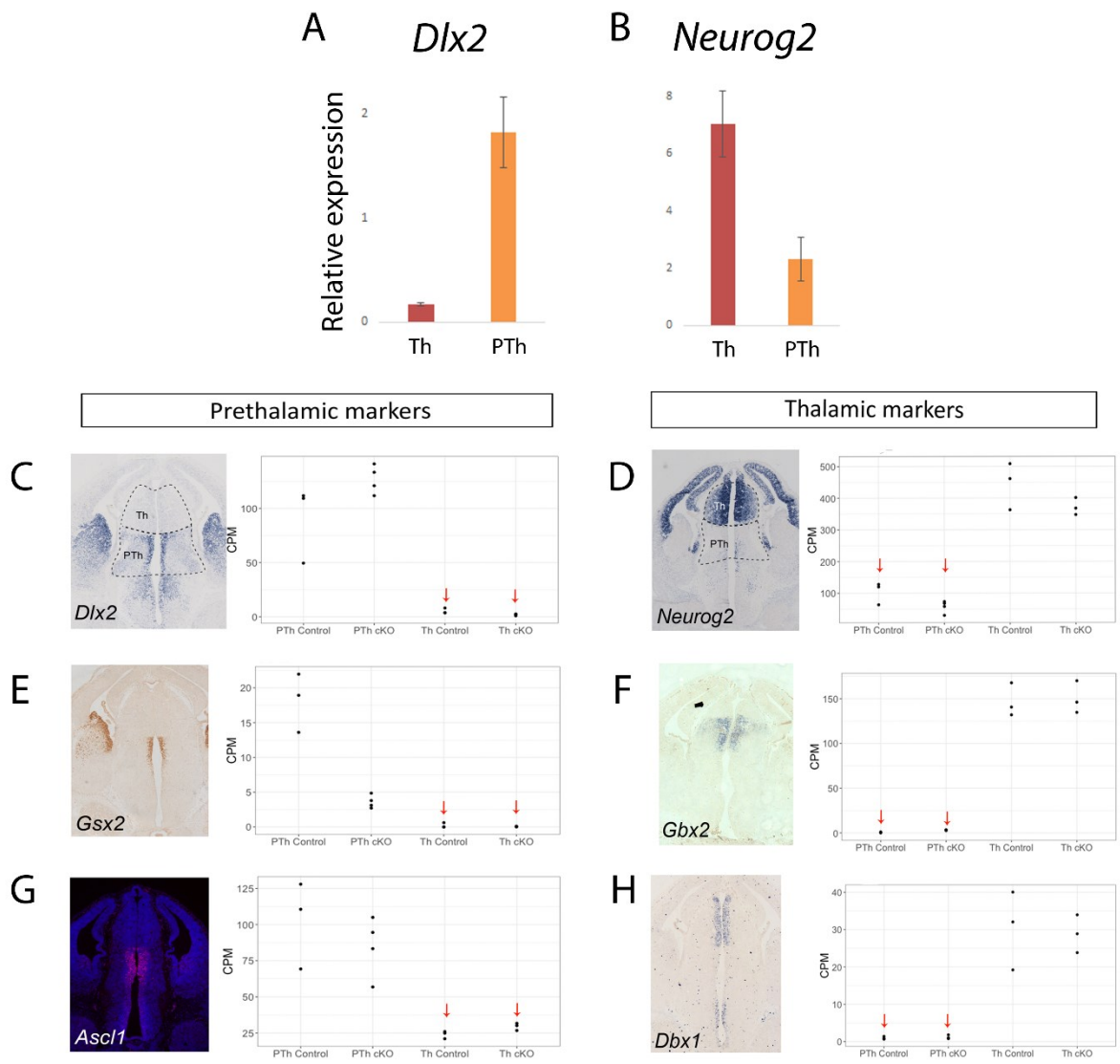

Figure S2 Quality control of samples for RNA-seq. Related to Figure 2

(A,B) Quantitative real-time PCR (qRT-PCR) to measure the levels of expression of *Dlx2* and *Neurog2* in E13.5 control thalamus (Th) and prethalamus (PTh) relative to *GAPDH*. Values are means ± sem; n=3 animals in all cases ($p<0.05$ Student's t-test).

(C-H) In situ hybridizations on control forebrains at E13.5 and data from control and *CAG*^{CreER} *Pax6* cKOs on counts per million reads (CPM) extracted from RNA-seq experiments for three thalamic and three prethalamic markers. Red arrows indicate low values for markers of each region in the other region.

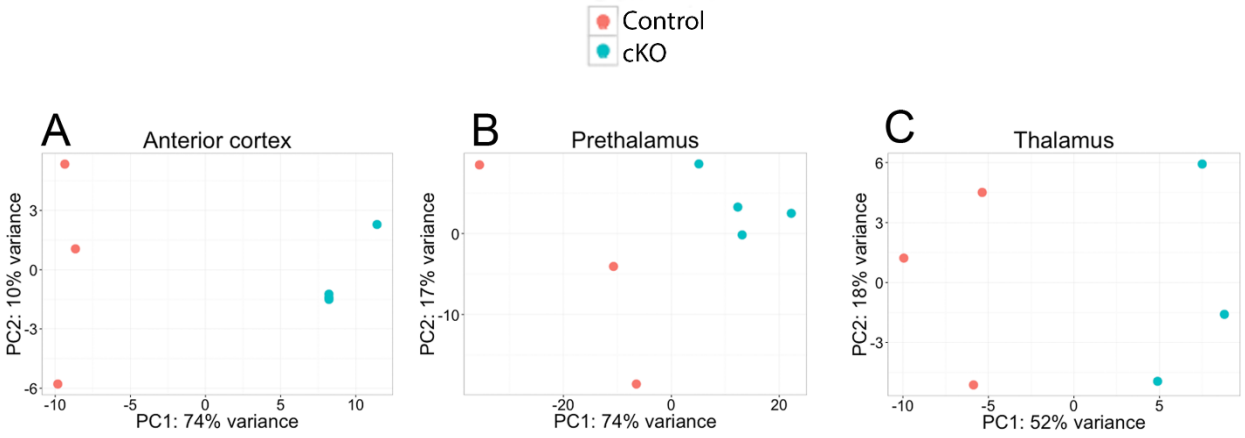

Figure S3 Principal component analysis on RNA-seq data. Related to Figure 2.

(A-C) Analysis of data from 3 samples from each of control and *CAG*^{CreER} *Pax6* cKO anterior cortex, control prethalamus and control and *CAG*^{CreER} *Pax6* cKO thalamus and 4 samples from *CAG*^{CreER} *Pax6* cKO prethalamus.

