Terminal neuron localization to the upper cortical plate is controlled by the transcription factor NEUROD2

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Excitatory neurons of the mammalian cerebral cortex are organized into six functional layers characterized by unique patterns of connectivity, as well as distinctive physiological and morphological properties. Cortical layers appear after a highly regulated migration process in which cells move from the deeper, proliferative zone toward the superficial layers. Importantly, defects in this radial migration process have been implicated in neurodevelopmental and psychiatric diseases. Here we report that during the final stages of migration, transcription factor Neurogenic Differentiation 2 (Neurod2) contributes to terminal cellular localization within the cortical plate. In mice, in utero knockdown of Neurod2 resulted in reduced numbers of neurons localized to the uppermost region of the developing cortex, also termed the primitive cortical zone. Our ChIP-Seq and RNA-Seq analyses of genes regulated by NEUROD2 in the developing cortex identified a number of key target genes with known roles in Reelin signaling, a critical regulator of neuronal migration. Our focused analysis of regulation of the Reln gene, encoding the extracellular ligand REELIN, uncovered NEUROD2 binding to conserved E-box elements in multiple introns. Furthermore, we demonstrate that knockdown of NEUROD2 in primary cortical neurons resulted in a strong increase in Reln gene expression at the mRNA level, as well as a slight upregulation at the protein level. These data reveal a new role for NEUROD2 during the late stages of neuronal migration, and our analysis of its genomic targets offers new genes with potential roles in cortical lamination.

The cerebral cortex of higher animals is composed of numerous excitatory and inhibitory neuron types organized into six anatomical and functional layers. Developmentally, excitatory neurons originate from neural stem cells that divide in the ventricular zone of the anterior neural tube, then migrate radially to the overlying cortical plate. While neural stem cells initially divide symmetrically during an increase of the proliferative population, the division mode transitions to asymmetric division as embryogenesis progresses. One daughter cell from each cell division remains at the ventricular zone, while the other daughter exits cell cycle and initiates a complex mode of radial migration to the border between the cortical plate (CP) and the marginal zone (MZ). As development progresses, each wave of migrating neurons reaches the surface by passing through the nascent cortex in an inside-out manner, eventually giving rise to the layered structure of the cerebral cortex. Consequently, early-born neurons populate the deep layers, and late-born neurons settles in the superficial layers. The precise control of the radial migration process is essential for the proper formation of the six cortical layers. Importantly, in mice and humans, mutations, infections or environmental agents that perturb cortical migration cause cortical structural malformations, intellectual disability, neuropsychiatric diseases, and seizures.

A closer examination of the radial migration process reveals a series of different modes of cellular motility, as opposed to a uniform directed movement from the ventricular zone toward the superficial marginal zone. Initially neurons are multipolar in morphology as they emerge from the ventricular zone, which is followed by a transition to a bipolar morphology and locomotion on radial glia fibers through the intermediate zone and much of the cortical plate. Upon approaching the border between the cortical plate and the marginal zone,
they terminally translocate from the radial glia fibers. Terminal translocation is accompanied by positioning of neuronal cell bodies in the uppermost layer of the cortex, also termed as the primitive cortical zone. During this developmental period, the marginal zone is populated by Cajal-Retzius cells which secrete the extracellular glycoprotein REELIN, considered a stop signal for migrating neurons. Migrating neurons express the REELIN receptors VLDLR and LR8 (APOER2), as well as the intracellular adaptor molecule DAB1, which conveys receptor activation to downstream signaling pathways. Ligand binding induces the endocytic internalization of REELIN and phosphorylation of DAB1. Activation of REELIN signaling in neurons induces anchorage of nascent primary dendrites to the extracellular matrix of the marginal zone, stabilization of the neuronal skeleton, and termination of migration. Loss of REELIN, VLDLR, APOER2 or DAB1 function causes profound defects in cortical lamination; the cortical layers develop in an inverted pattern, with early- and late-born neurons being located in superficial and deep layers, respectively.

Individual steps of radial migration are controlled by the combinatorial expression of specific transcription factors (TFs). Prominent among these TFs are the bHLH family members, including the Neurogenin and the NeuroD proteins. In addition to their roles in neuronal cell fate specification and neuronal differentiation, these factors have also been shown to control specific steps of cortical radial migration described above. A key member of the NeuroD family of neurogenic bHLH TFs is NEUROD2. The Neurod2 gene is highly expressed in the developing cortex, and its expression persists, albeit at low levels, into adulthood in cortical excitatory neurons. The gene is highly expressed in nascent primary dendrites to the extracellular matrix of the marginal zone, stabilization of the neuronal skeleton, and termination of migration. Loss of REELIN, VLDLR, APOER2 or DAB1 function causes profound defects in cortical lamination; the cortical layers develop in an inverted pattern, with early- and late-born neurons being located in superficial and deep layers, respectively.

