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Reduced chromatin accessibility correlates with resistance to Notch activation

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The Notch signalling pathway is a master regulator of cell fate transitions in development and disease. In the brain, Notch promotes neural stem cell (NSC) proliferation, regulates neuronal migration and maturation and can act as an oncogene or tumour suppressor. How NOTCH and its transcription factor RBPJ activate distinct gene regulatory networks in closely related cell types in vivo remains to be determined. Here we use Targeted DamID (TaDa), requiring only thousands of cells, to identify NOTCH and RBPJ binding in NSCs and their progeny in the mouse embryonic cerebral cortex in vivo. We find that NOTCH and RBPJ associate with a broad network of NSC genes. Repression of NSC-specific Notch target genes in intermediate progenitors and neurons correlates with decreased chromatin accessibility, suggesting that chromatin compaction may contribute to restricting NOTCH-mediated transactivation.
Notch signalling plays a key role in cell fate transitions during the differentiation of neural lineages, from neural stem cells (NSCs) to postmitotic neurons. Upon ligand binding, the NOTCH receptor is cleaved and the intracellular domain (NICD) translocates to the nucleus where it associates with its cofactor, RBPJ, to activate target gene expression (Fig. 1a). How cell-type specific binding patterns, in vivo and genome-wide, would help to reveal how cell-type specific target gene expression is achieved. Here we used TaDa after in utero electroperoration (IUE) to map NOTCH and RBPJ binding in the developing mouse brain (Fig. 1b–e). TaDa was originated to enable cell type-specific profiling without cell isolation, allowing genome-wide profiling of DNA- or chromatin-binding proteins without cell sorting, fixation or affinity purification. TaDa is extremely sensitive and highly reproducible in Drosophila, as well as in mammalian cells, starting from fewer than 10,000 cells. Nonetheless, DamID and TaDa were considered incompatible with transient transfection: the Dam-fusion protein methylates plasmid DNA when expressed in bacteria. The methylated plasmid DNA co-amplifies with genomic DNA, comprising a substantial proportion of the sequencing library. To overcome this, an intron was introduced into the coding sequence of the Dam methylase to prevent plasmid methylation in bacteria without affecting methylation in eukaryotes (intronDam18; Fig. 1g; Supplementary Fig. 1a, b; see Methods). To test this, we expressed Dam methylase alone under the control of an ubiquitous promoter (CAG; mouse embryonic stage E13.5), which should reveal regions of accessible chromatin (CaTaDa15,30). IUE of intronDam-plasmids generated reproducible methylation patterns in single brain hemispheres at E17.5 (Supplementary Fig. 2a; Supplementary Fig. 3a) without seemingly affecting neurogenesis (DamID-seq14,15; Supplementary Fig. 2b–d). The in utero Dam-methylation peaks corresponded with previously published ATAC-seq peaks from E13.5 mouse forebrain (Supplementary Fig. 2e, f), consistent with preferential methylation of accessible chromatin by untethered Dam methylase30,33.

We designed a Cre-inducible construct for conditional expression and to prevent leaky expression (‘non-leaky’ floxDam). The...
Notch pathway activation in RGCs is not sufficient to prevent neurogenesis entirely. Notch and RBPJ are key regulators of RGC proliferation and prevent the transition of RGCs into IPCs and postmitotic neurons. Notch1 mRNA levels decrease significantly upon transition from RGCs to IPCs (34,35) (Fig. 1k, l), or the NOTCH-target gene Hes1 (Fig. 1m). We found the binding patterns of NOTCH-NICD, rather than full length NOTCH, we performed TaDa (Supplementary Fig. 6a, and 7) for the fourth dataset (Fig. 2c, d). The most highly enriched motif under the RGC-NICD peaks matches the known consensus binding site of RBPJ: 5′-TTCCCA-3′ (p = 1−199, 44.82% of peaks; Fig. 2e; Supplementary Fig. 5i). In addition, consensus binding sites for homeobox transcription factors, in particular LHX2, were significantly enriched at Notch and RBPJ peaks in both RGCs and IPCs peaks (Fig. 2e; Supplementary Fig. 5j). LHX2 is necessary for the activation of several Notch target genes in the developing retina (44) and other data suggest a similar requirement in cortical NSCs, possibly through increasing enhancer accessibility by establishing a permissive chromatin state.2,45