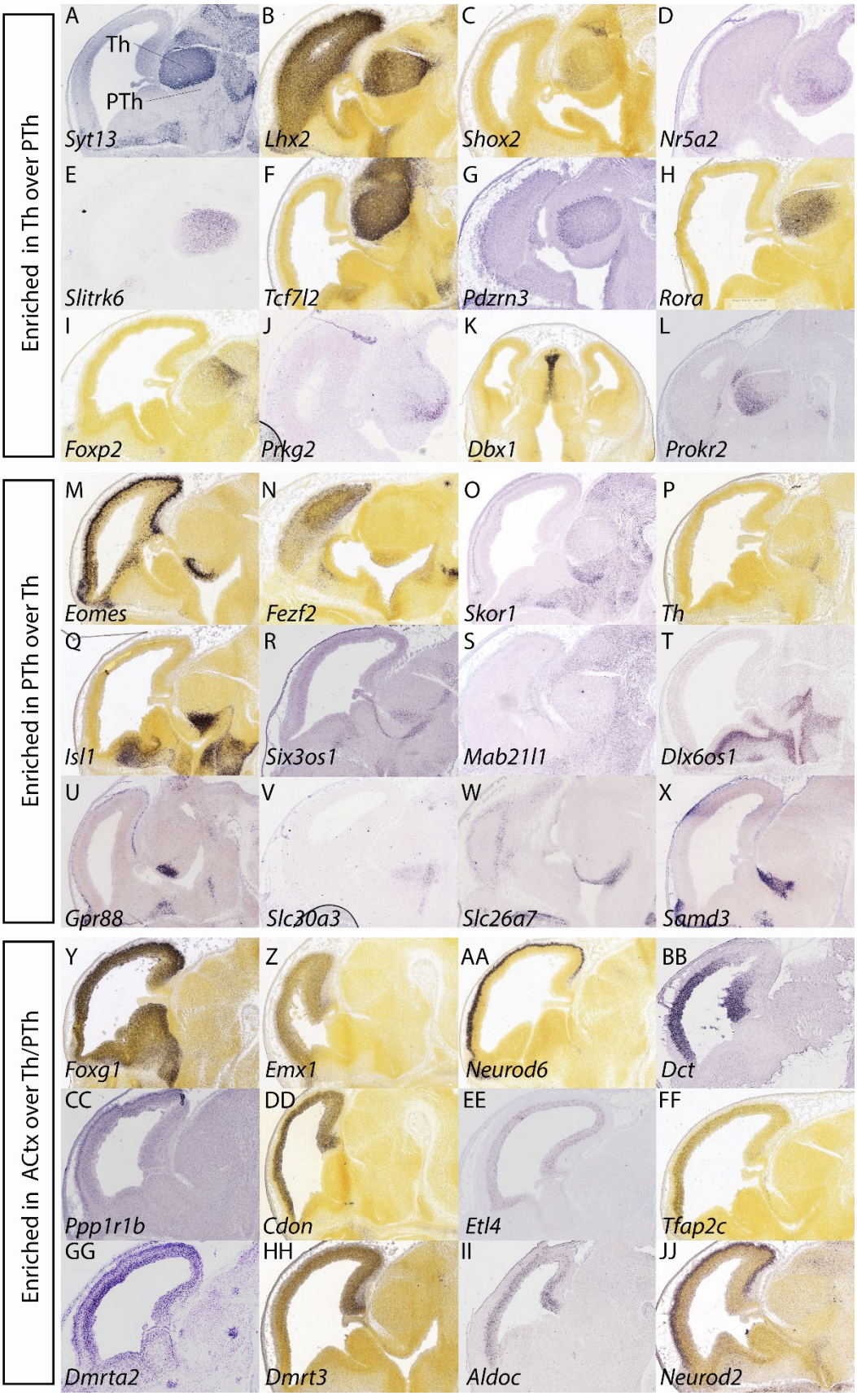
Enriched in Th over PTh
A
Th
PTh
Syt13
B
Lhx2
C
Shox2
D
Nr5a2
E
Slitrk6
F
Tcf7l2
G
Pdzrn3
H
Rora
I
Foxp2
J
Prkg2
K
Dbx1
L
Prokr2
Enriched in PTh over Th
M
Eomes
N
Fezf2
O
Skor1
P
Th
Q
Isl1
R
Six3os1
S
Mab21l1
T
Dlx6os1
U
Gpr88
V
Slc30a3
W
Slc26a7
X
Samd3
Enriched in ACtx over Th/PTh
Y
Foxg1
Z
Emx1
AA
Neurod6
BB
Dct
CC
Ppp1r1b
DD
Cdon
EE
Etl4
FF
Tfap2c
GG
Dmrta2
HH
Dmrt3
II
Aldoc
JJ
Neurod2

Figure S4 Expression patterns of genes showing the greatest inter-regional differential expression in control embryos. Related to Figure 1 and Table S1.

(A,D,E,G,J,L,O,R-X,BB,CC,EE,GG,II) From Genepaint, http://www.genepaint.org/

(B,C,F,H,I,K,M,N,P,Q,Y-AA,DD,FF,HH,JJ) From Allen Brain Atlas http://developingmouse.brain-map.org/.

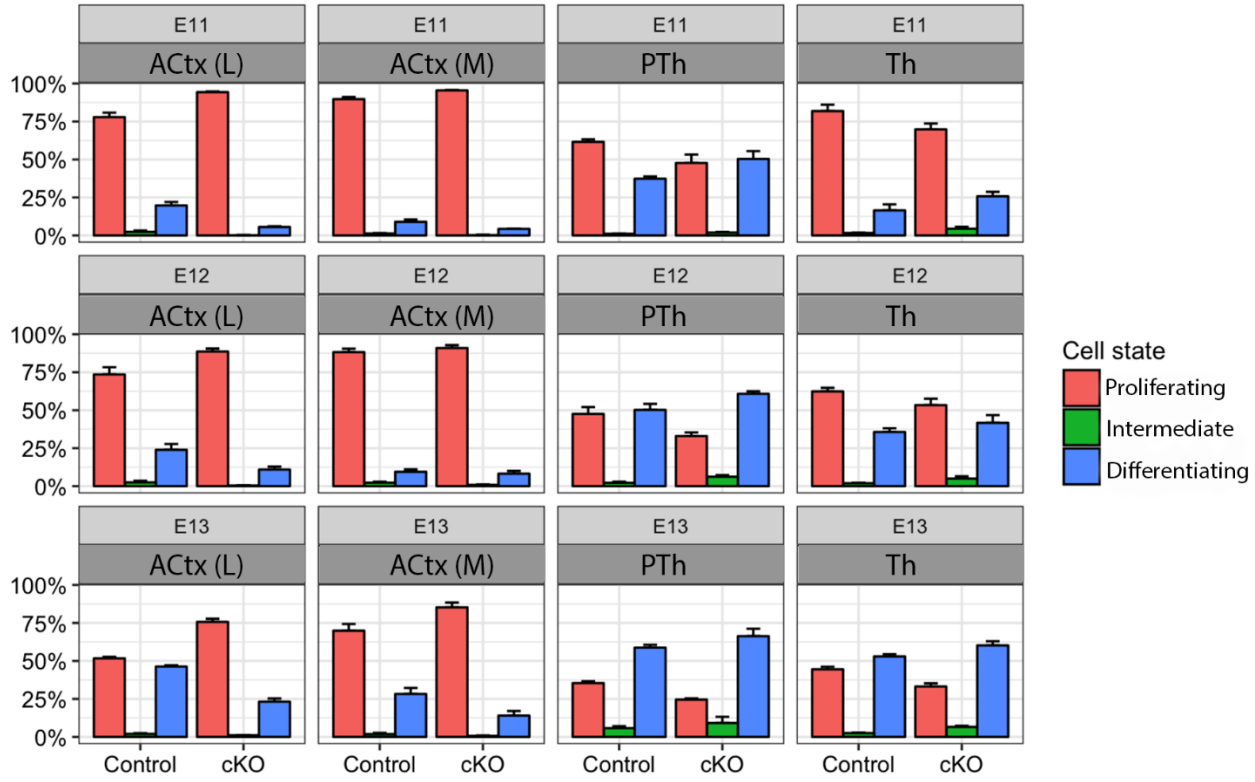

Figure S5 Effects of Pax6 deletion on proportions of different cell types in forebrain regions with age in control and *CAG^{CreER} Pax6* cKOs. Related to Figure 4.