We previously characterized the genetic targets of NEUROD2 in the cerebral cortex at two developmental timepoints: embryonic day 14.5 (E14.5), representing the peak of neurogenesis and migration in the mouse cortex; and postnatal day 0 (P0), representing the onset of neuronal differentiation, dendritic growth and synaptogenesis. Here, we carry out a comparative analysis of these two datasets and overlay it with transcriptionomics analysis of primary neurons in which Neurod2 expression is knocked down. We find that NEUROD2 exhibits quantitative and qualitative differences in target-selectivity at these two developmental timepoints. From our postnatal dataset, we identify several gene targets with known roles in neuronal projection development, such as the Ca2+/Calmodulin-dependent Kinase IV (Camk4) gene, and we demonstrate that suppressing Neurod2 expression in primary cortical neurons causes dendritic differentiation defects. Using our embryonic dataset, we identify numerous NEUROD2 targets with known functions in the Reelin signaling pathway, such as Reln, Dab1, and Lrp8. Furthermore, suppressing Neurod2 expression by in utero shRNA electroporation caused a defect in cellular positioning of neurons to the primitive cortical zone. Our results point to NEUROD2 as a regulator of the terminal stage of radial migration that acts, at least in part, by regulating genes functioning in the Reelin pathway. Our dataset also provides novel candidate genes that might have functions in different aspects of cortical radial migration. Future experiments resucing individual target genes with roles in neuronal migration in a Neurod2-deficient background will uncover the precise mechanism by which this TF orchestrates critical pathways for proper cortical lamination.

Results

**NEUROD2 binds to overlapping and unique gene-sets in embryonic and postnatal cerebral cortex tissue.** To evaluate whether NEUROD2 interactions with specific genomic sites are temporally regulated, we compared NEUROD2 binding profiles between embryonic day 14.5 (E14.5) and postnatal day 0 (P0). We retrieved binding scores from our previously published NEUROD2 ChIP-Seq datasets that represent the amount of relative TF binding to target sequences, then carried out a comparative analysis using the E14.5 and P0 results. Based upon this comparison, we identified a large number of binding sites that were exclusively targeted at E14.5, and fewer sites that were targeted at both developmental stages or were specific to P0 (Supplemental Material 1) (Fig. 1A). For both datasets, we had previously reported that a majority of NEUROD2 binding sites were not located at promoters, but rather at distant regulatory elements. Therefore, we decided to employ a target gene prediction approach, called the Closest Gene Method, which generates a score based on the number of TF binding sites and their proximity to the transcription start site of each gene. We calculated Closest Gene scores for the P0 dataset and compared it to the E14.5 dataset that we had previously published (Supplemental Material 2). Genes with a Closest Gene score above a threshold value were predicted as targets. Comparison of these predicted targets between two developmental stages revealed a large number of E14.5-specific genes, as well as a smaller number of target genes expressed only in the P0 developmental stage or at both stages (Fig. 1A).