Chromatin accessibility correlates with Notch and RBPJ binding. Peaks from the four datasets (n = 20,811) were clustered using an optimisation approach based on k-means (Supplementary Fig. 6; Supplementary Data 3), revealing eight robust subsets of peaks (Fig. 3a, b). Consistent with the prevailing view of how Notch regulates target gene expression (46), many binding sites were characterized by binding of both NOTCH and RBPJ in RGCs (cluster 4), or in both RGCs and IPCs (clusters 2, 6 and 7) (Fig. 3a–c). The majority of peaks, however, showed preferential binding of either NOTCH or RBPJ, but not both: NOTCH alone in RGCs (cluster 1), RBPJ alone in RGCs (clusters 5 and 8), or RBPJ alone in IPCs (cluster 3) (Fig. 3a–c). To confirm that these results were not due to expression of NICD, rather than full length NOTCH, we performed TaDa with full length NOTCH (NOTCH-FL) and compared these results with NICD. We found the binding patterns of NOTCH-FL and NICD in RGCs and IPCs to be remarkably consistent across the 8 clusters (Supplementary Fig. 7).

To assess whether chromatin state might influence NOTCH/RBPJ binding, we assayed chromatin accessibility using an unthegrated Dam methylase (CaTaDa (53,50). To compare our results with previously published ATAC-seq results (32), Clusters where NOTCH and RBPJ co-localized (clusters 2, 4, 6, 7), were

Fig. 2 Cell-type specific in utero TaDa of NOTCH and RBPJ. a IUE of plasmids with destabilized fluorescent proteins under control of Hes5 and Tal promoters (n = 1). Staining for DAPI (nuclei, blue), GFP (green) and mCherry (red). Scale bar, 20 μm. b Constructs for in utero TaDa. c–d NOTCH and RBPJ binding peaks near known target genes (grey shading) in RGCs and IPCs. Arrowheads indicate Hes5-promoter present in pHes5-plasmids. e Motif detection at RBPJ binding peaks in RGCs.
more accessible than those where only one of the two factors were bound (clusters 1, 3, 5, 8) (Fig. 3d; Supplementary Fig. 8a). Comparative motif analysis with i-CisTarget revealed overall high similarity between most peak clusters (Supplementary Fig. 8b) and a strong overlap with embryonic brain-specific regulatory regions (ENCODE) (Supplementary Fig. 8c). In contrast, the binding sites with the lowest average accessibility (clusters 3 and 8) (Fig. 3d; Supplementary Fig. 8a) were associated with a different set of motifs and genomic features than the other clusters (Supplementary Fig. 8b,c). These peaks are characterized by RBPJ binding without Notch (Fig. 3a–c) and are reminiscent of previously identified constitutive RBPJ binding sites. This may point to a role for RBPJ in maintaining transcriptional repression or, conversely, as part of a pioneering complex to unmask enhancers during differentiation. Interestingly, RBPJ binding motifs could not be detected under the IPC-RBPJ-specific peaks (cluster 3) (Supplementary Fig. 8d) suggesting that other factors, possibly homeobox transcription factors (Fig. 2c; Supplementary Fig. 8d), may recruit RBPJ to regulate target gene expression upon differentiation. Together, these data suggest that, in parallel to NOTCH/RBPJ-binding, differences in chromatin accessibility and co-factor occupancy may influence NOTCH/RBPJ-target gene expression (Fig. 3e).

Notch/RBPJ are able to bind at NSC-genes when expressed ectopically in intermediate progenitors. When NOTCH/RBPJ were bound together in RGCs, they could also be detected after ectopic expression in IPCs, where they mostly co-localized (55% 75% 73%; clusters 2, 6, 7) (Fig. 3a–c). Genes near these peaks showed the strongest enrichment for GO terms related to neurogenesis and Notch signalling (Supplementary Fig. 9a, b). This could suggest that NOTCH/RBPJ may be able to bind at NOTCH-regulated NSC-genes during differentiation. To determine on a genome-wide basis how NOTCH/RBPJ binding dynamics correlate with transcriptional changes during neurogenesis, we intersected the eight NOTCH/RBPJ peak clusters with published RNA-seq data from sorted RGCs and IPCs at E13.5 (Fig. 4a, b; Supplementary Fig. 10a–e), and with two independent single-cell RNA-seq datasets at E13.5 and E14.5 (Fig. 4c, d; Supplementary Fig. 10f–q). Peaks with constitutive NOTCH/RBPJ binding potential (cluster 6) were highly enriched near