Counts in ACtx were in lateral (L) and medial (M) regions. Proliferating cells were BrdU+, Ki67+, Tuj1-; differentiating cells were BrdU+, Ki67-, Tuj1+; intermediate cells were BrdU+, Ki67-, Tuj1-.

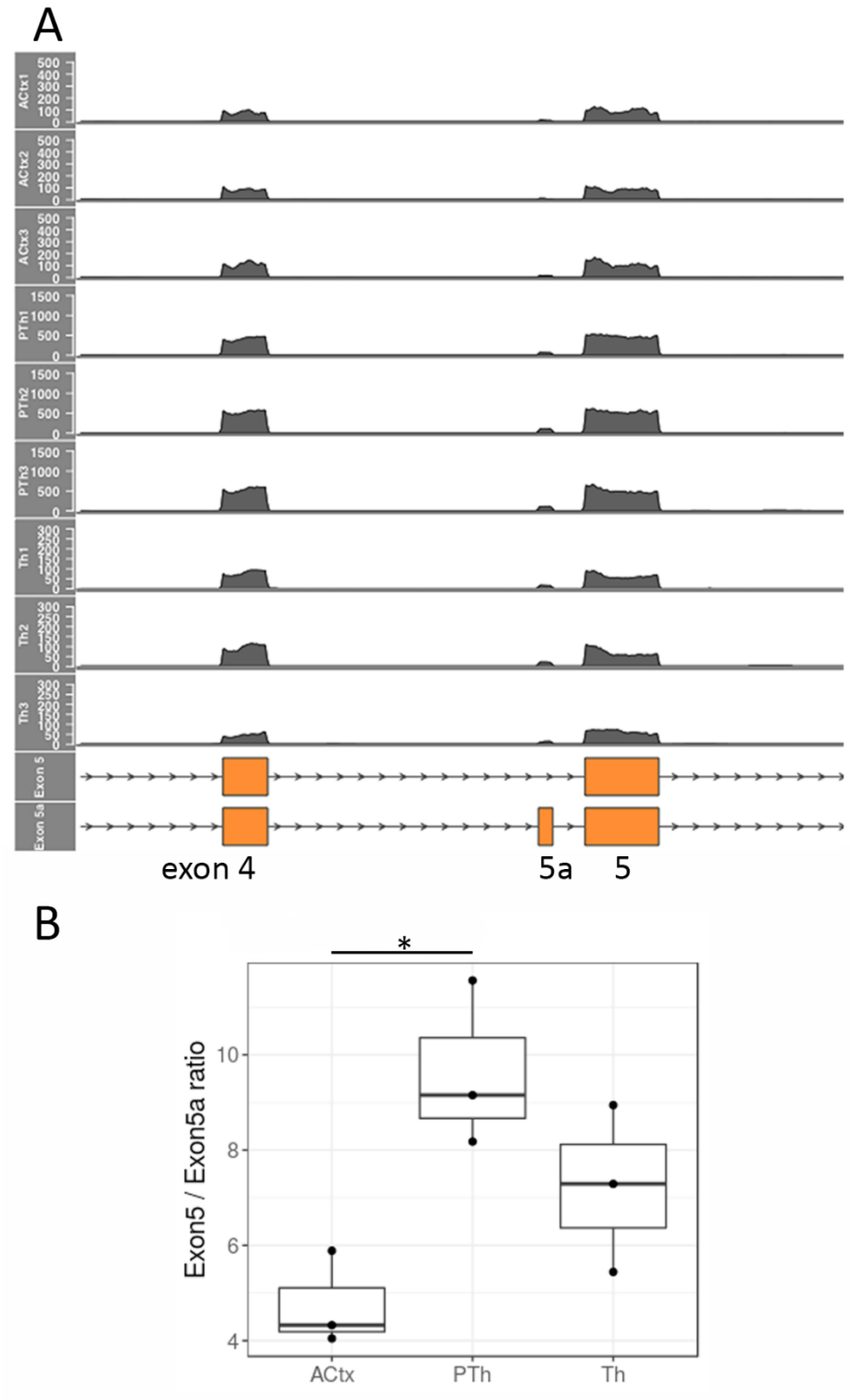

Figure S6 *Pax6/Pax6(5a)* ratio in ACtx, Th and PTh in control embryos.

(A) Counts per base read coverage of exons 4, 5a and 5 of *Pax6* from each sample.

(B) Ratios between the average coverage per base in exons 5 and 5a for each sample superimposed on box and whisker plots. ANOVA followed by post hoc Tukey comparison showed a significant difference between the ratios in ACtx and PTh (p=0.02).