In order to determine whether NEUROD2 gene targets at embryonic and postnatal timepoints represented functionally distinct gene groups, we carried out gene ontology analysis. Not surprisingly, at both tested developmental stages, genes related to nervous system development were significantly enriched (Fig. 1B). However, genes associated with migration of different types of neurons were specifically enriched at E14.5. To gain better insight into the signaling pathways regulated by NEUROD2, we performed pathway analysis at the two stages using the Reactome database. In addition to pathways whose significance is not immediately clear to us, our results also demonstrated that embryonically expressed NEUROD2 might control pathways related to cellular adhesion, synaptic connectivity, migration, and postnatal neuron projection development. Furthermore, NEUROD2 appeared to impinge upon calcium-mediated transcription, including CaMKIV transactivation of CREB (CAMP-response element binding protein) (Fig. 1C, Supplementary Material 3). Collectively these data suggested that NEUROD2, a TF that is expressed throughout the lifetime of cortical excitatory neurons, may regulate distinct functions during different developmental events.
NEUROD2 is required for dendritic differentiation of cortical neurons. Since our analysis of our postnatal dataset suggested a role for NEUROD2 in neuronal projection development, as well as of CaMKIV-mediated transcription, a known regulator of dendritic differentiation, we asked whether neurons silenced for Neurod2 also exhibited generalized dendritic arborization defects. Toward this aim, we cultured primary cortical neurons and transfected them with a short hairpin RNA targeting NeuroD2 transcripts (shNeurod2-1) at low efficiency to achieve knockdown in isolated neurons (Fig. 2A). We then quantified dendritic arborization of transfected cells, also marked by EGFP expression, by Sholl analysis, an approach that reports upon the number of dendrite branches as a function of distance from the neuronal soma. Our results demonstrated that while the number of primary dendrites protruding from the soma were comparable between the two conditions, a significant reduction in higher order dendritic branches was observed in neurons where Neurod2 expression was silenced (Fig. 2A, B). While this experiment pointed to a requirement for NEUROD2 in dendritic arborization in cortical neurons, future experiments will uncover which specific target genes are functioning in dendrite arbor development.
RNA-Seq analysis reveals NEUROD2-regulated target genes in primary cortical neurons. To further focus our attention upon genes for which expression is regulated by NEUROD2, we silenced Neurod2 expression in primary cortical cultures using two validated shRNAs and analyzed gene expression changes. When gene expression was compared to cells treated with a control shRNA, we found that 25 genes were down-regulated and 9 genes were up-regulated upon silencing of Neurod2 (Supplemental Material). As expected, we detected Neurod2 as one of the significantly downregulated genes. Genes perturbed by Neurod2 depletion included those expressing DNA binding proteins (Arid5a, Ddit4, Ubn2, Trim66, Zgpl1, and Sp8); regulators of extracellular matrix, adhesion and synaptogenesis (Thbs1, Reln, Col3a1, Col1a1, and Ank1); metabolic genes (Shmet2, Tkt, Pck2, and Pde12); signaling molecules (Chac1 and Sesan), and a translational regulator (Rps6ka4). Next, we asked whether any of the regulated targets were also directly bound by NEUROD2 as revealed by our ChIP-Seq data. We found that 14/34 genes harbored binding sites for NEUROD2, with the remaining (20/34) potentially represented secondary or off-target effects (Supplemental Material). Among the direct and regulated targets of NEUROD2, we were intrigued to identify Reln, a gene with essential roles in neuronal migration, dendrite development and synaptogenesis. Our previous findings had also implicated NEUROD2 in regulating the signaling pathway activated by this extracellular matrix protein.

Next we predicted the protein interactome of REELIN using the String database and asked whether additional NEUROD2 targets were found in this network. As well as the previously reported targets, REELIN receptor Lrp8, intracellular adaptor molecule Dab1, and membrane-associated tyrosine kinase Fyn, we found that we had also identified additional REELIN-interacting NEUROD2 targets, such as Itgb1, Tbr1 and Kenj6 (Fig. 3B). Furthermore, in order to achieve a comprehensive view of NEUROD2 targets with roles in cortical lamination,
NEUROD2 regulates the expression of Reln gene in cortical neurons. In mice, Reln gene consists of 65 exons, spanning approximately 460 kb of genomic sequence. Upon inspection of NEUROD2 binding sites along this gene we identified four prominent peaks at introns 21, 22, 55 and 63. Initially, we confirmed binding to all four peak sequences by ChIP followed by quantitative PCR (ChIP-qPCR) using cortical tissue from E14.5 and P0 pups. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex. We normalized our qPCR signal individually from all four peaks to the amount of input DNA and compared to negative control samples where immunoprecipitation was carried out with an antibody against GFP. Our results suggested quantitatively more binding to all four intronic sequences from E14.5 cortex when compared to samples from P0 cortex.
Neurod2 cDNA (Fig. 4E,F). Taken together, our results suggest that NEUROD2 strongly suppresses expression of Reln mRNA in cortical neurons. However, this regulation of gene expression at the mRNA level only modestly affects levels of REELIN protein.

Next we determined how REELIN protein levels were affected in the embryonic cortex upon silencing Neurod2 expression via in utero electroporation of shNeurod2-1. We electroporated E14.5 embryos, fixed the cortices at E17.5 using paraformaldehyde, and carried out immunofluorescence staining against REELIN in cortical sections. In agreement with previous findings, we observed high levels of REELIN protein in the MZ, but not in the CP or the SVZ-IZ of control samples (Fig. 5A,B). Our quantification of immunofluorescence signal intensity revealed no difference within the CP and SVZ-IZ when comparing control and Neurod2 knockdown samples (Fig. 5D and E). Interestingly, in embryos electroporated with shNeurod2-1, we observed a small but significant upregulation of REELIN protein in the MZ, generally localized to the cell body (Fig. 5A–C). These results raised questions about the cellular source of this ectopic REELIN expression. Even though we observe a small number of neurons over-migrating into the MZ in neurons transfected with shNeurod2-1, the vast majority of neurons are localized to the CP. Whether this additional REELIN protein in the MZ is derived from the projecting dendrites of cortical neurons, from over-migrating neurons, or instead is a secondary response of the CR cells to reduction of Neurod2 expression in cortical neurons is currently unclear, but this topic is worthy of future investigation.