Fig. 3 Dynamic NOTCH/RBPJ binding during neurogenesis. a–c k-means clustering of all regions bound by NOTCH or RBPJ in RGCs or IPCs results in 8 clusters corresponding to distinct binding patterns of RBPJ (blue) and NICD (yellow). Colour scale indicates TaDa binding intensity. d pCAG-iDam average signal (±s.e.m., shaded area) on NOTCH/RBPJ TaDa peak clusters. e Peak clusters indicate chromatin accessibility and binding mode.
Fig. 4 Ectopic NICD can bind to RGC-specific genes during differentiation. a–d Bulk RNA-seq a and single-cell RNA-seq c of RGCs and IPCs. Enrichment of NOTCH/RBPJ peak clusters b, d near genes differentially expressed in RGCs as compared to IPCs. e, f NOTCH and RBPJ binding profiles in RGCs and IPCs near RGC-specific genes. Peak loci and corresponding peak cluster (coloured shading corresponds to clusters in Fig. 3) shown. h–j IUE of pCIG-N1AECID stained for GFP (green) and the indicated RGC-specific NOTCH/RBPJ target gene (Sox9, Gli3, Prdm16; red; n = 1). Arrowheads indicate differentiating electroporated cells in the SVZ/IZ. Scale bars 50 μm.

The strong enrichment of cluster 6 peaks, NICD and RBPJ binding in RGCs and IPCs near genes that are downregulated upon differentiation, was surprising given that downregulation of NOTCH-target genes is thought to result from the loss of NICD binding31, 131 genes that were shown to be expressed specifically in RGCs37 (FDR < 10⁻⁵) were associated with cluster 6 peaks (Supplementary Data 4). The regions associated with cluster 6 peaks could also be bound in IPCs by full-length NICD, as assayed by TaDa (Supplementary Fig. 7b) and by transient transfection experiments, we were able to achieve ectopic NICD expression in vivo in RGCs, IPCs and postmitotic neurons by in utero TaDa with untethered Dam (Fig. 5a, b)15, 30, 33. This generated cell-type specific accessibility profiles comparable to whole-brain ATAC-seq32 (Supplementary Fig. 11a, b). Surprisingly, the profiles showed strong similarity between different cell types, particularly between RGCs and IPCs (Fig. 5c). Most NOTCH/RBPJ peak clusters also did not show dynamic changes in chromatin accessibility between RGCs and IPCs (Fig. 5d; Supplementary Fig. 11d, f, g). On cluster 4 peaks, for example, which have decreased binding of NOTCH/RBPJ upon differentiation, average Dam-accessibility was indistinguishable between RGCs and IPCs, and only decreased upon differentiation into postmitotic neurons (Supplementary Fig. 11f). This demonstrates that chromatin accessibility and cell-type specific binding dynamics of NOTCH/RBPJ appear to be regulated independently.

In contrast, near RGC-specific genes, the average accessibility on the Notch/RBPJ co-binding sites (cluster 6) decreased between RGCs and IPCs (Fig. 5e, f; Supplementary Fig. 11f–h), as did accessibility across the upstream regulatory regions of NOTCH/ RBPJ-bound genes (Fig. 5f; Supplementary Fig. 11e). This suggests that chromatin accessibility may help to restrict Notch activity during neurogenesis, for example, at loci where NOTCH/ RBPJ might still be bound.

Discussion

Intercellular signalling is mediated by signalling pathways that are used iteratively throughout development and disease. How the same signal is interpreted differently depending on cellular context remains largely unanswered. This has been due, in part, to the need for sensitive tools for cell type specific genome-wide chromatin profiling in vivo. Targeted DamID enables genome-wide profiling of DNA- or chromatin-binding without cell isolation, fixation or affinity purification13,15–18. Using vectors that overcome background plasmid methylation that can interfere with transient transfection experiments, we were able to achieve genome-wide profiling in limiting cell numbers in vivo, without