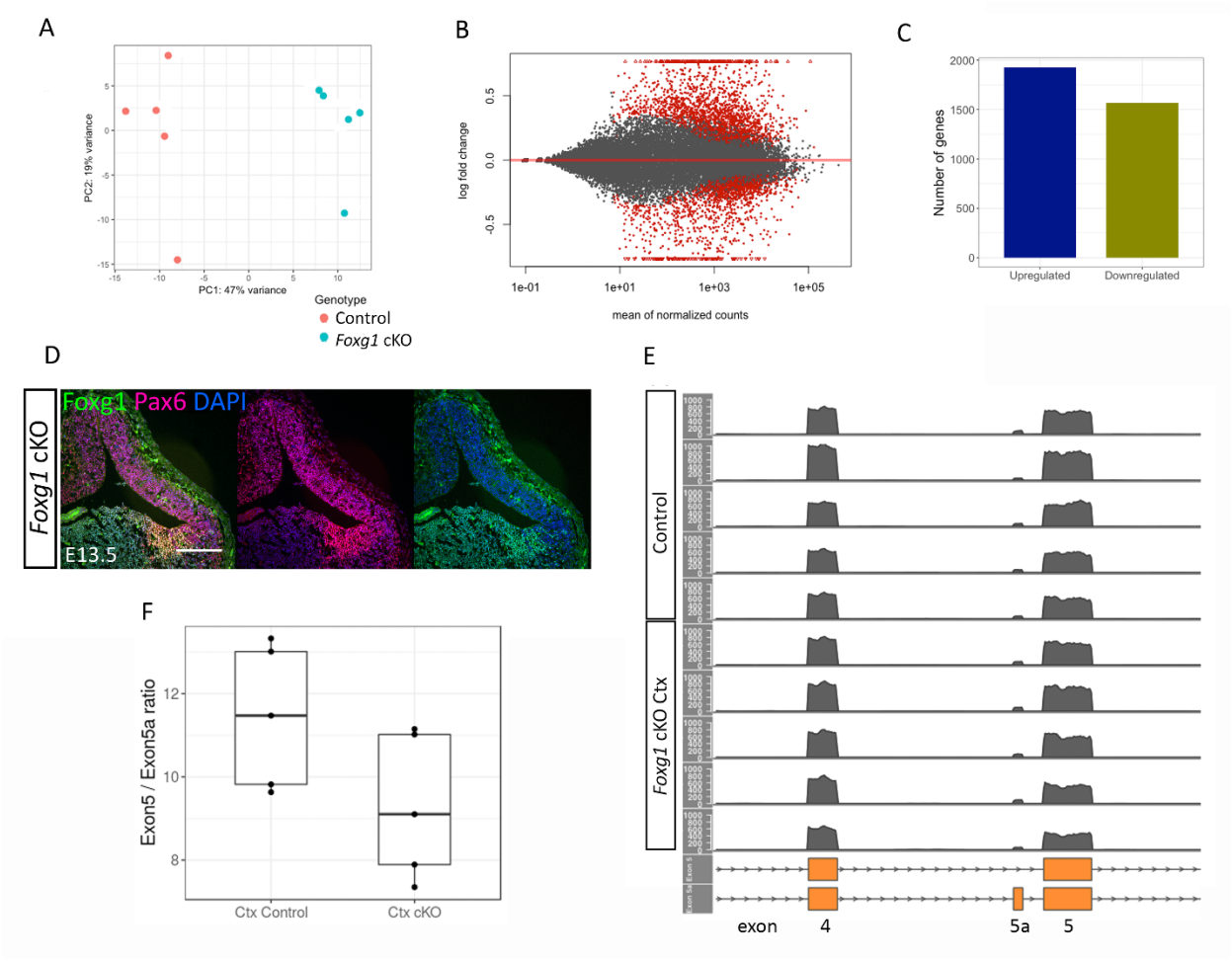

Figure S7 Effects of Foxg1 deletion from the cortex. Related to Figure 6.

(A) Principal component analysis of RNA-seq data from 5 control and 5 *Foxg1* cKO samples.

(B) MA plots of $\log_2$ fold changes in the expression of each gene against its average expression level; red dots indicate statistically significant changes between genotypes (adjusted p values <0.05).

(C) Numbers of significantly (adjusted p<0.05) upregulated and downregulated genes in *Foxg1* cKO cortex.

(D) Expression of Foxg1 and Pax6 in *Foxg1* cKO. Panels show merged, Pax6 and Foxg1 staining. Scale bar: 0.25mm.

(E) Counts per base read coverage of exons 4, 5a and 5 of *Pax6* from each control and *Foxg1* cKO sample.

(F) Ratios between the average coverage per base in exons 5 and 5a for each sample superimposed on box and whisker plots. There was no significant difference between genotypes.

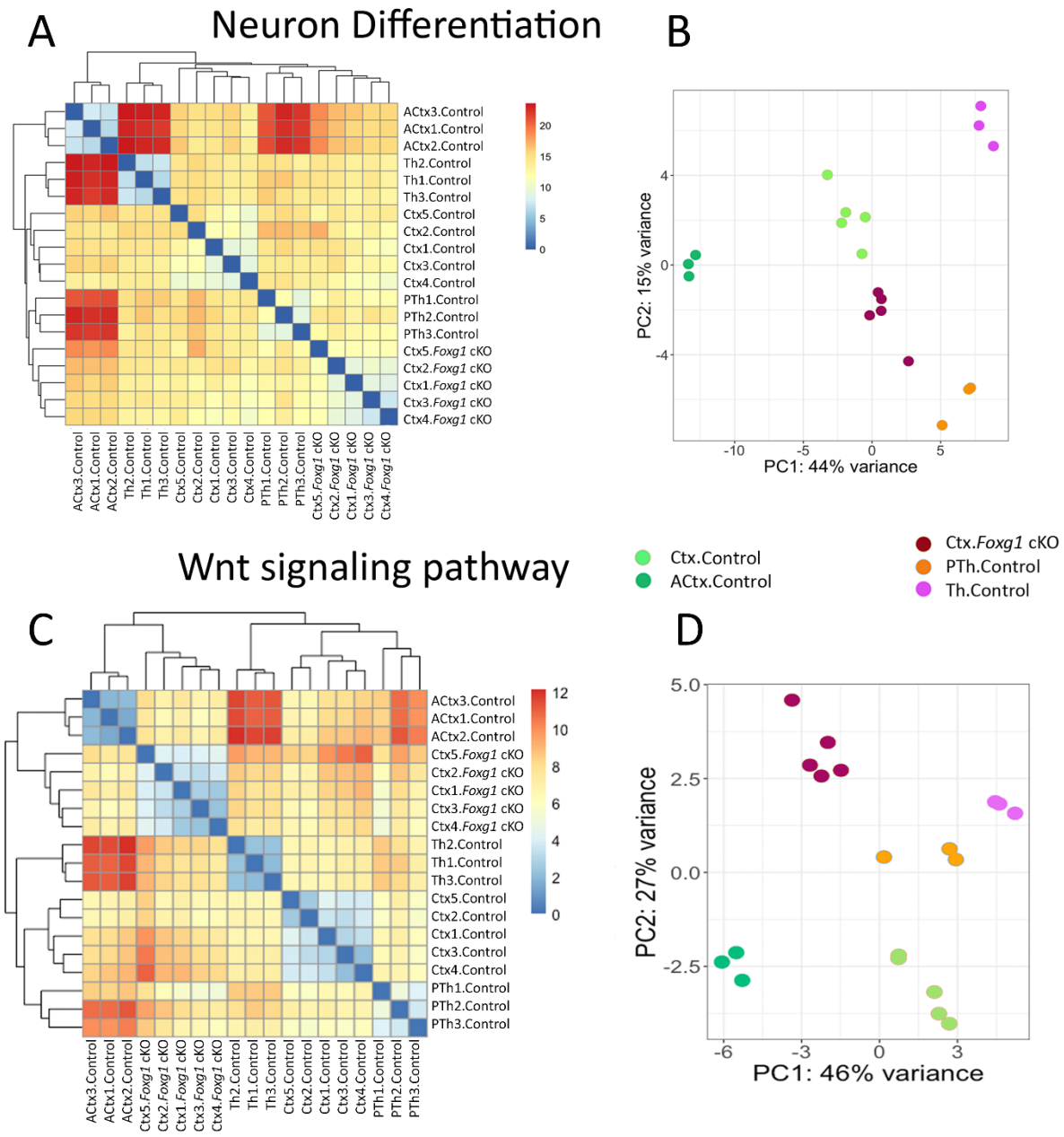

Figure S8 Clustering of RNA-seq data on genes annotated by the Neuron Differentiation and Wnt signalling pathway GO terms. Related to Figure 6.