**NEUROD2 is required for the terminal stages of cortical radial migration.** Our comprehensive analysis uncovering NEUROD2 target genes functioning in neuronal migration, along with focused experiments on regulation of Reln gene expression, prompted us to test whether cortical migration is affected upon suppressing
**Figure 5.** *In vivo* quantification of REELIN protein levels upon *Neurod2* silencing in the embryonic cortex. 

(A) Embryos were electroporated with non-silencing or sh*Neurod2*-1 shRNA and TdTomato at E14.5, cortices were harvested at E17.5 and confocal images were acquired after REELIN immunofluorescent staining. REELIN levels were not significantly altered within the CP, SVZ-IZ and VZ. However we observed a slight but significant increase in REELIN protein within the MZ. Scale bar: 100 µm. (B) Higher magnification images of those displayed in (A). Scale bar: 50 µm. (C–E) Quantification of images displayed in (A,B). A total of 21 images acquired from n = 3 embryos per group were used for quantification. *p = 0.02 by unpaired two-tailed t-test. Abbreviations: MZ (marginal zone), SVZ-IZ (subventricular-intermediate zone), CP (cortical plate), NS (non-silencing), ns (non-significant).
Neurod2 expression. Toward this goal, we silenced Neurod2 in neural progenitor cells by in utero transfection of shNeurod2-1 and a tdTomato marker in E14.5 embryos, then quantified the number of tdTomato-positive neurons in different cortical layers at E17.5 by confocal microscopy (Fig. 6A,B). In control samples, we observed tdTomato-positive neurons at expected locations within the ventricular zone, along the subventricular and intermediate zones, and at the cortical plate. In samples in which we silenced Neurod2 expression, we did not observe gross defects in migration, and neurons generally appeared to migrate from the ventricular zone toward the cortical plate in a manner comparable to control samples. Specifically, multipolar migration and glial-guided migration appeared intact. However, we observed a reduction in the number of tdTomato-positive neurons in the primitive cortical zone (located immediately below the marginal zone), suggesting that NEUROD2 depletion leads to a defect specific to the terminal stage of migration (Fig. 6A,B). In Neurod2 knockdown neurons, concomitant with a significant reduction of neurons localized to the primitive cortical zone, we occasionally also observed neurons which had over-migrated into the MZ of the shNeurod2-1 transfected samples. Scale bar: 50 µm. Bars represent S.E.M. p < 0.0001 by two-way ANOVA and *p = 0.0165 and **p = 0.0015 by post hoc Sidak’s test.

**Discussion**

Here we report a comparative analysis of the genetic targets of NEUROD2 and reveal a new role for this TF in the terminal stage of cortical radial migration. We had previously reported NEUROD2 targets identified by ChIP-Seq experiments both in embryonic and postnatal cerebral cortices26,27, and here we have complemented these earlier studies by a comparative analyses of these two developmental timepoints. Furthermore, in order to focus upon targets whose expression is regulated by NEUROD2, we silenced its expression in primary cortical neurons and carried out an RNA-Seq analysis. Our comprehensive and comparative analyses of these two ChIP-Seq and RNA-Seq datasets converged upon the Reln gene. We show that in mice, NEUROD2 binds to several different introns of Reln at both embryonic and postnatal stages, and this binding correlates with reduced levels of Reln mRNA. Even though NEUROD2 has typically been considered as a transcriptional activator, our studies point to a potentially dual function for NEUROD2; in addition to its previously reported transcription activating function25,42,43, it may also act as a repressor in a context-dependent manner. In support of our findings, Reln expression was also previously shown to be upregulated in the embryonic cortex of Neurod2/6 double knockout mice44. However, whether this effect is a direct consequence of NEUROD2 binding to Reln introns, or instead is a secondary response, awaits confirmation by site-directed genome editing of these binding sites.

NEUROD2’s potential capacity to function as a transcriptional repressor agrees well with our previous results demonstrating that NEUROD2 inhibits expression of the Stim1 gene to control calcium signaling in cortical neurons26. Furthermore, several of the NEUROD2 binding peaks both in Reln and Stim1 genes have also been
identified in ChIP-seq experiments carried out for the chromatin insulating factor CTCF (encodeproject.org), suggesting that NEUROD2 might be involved in establishing or maintaining higher order architecture of chromatin loops. In support of this hypothesis, NEUROD2 was recently shown to function as a major organizer of cell-type specific chromatin folding in cortical neurons, suggesting that its transcription activating or repressing functions might in fact be directly related to its role in establishing or maintaining transcriptionally isolated domains. The mechanistic details of how NEUROD2 regulates transcriptional activity in distinct genetic loci are of great interest for future investigations.