NSC genes bound by NOTCH/RBPJ become inaccessible upon differentiation. To assess whether changes in chromatin accessibility during differentiation correlate with differential expression of NOTCH/RBPJ target genes, we assayed cell-type specific chromatin accessibility in vivo in RGCs, IPCs and postmitotic neurons by in utero TaDa with untethered Dam (Fig. 5a, b)15, 30, 33. This generated cell-type specific accessibility profiles comparable to whole-brain ATAC-seq32 (Supplementary Fig. 11a, b). Surprisingly, the profiles showed strong similarity between different cell types, particularly between RGCs and IPCs (Fig. 5c). Most NOTCH/RBPJ peak clusters also did not show dynamic changes in chromatin accessibility between RGCs and IPCs (Fig. 5d; Supplementary Fig. 11d, f, g). On cluster 4 peaks, for example, which have decreased binding of NOTCH/RBPJ upon differentiation, average Dam-accessibility was indistinguishable between RGCs and IPCs, and only decreased upon differentiation into postmitotic neurons (Supplementary Fig. 11f). This demonstrates that chromatin accessibility and cell-type specific binding dynamics of NOTCH/RBPJ appear to be regulated independently.

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disruption of the native tissue environment. We profiled NOTCH and RBPJ binding in RGCs and IPCs in the developing mouse cerebral cortex after IUE. We found that NOTCH/RBPJ binding patterns in specific cell types correlate with chromatin accessibility although we also found many putative binding sites of NOTCH or RBPJ in relatively inaccessible chromatin regions. Enrichment of homeobox transcription factor binding motifs at NOTCH or RBPJ in relatively inaccessible chromatin regions. 

To assess the transcriptional activity of the putatively bound loci, we screened publicly available cell-type specific transcriptional data sets and found that ectopically expressed NOTCH and RBPJ could bind, perhaps only transiently, to RGC-specific genes in IPCs. Therefore, additional mechanisms may ensure that RGC-specific NOTCH target genes are inactivated to enable neurogenesis if NOTCH were to remain present during or after the transition of RGC to IPC. One such mechanism could be a reduction in chromatin accessibility at the regulatory regions of Notch target genes which, when paired with a decrease in NOTCH/RBPJ expression, could facilitate efficient repression of RGC-specific genes. Nevertheless, given that NOTCH/RBPJ can bind their target genes in IPCs, we hypothesize that the binding events occurring in RGCs could be maintained in IPCs, making chromatin accessibility changes essential for blocking the activation of RGC-specific NOTCH target genes.

Our data represent a population level view of NOTCH activity and it remains to be determined whether there is heterogeneity of NOTCH/RBPJ binding within cell types at the single cell level. With recent advances in single-cell technology, future experiments may be able to better characterize variability in NOTCH activity within cell populations in the developing cortex. The factors that determine the competence of cells to respond to Notch pathway activation have broad relevance, not only to the multitude of developmental processes in which Notch is involved, but also to pathologies such as cancers. Chromatin accessibility and the tumour cell of origin within the NSC lineage may influence the progression of specific brain tumour subtypes.

**Methods**

**Constructs**

*Intron-dam constructs.* For intron1Dam, the sequence of intron 3 of mouse IgH56 was inserted between helix 3 and 4 of the DNA-binding domain of the Dam methylase57 in pPB-PGK-mcherry-Dam17. mCherry-intron1Dam-SV40polyA was

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*Fig. 5 Chromatin accessibility correlates with NOTCH target gene expression. a, b* Constructs for *in utero* chromatin accessibility TaDa and cell type specific expression pattern. *c* Chromatin accessibility profiles at the Pax6 locus (bottom), and the corresponding NOTCH/RBPJ binding profiles (top). *d–f* Accessibility (average signal ± s.e.m., shaded area) at all NOTCH/RBPJ peak regions *d* on NOTCH/RBPJ cluster 6 peaks *e* and on cluster 6-bound RGC-specific genes *f* in the indicated cell types. *g* Heatmap of accessibility in the region highlighted in *c* (colour scale indicates TaDa binding intensity. GATC sites and NOTCH/RBPJ peaks are indicated. *h* Model of NOTCH target gene repression during differentiation after ectopic expression of NICD-Dam.
cloned into pCAG-IREs-GFP (pCIG, gift from P. Vanderhaeghen) to give pCAG-mcherry-intron1ΔDam. For intron2ΔDam a modified version of the Promega chromatic intron sequence was sub-cloned into pCAG-mcherry- intron1ΔDam, replacing intron 1 and modifying the exon junctions. intron2ΔDam was more efficient, as assessed by RT-PCR, and was used for most experiments.