(A,C) Heatmaps of hierarchical clustering of RNA-seq data from samples of control cortex, control ACtx, control PTh, control Th and *Emx1*^{CreER} *Foxg1* cKO cortex.

(B,D) Principal component (PC) analysis on the same RNA-seq data as in A,C).

Transparent Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Price (David.Price@ed.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice colony maintenance and transgenic lines

To generate a conditional tamoxifen-inducible deletion of *Pax6* throughout the embryo, we combined lines carrying a *CAGGCre-ER*^{TM} allele (Hayashi and McMahon, 2002), a green fluorescent protein (GFP) reporter allele (Sousa et al., 2009) and *Pax6*^{loxP} alleles (Simpson et al., 2009). Pregnant mice were given 10mg of tamoxifen (Sigma) by oral gavage on embryonic day 9.5 (E9.5) to induce *Pax6*^{loxP} deletion and embryos were collected on E11.5, E12.5 and E13.5. Embryos heterozygous for the *Pax6*^{loxP} allele (*Pax6*$^{fl/+}$;*CAGG*^{CreER}) were used as controls since previous studies have shown no detectable defects in the forebrain of *Pax6*$^{fl/+}$ embryos (Simpson et al., 2009). Embryos carrying two copies of the floxed *Pax6* allele (*Pax6*$^{fl/fl}$;*CAGG*^{CreER}) were the experimental conditional knock-out (cKO) group. DTy54, a YAC transgene that expresses GFP in Pax6 expressing cells (Tyas et al., 2006), was crossed into some animals to guide the diencephalic dissections.

To delete *Foxg1* in cortex, we combined lines carrying an *Emx1-CreER*^{TM} allele (Kessaris et al., 2006), a GFP reporter allele (Sousa et al., 2009) and *Foxg1*^{loxP} alleles (generously donated by Drs. Goichi Miyoshi and Gord Fishell; Miyoshi and Fishell, 2012). Pregnant females were given 10mg of tamoxifen by oral gavage at E9.5 and embryos were collected at E13.5 and E15.5. *Foxg1*$^{fl/+}$;*Emx-CreER*^{TM} embryos were considered controls and *Foxg1*$^{fl/fl}$;*Emx-CreER*^{TM} embryos were the experimental cKO group.

To simultaneously delete *Pax6* and *Foxg1* in the cortex, we combined lines carrying an *Emx1-CreER*^{TM} allele (Kessaris et al., 2006), a GFP reporter allele (Sousa et al., 2009) and both *Pax6*^{loxP} alleles (Simpson et al., 2009) and F*oxg1*^{loxP} alleles (Miyoshi and Fishell, 2012). Pregnant females were given 10mg of tamoxifen by oral gavage at E9.5 and embryos were collected at E13.5 and E15.5.

The day the vaginal plug was detected was considered E0.5.

Animals were bred according to the guidelines of the UK Animals (Scientific Procedures) Act 1986 and all procedures were approved by Edinburgh University's Animal Ethics Committee.

METHOD DETAILS

Tissue processing for RNA-seq

We bisected embryonic brains (E13.5) along the midline and processed one half of the brain for immunohistochemistry to confirm Pax6 or Foxg1 loss in cKO samples. The other half was dissected further. For *Pax6*-cKO and corresponding control embryos, we dissected the thalamus (Th), prethalamus (PTh) and anterior half of the cerebral cortex (ACtx) (Figure S1) and extracted total RNA with an RNeasy Plus micro kit (Qiagen). We sequenced three biological replicates for ACtx and four biological replicates for Th and PTh. Each replicate consisted of samples pooled from three (ACtx and Th) or five (PTh) embryos of the same experimental group. We used embryos from 14 different litters, pooling control and experimental embryos from the same litter whenever possible. Poly-A mRNA was purified and TruSeq RNA-seq libraries were prepared and sequenced with Illumina HiSeq v4 (50 base paired-end reads for ACtx samples; 125 base paired-end reads for Th and PTh samples). For *Foxg1*-cKO and corresponding control embryos, we dissected only cortical tissue. Total RNA extraction and RNA-seq library preparation was performed as above (150 base paired-end reads). Five biological replicates were included, each consisting of two pooled cortices from littermate embryos.

We performed a post-processing quality control of samples through a series of principal component analysis (PCA) to see which samples satisfy the criteria of minimal within-group variance. Certain samples (1 PTh control, 1 Th control and 1 Th Pax6 cKO) did not satisfy the criteria since PCA resulted in them clustering out of their groups. We decided to remove these samples from further analysis in order to minimise unwanted technical variance.

Tissue processing for immunohistochemistry and *in situ* hybridization (ISH)

Embryos were dissected in cold phosphate buffered saline (PBS), their heads were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in PBS, cryoprotected in 30% sucrose and embedded in a mixture of 30% sucrose and OCT compound (50:50). Cryostat sections (5 or 10µm) were obtained and stored at -20°C until processed.

Immunohistochemistry

Cryo-sections were let to stabilize at room temperature for at least 2 hours and then washed three times in PBST (1X PBS with 0.1% Triton X-100, Sigma). To block endogenous peroxidase, sections were treated with 3% H_2O_2 for 10 minutes. After two PBS washes, antigen retrieval was performed by immersing the sections in Sodium Citrate buffer (10mM, pH6) heated at approximate 90°C using a microwave for 20 minutes. Once the solution cooled down sections were washed twice in PBST. After a 20 minutes pre-incubation in 20% Normal Goat Serum (Invitrogen), sections were incubated with the primary antibody overnight at 4°C. Biotin-coupled secondary antibodies were incubated for 1 hour at room temperature followed by a 30 minute incubation with Avidin-Biotin complex (ABC kit, Vector laboratories). Finally, diaminobenzidene (DAB, Vector Laboratories) reaction was used to obtain a brown precipitate and sections were mounted in DPX media (Sigma).

For immunofluorescence a cocktail of primary antibodies were incubated overnight at 4°C. Secondary antibodies were incubated at room temperature for one hour. For Ki67, Foxg1 and Tbr1 detection we used Streptavidin signal amplification (biotin-coupled secondary antibody followed by 30 minute incubation with Streptavidin Alexa Fluor™ 488, 546 or 647 conjugate; Thermo Fisher Scientific). Sections were counterstained with DAPI (Thermo Fisher Scientific) and mounted in ProLong Gold Antifade Mountant (Thermo Fisher Scientific).

Details of the antibodies used in this study can be found in Key Resources Table.

In situ hybridization

In vitro transcription of digoxigenin-labeled probes was done with DIG RNA-labeling kit (Sigma-Aldrich).