**Flodam construct.** Lox71 and Lox61 sites were inserted into the Nhel site upstream of mCherry and the XmaI site inside intron2, respectively. The two Lox sites and intervening sequence were then inverted to give pCAG-Flodam2Δ.

**Promoter-fragments.** A 764 bp fragment of the mouse Hes5-promoter and 5′UTR38, and a 1097 bp fragment of the mouse Tuba1a-promoter, Tau42,43,44, were amplified from genomic DNA and cloned into SpeI-HindIII cut pCAG-mcherry-intron2ΔDam. pPhes5-iDam-GFP was removed from pND1-IREs-GFP (gift from F. Polleux) and replaced with Cre to generate pNeuro1-Cre. pTA1-mcherry-NLS-DR was cloned by Gibson assembly from Addgene plasmid 8460338, pCAG-Venus was generated by removing the U6-shRNA cassette from pSVC2 (gift from F. Polleux).

**Ectopic Notch expression.** For the IUE experiments with constitutively active Notch the plasmid pCAG-NΔECD was used, which consists of pCAG-NAECs-GFP and was a gift from G. Del Sal63. As a control, an empty pCAG-IREs-GFP plasmid was used (pCIG, gift from P. Vanderhaeghen).

**Dam-fusion proteins.** The mouse Notch1 intracellular domain with the transmembrane domain (NTM0) or without the transmembrane domain (NTM380) was amplified from pCAG-NΔECD (pCAG-NAECs-GFP, gift from G. Del Sal63). Mouse full-length NOTCH was from Addgene 4172838. RBPJΔ1 was amplified from cDNA of Human derived NSCs. mCCherry(2), mammalian codon-optimized version of mCherry, was from Addgene 8460538. Plasmids for IUE were prepared from Dam-negative bacteria with Endofree Plasmid Maxi kit (Qiagen 12362). Molecular weight ladder is Hyperladder 1 kb (BioLynx BIO-33026).

**in utero electroporation.** All mouse husbandry and experiments were carried out in a Home Office-designated facility, according to the UK Home Office guidelines upon approval by the local ethics committee (project licence PPL70/8277). Experiments were done in wild-type MF1 mice. Timed natural matings were used, where noon of the day of plug-identification was E0.5. IUE was performed as previously described69 at E13.5 with 50 µl micropipette pulses (RTF380) using CUY65050s electrodes (Sonidel). DamID plasmids were injected at 1 µg/µl together with pCAG-Venus at 0.25 µg/µl. All other plasmids were injected at 1 µg/µl. Embryos were harvested after 24 hours (E14.5) for TaDa with Phes5 and pTA1, after 72 hours (E16.5) for TaDa with pND1-Cre and after 96 hours (E17.5) for TaDa with pCAG-intronDam. For RGoChIP data, we combined samples from Phes5-mCherry-NICD-intron2ΔDam, pHes5-mCherry-NICD-intron2ΔDam and pHes5-mCherry-NΔE-intron2Dam. IUE of pPhes5-NAECs-intron2ΔDam did not prevent differentiation, consistent with the very low levels of translation of the Dam-fusion protein13,17,28.

**Immunostaining and imaging.** For immunostaining, embryos and tissue were processed as previously described49 and staining was performed on 100 µm thick vibratome sections in PBS with 0.3% Triton (PBST) and 3% BSA (Sigma A3608). Antiseras were as follows: chicken anti-GFP 1/1000 (Abcam ab13970), rabbit anti-RFP 1/500 (Abcam ab62341), goat anti-Sox2 1/500 (R&D AF2018), rabbit anti-Sox9 (Millipore AB5535), rabbit anti-Pdm16 1/200 (gift from P. Seale67), rabbit anti-Prdm16 1/200 (gift from P. Vanderhaeghen), rabbit anti-Prdm16 1/200 (gift from P. Seale67), goat anti-Sox2 1/500 (R&D AF2018), rabbit anti-iDam; 3× pTA1-iDam-RBPJ; 4× pTA1-Notch-iDam; 4× pHes5-NotchFL-iDam; 2× pTA1-NotchFL-iDam) were normalized against separate Dam-only replicates (7× pCAG-iDam; 5x pHes5-iDam; 4x pTA1-iDam, 3x pCAG-floxDam) with a modified version of the damidseq pipeline48 (RPM normalization, 300 bp bins) and all peaks were clustered using R (v2.17.2) and GO terms were associated with indicated signalling pathways or biological processes were highlighted in the same colors in bar- and violin/dot-plots.

**Motif detection.** Comparative motif analysis was performed with i-cisTarget49. Enriched features were detected by uploading coordinates for all peaks of individual Notch/RBPJ k-means clusters to the i-cisTarget online platform (Gene annotation: RefSeq r70, Database: v5.0) after conversion to mm9 with the liftOver function of rtracklayer (v1.42.2). Normalized enrichment scores across all features and all k-means clusters were converted to z-scores, subjected to unsupervised clustering to identify stable binding patterns. The optimal cluster number was determined with the help of the R packages factoextra (v1.0.5), clValid (v0.6-6) and mclust (v5.4.5). k-means as the clustering approach was chosen and its parameters optimised by calculating silhouettes with the R packages clValid (v0.6-6) and mclust (v5.4.5). The depth branch was reduced to 4 for simplicity with the cut function of the stats package and the R dendextend package (v1.0.0). De novo motif analysis on binding sites was performed with Homer50 (findMotifsGenome.pl) and motifs consisting of 5' GATC-3' were manually removed from the results. Known motif enrichment was analysed with AME of the MEME suite with the following scoring method: Average odds score; Fisher's exact test, E-value < 10^-08; Motif databases: Jaspar Core Vertebrae non-redundant, UniProteome Mouse, Jolma2013 Human and Mouse. Sequences corresponding to all peaks were first retrieved from the Biostrings genome object for the UCSC mm10 genome (hg38/mm10.ucsc.mm10.fa) and converted to v2.10.0. The getSeq command of the Biostrings R package (v2.50.2).

**Comparative GO term analysis.** Gene Ontology term enrichment of genes associated with peaks was done using the broadrench command of chipenrich (v2.10.0) for all available mm10 genomics in chipenrich.data (v2.10.0) (locusdef = nearest_tt). Gene identifiers were converted with AnnotateDbi (v1.48.0) based on the org.Mm.eg.db (v3.10.0) database. Enrichment of GO terms annotated to biological processes (GO BP) was visualized by plotting -log10-transformed p-values, if the calculated p-value was <0.01 in at least one of the compared conditions GO terms were manually curated with associated signaling pathways or biological processes were highlighted in the same colors in bar- and violin/dot-plots.
**Genome wide correlation.** Mapped reads (see DamID-seq data processing) were extended to 150 bp with bamCoverage from the deepTools suite (v3.1.3). The resulting *b*edGraph files were read into R, reads were then binned into peaks if they shared one or more chromosomal locations or bins or into the regions covered by all NOTCH/RBPJ peaks and Pearson Correlation Coefficients were calculated with the cor command of the R stats package (v3.6.1). Coefficients for all pairwise comparisons were plotted as tiled heatmap.

**Bulk RNAseq data processing.** Bulk RNAseq datasets for mouse cortex cell types were acquired from GEO (GSE65000) and trimmed with TrimGalore (v0.4.5). *fastq.gz files were pseudaligned with kallisto (v0.45.0; b 100–single -1 200 -s 30) and statistical analysis was performed with sleuth (v0.30.0) by specifying full models for pairwise combinations of cell types. Reads were aggregated per gene rather than transcript while preparing the sleuth object with the sleuth_prep command (i.e., gene-mode = TRUE). Significance of differential expression was determined via q-values derived from Wald tests (sleuth_wt) based on the full model. Raw data from bulk RNAseq upon NICD overexpression was not publicly available, but genes with a p-value for differential expression <0.05 were extracted from the Supplementary Information.