Cryo-sections were processed for ISH using standard protocols. Digoxigenin-labelled probes used were *Ccnd1* (kindly donated by Dr. Ugo Borello, INSERM, France), *Dlx2* (kindly donated by Dr. John L.R. Rubenstein, USCF, USA), *Neurog2* (kindly donated by Dr Thomas Theil, University of Edinburgh, UK), *Gbx2* (kindly donated by Dr. Alexandra L. Joyner, HHMI,USA), *Gsx2* (kindly donated by Dr. Kenneth Campbell, Cincinnati Children's Hospital Medical Center, USA), *Dbx1* (kindly donated by Dr. Luis Puelles, University of Murcia, Spain), *Lef1* (kindly donated by Dr. J. Galcerán, University of Alicante, Spain), *Sfrp2* (kindly donated by Dr. Jeremy Nathans, JHU, USA), *Dkk3* (synthetized in the lab from cDNA using primers

specified in Witte et al., 2009), *Foxg1* (kindly donated by Dr. Thomas Theil) and *Ascl1* (kindly donated by Dr. Francois Guillemot, Francis Crick Institute, UK).

Some slides were sequentially processed for fluorescent ISH (*Sfrp2*) followed by immunofluorescence (Pax6, Biolegend).

Genotyping of mutant lines

We dissected tissue from the tails of each embryo, extracted DNA and performed PCR amplification to detect the alleles of interest.

For the detection of the floxed Pax6 allele, PCR reaction was performed in a final volume of 25µl containing 1.5µl of extracted DNA, 0.5mM primer mix (Simpson et al. 2009, forward primer: 5'-AAA TGG GGG TGA AGT GTG AG-3'; reverse primer: 5'-TGC ATG TTG CCT GAA AGA AG-3'), 0.5 mM dNTPs mix, 1X PCR reaction buffer and 5U/µl Taq DNA Polymerase (Qiagen). PCR was performed with 35 cycles and a Tm of 59°C. The PCR product was subsequently run in a 2% agarose gel. Wild type allele results in a fragment of 156bp and floxed allele fragment was 195bp, therefore two bands indicated the heterozygous condition (used as controls) and one strong 195bp band identified the homozygous floxed allele condition (*Pax6* KOs).

For genotyping the floxed Foxg1 allele PCR reaction was performed in a final volume of 50µl containing 4µl of extracted DNA, 0.4mM primer mix (forward primer: 5'-TTGCTACATGCCTTGCCAG-3' ; reverse primer: 5'-TCCAGCATCACCCAGGCGTC-3'), 0.2 mM dNTPs mix, 1X PCR reaction, 5% DMSO, and 5U/µl Taq DNA Poltymerase (Qiagen). PCR was performed with 34 cycles and a Tm of 58°C. The PCR product was subsequently run in a 2% agarose gel. Wild type allele results in a fragment of 190bp and floxed allele fragment was 230bp, therefore two bands indicated the heterozygous condition (controls) and one 230bp band identified the homozygous floxed allele condition (*Foxg1* KO).

Western blot analysis

Embryonic tissue was triturated in 50-200µl TENT (Tris-EDTANaClTritonX100 buffer) with protease inhibitors and homogenised. Cells were allowed to lyse for 20 minutes at 4°C, cellular debris was pellet by centrifugation at 13,000rpm for 15 minutes at 4°C and supernatant, containing cellular proteins, was decanted. All protein samples were resolved on 12% pre-cast tris-glycine gels. Protein (50mg in 20ml 4X sample loading and TENT buffer)

was denatured at 95°C for 5 minutes and gels were run for 1 hour 40 minutes at 150V; each included a molecular weight ladder. Proteins were transferred onto a nitrocellulose membrane (225mA at room temperature for 2½ hours). Transfer efficiency was determined by staining the membrane in Ponceau's solution followed by two successive de-stains in ddH2O. Membranes were blocked in TBS-tween with 10% dried milk for 1 hour at room temperature. Primary antibodies were Serum 13 anti-Pax6 homeodomain (Carriere et al., 1993). Following incubation in primary antibody, membranes were washed 3 times in TBS-tween to remove unbound antibody. Secondary antibodies were centrifuged and diluted in 10ml blocking buffer, and allowed to bind for one hour at room temperature on a shaking platform. All secondary antibodies were conjugated to horseradish peroxidase to allow chemiluminescent detection of bound primary antibody. Membranes were washed 3 times in TBS-tween, to remove unbound secondary antibody. Bound antibody was then detected using the ECL+ chemiluminescent detection kit. Membranes were bathed for 5 minutes in ECL+ substrate, which was broken down by horseradish peroxidase, causing the emission of light. Signal was then detected by exposure to photographic film for 45 minutes. Membranes were washed 3 times in TBS-tween after chemiluminescent detection and re-probed for β-actin. Densitometric analysis of gel films was performed using a BioRad GS-710 densitometer. Gels were scanned at a high resolution in greyscale and analysis was performed using the Quantity One software (BioRad; rolling disc with background subtraction, peak area was taken as a measurement of band intensity). The relative intensity of each band was determined, and divided by the intensity of the β-actin band obtained for that sample. For each gel, the intensity of the WT bands were then averaged, and assigned an arbitrary value of 1. The intensity of the non-WT bands on the gel were divided by that of the WT samples.

Microscopy and imaging

ISH and DAB images were taken with a Leica DMNB microscope coupled to a Leica DFC480 camera. Fluorescence images were taken using a Leica DM5500B automated epifluorescence microscope connected to a DFC360FX camera. Images of embryo dissections were taken with a Leica MZFLIII fluorescence stereomicroscope. Image panels were created with Adobe Photoshop CS6.

Bromodeoxyuridine (BrdU) injections

Pregnant females, previously gavaged with tamoxifen at E9.5 to induce Pax6 deletion (see methods above), were intraperitoneally injected with a single dose of BrdU (10ug/ul, Thermo Fisher Scientific) at E10.5, E11.5 or E12.5 and embryos were collected 24 hours after the injection (E11.5, E12.5 or E13.5, respectively).

Quantitative Real Time PCR (qRT-PCR)

We extracted total RNA with RNeasy Plus micro kit (Qiagen) from Th, PTh and Ctx. cDNA was synthesized with a Superscript reverse transcriptase reaction (Thermo Fisher Scientific) and we performed qRT-PCR using a Quantitect SYBR Green PCR kit (Qiagen) and a DNA Engine Opticon Continuous Fluorescence Detector (MJ Research). We used the following primer pairs: Dlx2, 5'- CCAAAAGCAGCTACGACCT-3' and 5'-GGCCAGATACTGGGTCTTCT-3'; Ngn2, 5'-CAAACTTTCCCTTCTTGATG-3' and 5'-CATTCAACCCTTACAAAAGC-3'; Wnt8b, 5'-AACGTGGGCTTCGGAGAGGC-3' and 5'-GCCCGCGCCGTGCAGGT-3'; Ccnd1, 5'-GAAGGGAAGAGAAGGGAGGA-3' and 5'-GCGTACCCTGACACCAATCT-3'. We calculated and plotted the abundance of each transcript relative to GAPDH expression levels. For all samples we used three biological replicates consisting on tissue dissected from embryos belonging to three different litters. Controls and experimental embryos were from the same litter whenever possible. For each biological replicate we run three technical replicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-seq data analysis

Read alignment and counting

RNA-seq reads from each sample were mapped using STAR 2.4.0i (Dobin et al., 2013) to the mm10 mouse genome build downloaded from Ensembl77 (Aken et al., 2016) in October 2014. STAR was run with default options, allowing maximum multi-mapping to three sites. The number of reads mapped to each gene was counted using featureCounts v1.4.5-p1 (Liao et al., 2014) from the Subread package. Default options were used with a requirement that both reads needed to be properly mapped over exons to be counted, including reads which aligned over splice junctions.

Differential expression (DE) analysis

DE analysis was done in R (R Core Team, 2016) to assess the significance of differences in gene expression levels in cKO samples over control samples. We used two different R

packages: DESeq2 1.8.1 (Love et al., 2014) and edgeR 3.1.12 (Robinson et al., 2009), both run with default parameters. Appropriate filtering of low expressing genes was performed manually for edgeR, where genes with CPM values were lower than 1 were removed. For DESeq2, independent filtering of low expressed genes was performed before multiple testing corrections. DE was considered probable at FDR ≤ 0.05 for edgeR and an adjusted p-value ≤ 0.05 for DESeq2. Some genes were identified as differentially expressed by either DESeq2 alone or edgeR alone, while most of the genes identified as differentially expressed were identified by both (78.56% in ACtx, 73.96% in PTh and 70.60% in Th). After examining the expression levels of those genes by plotting counts per million (CPM) mapped reads in controls versus cKO, we decided to accept genes identified by either package as differentially expressed, attributing the difference in detection to the underlying methods (low-expression filtering, expression normalization and multiple testing correction) of each package. Accordingly, we included all genes identified by either DESeq2 or edgeR in our subsequent analysis.

Functional analysis

Lists of genes were analysed for Gene Ontology (GO) term enrichment using DAVID 6.8 Beta (Huang et al., 2009a, 2009b). Enrichment statistics were calculated for biological process terms from DAVID GO FAT database (GO_BP_FAT category).

Plots generated in R

Plots were generated with ggplot2 package (Wickham, 2016) and heatmaps were generated with pheatmap package. MA-plots were generated using the plotMA() function from DESeq2, modified so that it also includes the DE results from edgeR. PCA plots were generated using prcomp() function from base R.

Sample clustering methods

Expression values were transformed from raw read counts using variance stabilizing transformation described in DESeq2 and samples were hierarchically clustered using dist() and hclust() functions from base R, using parameters for Euclidean distance and Ward's linkage method (Ward, 1963). To cluster samples from experiments in which *Pax6* or *Foxg1* were deleted, variance stabilizing transformation was followed by the application of the ComBat function from R package sva (Leek et al., 2016) to correct for batch effects that might influence the comparison of results from these two sets of experiments. These

samples were clustered using the hcluster() function from amap R package (Lucas, 2014), with same distance and linkage methods as above. Within each tissue, $\log_2$-fold changes (LFCs) in gene expression between genotypes were calculated from average counts per million (CPM) mapped reads in cKO samples over average CPM mapped reads in control samples, using the following formula:

$$LFC = log_2\left(\frac{\frac{\sum_{cKO} CPM}{N_{cKO}}}{\frac{\sum_{Control} CPM}{N_{Control}}}\right)$$

Genes were clustered hierarchically by Pearson's correlation as a distance measure and Ward's linkage method.

Image analysis and quantification

BrdU quantification and calculation of proliferation/differentiation indexes

We counted the total number of BrdU-labelled cells and classified them into three different categories according to their expression of Ki67 (proliferation category), Tuj1 (differentiation category) or none (G0 category). We calculated the fractions of proliferative cells, cells in G0 and differentiated cells by dividing the number of cells in each of the three categories by the total number of BrdU-labelled cells. Cell quantification was performed on 40x magnification coronal microphotographs using the cell counter plug in from Fiji (Image J) (Schindelin et al., 2012).

We quantified three biological replicates (three embryos from three different litters, n=3) for each tissue (Actx, Thal, Pthal), genotype (control, *Pax6* cKO) and age (E11, E12, E13), being controls and experimental embryos pairs from the same litter. For each embryo we analysed three different rostro-caudal sections in the case of cortical tissue and four rostro-caudal sections for diencephalic tissues. For each tissue and condition, we counted an average of 989 cells.

The data from all ages, regions and genotypes was statistically assessed to test the effects of Pax6 inactivation on proliferation and differentiation depending on age and tissue. Data was fitted to a generalized mixed linear model using the glmer() function from lme4 R package (Bates et al., 2015). Counts of cells in proliferation and counts of cells exiting the cell cycle were set as outcome variables, with genotype, age and tissue set as interacting fixed effects and litter and embryo set as nested random effects. Function argument 'family' was set to

'binomial' due to two possible outcomes of cell state. P-values of fixed effects and their interactions were obtained using the Anova() function from car package (Fox and Weisberg, 2011) with argument type = 3 to specify the usage of Type III Wald chisquare tests. Contrasts of interest were tested using lsmeans() function from lsmeans package (Lenth, 2016).

Quantification of proliferation

To quantify proliferation in the cortices of *Foxg1* cKOs and *Pax6 Foxg1* cKOs we selected an area of the cortex (indicated in Figure 8) and counted the total number of cells (DAPI-positive) and the number of proliferating cells (Ki67-positive). Proliferation fraction was calculated by dividing the number of proliferating cells by the total number of cells.

Cells were counted in 40x magnification coronal microphotographs using cell counter plug in from Fiji (Image J) (Schindelin et al., 2012). We quantified three independent embryos from three different litters for each genotype (n=3). For each embryo we counted three different rostro-caudal sections. Differences across the two genotypes were assessed by one tail paired t-test and significance was considered when $p<0.05$.

Statistical analysis /RT-qPCR details

T-tests (n=3) were performed in Microsoft Excel. Statistical details of all experiments are specified in the text or corresponding figure legend.

Analysis of the splicing variants Pax6 and Pax6 (5a) ratio

We used summarizeOverlaps function from the GenomicAlignments R package (Lawrence et al., 2013) to count the number of reads aligning to genomic regions of Pax6 exons 5 and 5a. Read counts were divided by the number of bases of each exon (216b for exon 5, 42b for exon5a) to normalize for the exon length. Exon5/5a ratios for each sample were calculated as the normalized read counts in exon 5 over exon 5a. We then used aov and TukeyHSD functions from R stats package (base R) to test for significance in difference of exon5/5a rations between tissues and perform pairwise comparisons.

DATA AND SOFTWARE AVAILABILITY

The datasets generated in this study can be obtained from the European Nucleotide Archive (www.ebi.ac.uk/ena).

The accession number for the RNA-seq raw data from the Pax6 mutant experiments is ENA: PRJEB9747.

The accession number for the RNA-seq raw data from the Foxg1 mutant experiment is ENA: PRJEB21349.