**Single cell RNAseq data processing.** Single cell RNAseq datasets derived from mouse cortices at E13.5 and E14.5 were sourced from GEO as raw count matrices with the accession number (GSE107122 preselected for cortex-only cell types; GSE123335 combined matrix). GSE107122 (E13.5) was read into R as a Seurat Object via the corresponding R package Seurat (v2.3.4; min.cells = 100000), scaled and centred dependent on nUMI and normalized (scale.factor = 100000), scaled and centred dependent on nUMI and percent of reads aggregated on mitochondrial genes. Dimensionality reduction via PCA preceded clustering of cells using 5 dimensions as evaluated by JackStraw analysis. The Wilcoxon rank sum test was used to identify significantly differentially expressed genes for every cluster compared to all other clusters or for sets of clusters annotated to their respective cell types (IPCs, RGCs, neurons) via the FindAllMarkers command (logfc.threshold = 0, min.pct = 0, only.pos = FALSE, return.thresh = 1). GSE123335 (E14.5) was read into R as a dGMatrix via the methods package (v3.6.1) and processed by creating a Seurat Object with Seurat (v3.0.2; min.cells = 3, min.genes = 200). To properly integrate all 6 replicates combined in the matrix, they were first transformed by SplitObject, subsequently normalized via NormalizedData and further prepared for integration via Canonical Correlation Analysis with FindVariableFeatures and FindIntegrationAnchors. To avoid detection of differentially expressed genes, nfeatures was set to 18361 corresponding to the number of genes detected across all 6 scRNAseq replicates. Dimensionality reduction was performed with the runPCA and runTSNE commands based on 90 dimensions. Differential expression analysis was performed cluster-wise as indicated for GSE107122 data with the FindMarkers command.

**Statistical modelling of peak-expression correlation.** Associations between sets of peaks and significantly differentially expressed genes from bulk RNAseq or scRNAseq data were modelled as binomial distributed, since multiple peaks can be linked to the same gene. Based on the assumption that individual peaks have the highest likelihood to associate with the gene whose TSS is closest, associations of significantly and non-significantly differentially expressed genes with peaks as well as the total number of significantly and non-significantly differentially expressed genes were summed at a particular significance threshold (q-value, adjusted p-value). To test for enrichment of peak-to-gene associations, the binom.test command of the R stats package (v3.6.1) was used (alternative = less, confLevel = 0.95). This test was repeated across multiple gene expression significance thresholds and peak sets (cell-types, constructs, clusters). The resulting p-values were adjusted due to multiple testing with the Bonferroni correction as implemented in the p.adjust command of the R stats package (v3.6.1, method = bonferroni). Adjusted p-values and the number of peaks associated with expressed genes (peaksAtGenes) were used to visualize and average the binding intensities of peaks across all supplied coordinates. Signal from the mitochondrial genome, the IghE-intron, the Hes5-, Tuba1a- and NeuroD1-promoters were removed for p-values, ENCFF998QON.bam for alignments. Single cell and bulk RNAseq datasets were obtained from GEO (GSE107122, GSE123335 and GSE65000). Raw data from RBPI ChIP-seq and RNA-seq upon NICD overexpression in mouse cortex was not available anymore, but peak information was obtained from their Supplementary Table 5 (https://academic.oup.com/stmcls/article/30/4/741/6415703) and differential gene expression from their Supplementary Table 1 (https://academic.oup.com/stmcls/article/30/4/741/6415703). Source data are provided with this paper.

**Code availability** Scripts covering the outlined analyses are available on request.

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**Data availability** The data that support this study are available from the corresponding author upon reasonable request. Raw and processed sequencing data of all datasets in this study have been deposited on NCBI GEO under the accession code GSE152207. ATAC-seq data from E13.5 mouse brain was obtained from the ENCODE portal (ENCFF450ZSN bam for alignments). Single cell and bulk RNAseq datasets were obtained from GEO (GSE107122, GSE123335 and GSE65000). Raw data from RBPI ChIP-seq and RNA-seq upon NICD overexpression in mouse cortex was not available anymore, but peak information was obtained from their Supplementary Table 5 (https://academic.oup.com/stmcls/article/30/4/741/6415703) and differential gene expression from their Supplementary Table 1 (https://academic.oup.com/stmcls/article/30/4/741/6415703). Source data are provided with this paper.

**Code availability** Scripts covering the outlined analyses are available on request.
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Author contributions
J.v.d.A., S.W.C., R.K., A.P.A.D., O.L.-B., and A.H.B. designed the experiments; J.v.d.A., S.W.C., A.P.A.D., O.L.-B., and R.Y. performed the experiments; R.K. and J.v.d.A. performed bioinformatic analysis; J.v.d.A., S.W.C., R.K., A.P.A.D., O.L.-B., and A.H.B. wrote the paper; A.H.B. supervised the project.

Competing interests
The authors declare no competing interests.

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