ADDITIONAL RESOURCES

To interactively explore the Pax6 RNA-seq dataset visit https://pricegroup.sbms.mvm.ed.ac.uk/Pax6_diencephalon/

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Pax6 (1:200)	Biolegend	Cat# 901301, RRID:AB_2565003
Mouse monoclonal anti-Pax6 (1:10)	A gift from Prof. V van Heyningen, AD2.38 Simpson *et al.*, 2009	N/A
Rabbit polyclonal anti-Ki67 (1:200)	Abcam	Cat# ab15580, RRID:AB_443209
Rat monoclonal anti-BrdU (1:100)	Abcam	Cat# ab6326, RRID:AB_305426
Mouse monoclonal anti-Beta III tubulin (Tuj1) (1:200)	Abcam	Cat# ab18207, RRID:AB_444319
Rabbit polyclonal anti-Gsx2 (1:400)	Millipore	Cat# ABN162, RRID:AB_11203296
Mouse monoclonal anti-Foxg1 (1:100)	Hybridoma clone 17B12 Kindly donated by Dr. S. Pollard. Bulstrode *et al.*, 2017	N/A
Rabbit polyclonal anti-Tbr1 (1:1000)	Abcam	at# ab31940, RRID:AB_2200219
Goat anti-mouse biotinylated secondary antibody (1:200)	Vector laboratories	Cat# BA-9200, RRID:AB_2336171
Goat anti-rabbit biotinylated secondary antibody (1:200)	Vector laboratories	Cat# BA-1000, RRID:AB_2313606
Donkey anti-rabbit Alexa 568 secondary antibody (1:100)	Thermo Fisher Scientific	Cat# A10042, RRID:AB_2534017
Donkey anti-rat Alexa 488 secondary antibody (1:100)	Thermo Fisher Scientific	Cat# A-21208, RRID:AB_141709
Donkey anti-mouse Alexa 568 secondary antibody (1:100)	Thermo Fisher Scientific	Cat# A10037, RRID:AB_2534013
Streptavidin, Alexa Fluor 488 conjugate antibody	Thermo Fisher Scientific	Cat# S11223, RRID:AB_2336881
Streptavidin, Alexa Fluor 546 conjugate antibody	Thermo Fisher Scientific	Cat# S-11225, RRID:AB_2532130
Streptavidin, Alexa Fluor 647 conjugate antibody	Thermo Fisher Scientific	Cat# S-21374, RRID:AB_2336066
Chemicals, Peptides, and Recombinant Proteins		

Tamoxifen	Sigma-Aldrich	T5648; CAS: 10540-29-1
Critical Commercial Assays		
RNeasy Plus micro kit	Qiagen	74004
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific	18080093
QuantiTect SYBR Green PCR Kit	Qiagen	204143
Avidin-Biotin complex (ABC) kit	Vector Laboratories	PK6100
DAB peroxidase substrate kit	Vector Laboratories	SK4100
DPX Mountant for histology	Sigma-Aldrich	06522
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher Scientific	D1306
ProLong Gold Antifade Mountant	Thermo Fisher Scientific	P36930
DIG RNA Labeling Kit (SP6/T7)	Sigma-Aldrich	11175025910
Taq DNA Polymerase	Qiagen	201203
BrdU (5-Bromo-2´-Deoxyuridine)	Thermo Fisher Scientific	B23151
Normal Goat Serum	Thermo Fisher Scientific	31873
Normal Donkey Serum	Sigma-Aldrich	D 9663
Deposited Data		
Pax6 RNAseq raw data	This paper	European Nucleotide Archive (www.ebi.ac.uk/ena; ENA: PRJEB9747)
Foxg1 RNAseq raw data	This paper	European Nucleotide Archive (www.ebi.ac.uk/ena; ENA: PRJEB21349)
Experimental Models: Organisms/Strains		
Mouse: CAGGCre-ER^{TM}; RCE (GFP)	This paper Hayashi and McMahon, 2002 Sousa *et al.*, 2009	N/A
Mouse: Dty54	Tyas *et al.*, 2006	N/A
Mouse: Emx1-CreER^{TM}; RCE (GFP)	This paper Kessaris *et al.*, 2006 Sousa *et al.*, 2009	N/A
Mouse: Pax6^{loxP}	Simpson *et al.*, 2009	N/A
Mouse: Foxg1^{loxP}	Miyoshi *et al.*, 2007	N/A

Oligonucleotides		
Primers for the detection of floxed Pax6 allele. Forward: AAATGGGGGTGAAGTGTGAG. Reverse: TGCATGTTGCCT GAAAGAAG.	This paper Simpson *et al.*, 2009	N/A
Primers for the detection of floxed Foxg1 allele. Forward: TTGCTACATGCCTTGCCAG. Reverse: TCCAGCATCACCCAGGCGTC.	This paper	N/A
Primers for Dlx2 qRT-PCR. Forward: CCAAAAGCAGCTACGACCT. Reverse: GGCCAGATACTGGGTCTTCT.	This paper	N/A
Primers for Ngn2 qRT-PCR. Forward: CAAACTTTCCCTTCTTGATG. Reverse: CATTCAACCCTTACAAAAGC	This paper	N/A
Primers for Wnt8b qRT-PCR. Forward: AACGTGGGCTTCGGAGAGGC. Reverse: GCCCGCGCCGTGCAGGT.	This paper	N/A
Primers for Ccnd1 qRT-PCR. Forward: GAAGGGAAGAGAAGGGAGGA. Reverse: GCGTACCCTGACACCAATCT.	This paper	N/A
Software and Algorithms		
STAR 2.4.Oi	Dobin *et al.*, 2013	https://github.com/alexdobin/STAR
featureCounts v1.4.5-p1	Liao, Smyth and Shi, 2014	http://subread.sourceforge.net/
R software	R Core Team, 2016	https://www.r-project.org/
DESeq2 1.8.1 (R package)	Love, Huber and Anders, 2014	https://doi.org/doi:10.18129/B9.bioc.DESeq2
edgeR 3.1.12 (R package)	Robinson, McCarthy and Smyth, 2009	https://doi.org/doi:10.18129/B9.bioc.edgeR
ggplot2 (R package)	Wickham, 2016	https://github.com/tidyverse/ggplot2
sva (R package)	Leek *et al.*, 2016	https://doi.org/doi:10.18129/B9.bioc.sva
amap (R package)	Lucas, 2014	https://cran.r-project.org/package=amap
Lme4 (R package)	Bates *et al.*, 2015	http://lme4.r-forge.r-project.org/

car (R package)	Fox and Weisberg, 2011	https://cran.r-project.org/web/packages/car/index.html
lsmeans (R package)	Lenth, 2016	https://cran.r-project.org/web/packages/lsmeans/index.html
GenomicAlignments	Lawrence *et al.*, 2013	https://doi.org/doi:10.18129/B9.bioc.GenomicAlignments
DAVID 6.8 Beta	Huang, Lempicki and Sherman, 2009	https://david.ncifcrf.gov/
Fiji (Image J)	Schindelin *et al.*, 2012	https://fiji.sc/
Other		
Pax6 RNAseq analyzed data. Interactive app	This paper	https://pricegroup.sbms.mvm.ed.ac.uk/Pax6_diencephalon/

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