Past studies have suggested that NEUROD2 might have a role in regulating radial migration of excitatory cortical neurons. In addition to our current finding demonstrating NEUROD2 regulation of Rehn mRNA expression, our past work had shown that it also binds to promoters of several genes encoding downstream signaling proteins that transduce REELIN signals, such as Dab1 and Fyn, as well as the receptor gene Lrp8. To our surprise, upregulation of Rehn mRNA levels upon Neurod2 silencing does not correlate well with changes at the protein level. We observe a small but significant increase of REELIN in primary neurons, but not in embryonic sections, after Neurod2 suppression. Immunofluorescence staining is a semi-quantitative method and might not report accurately upon small changes in protein levels of REELIN. Taken together, we conclude that the major effect of NEUROD2 is most prominent at the mRNA level of Rehn.

The control of Rehn gene expression within a precise spatiotemporal window in the developing cortex appears to be a conserved and tightly regulated mechanism that depends upon several TFs. For example, double-knockout mice for the TFs Pbx1/2 also exhibit ectopic Rehn expression, leading to a dramatic inversion of cortical layering reminiscent of mouse models with defective REELIN signaling. Similarly, knockdown of the histone methyltransferase Ezh2, as well as deletion of Foxg1, result in derepression of Rehn gene expression and are associated with radial migration and cortical development defects. Finally, ectopic expression of Rehn cDNA by in utero electroporation into neural progenitors causes differentiation of neuronal aggregates in deep layers that are incapable of migrating towards the cortical plate. Taken together, these findings highlight the importance of maintaining a precise level of Rehn gene expression for the proper development of cortical layers. Our results suggest that NEUROD2 is an additional transcriptional regulator of the Rehn gene. Furthermore, deficiencies in NEUROD2 function might remove one layer of control over ectopic REELIN expression and may potentially sensitize migrating neurons to synthetic genetic interactions with other genes functioning in REELIN signaling.

Beyond our analysis of Rehn expression, we also generated a curated list of NEUROD2 binding targets with known roles in neuronal migration based on published literature. Interestingly, we identified a number of additional targets, including components involved in Ephrin signaling, members of the Cdk5 pathway, and additional TFs. Finally, in order to test whether NEUROD2-regulated transcription functions during radial migration of cortical neurons, we silenced Neurod2 expression in ventricular neural progenitors by in utero electroporation in mice and revealed that NEUROD2 is important for proper positioning of the neuronal soma upon arrival at the primitive cortical zone. These findings suggest that NEUROD2 might impact cortical lamination by regulating multiple components of the REELIN signaling pathway or other pathways. Finally, our dataset provides novel candidate genes that may function in different aspects of cortical lamination.

**Methods**

**Bioinformatic analyses of ChIP-Seq data.** Previously published NEUROD2 ChIP-Seq data were used to identify differential NEUROD2 binding to genomic regions at timepoints E14.5 and P0. These data were generated using three separate NEUROD2 antibodies (Abcam ab168932, ab104430 and ab109406). Histone ChIP-Seq datasets from E14.5 mouse embryonic cortex were produced by Bing Ren’s Laboratory, UCSD, USA (www.encodeproject.org). ChIP-Seq peaks were visualized using Trackster embedded within the Galaxy Project. Details of Closest Gene score calculations were previously described.

**Primary cortical cultures and Sholl analysis.** Primary cultures were prepared from E14.5 embryos as described. For Sholl analysis, primary cortical neurons were transfected with non-silencing or shNeurod2, or a non-silencing plasmid immediately before plating (Amaxa ™ P3 Primary Cell 4D-Nucleofector X Kit L; Lonza, CU133 program). The gene and knockdown efficiencies of shRNA plasmids were described previously. Total RNA was isolated with Absolutely RNA MicroPrep Kit (Agilent Technologies) at 5 d in vitro (DIV). The RNA samples were further used for either RNA-seq-directed library construction or for RT-qPCR. Library construction and sequencing
were carried out at Genewiz Inc. (New Jersey, USA). RNA-Seq libraries were sequenced on the Illumina HiSeq 2500 platform using a 50 bp single-end format. After quality filtering of reads, sequences were mapped onto the genome with TopHat2, assembled onto transcripts with Cufflinks24, and differential expression was determined with Cuffdiff25. Raw and processed RNA-Seq data are available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE131494. For RT-qPCR, cDNA was synthesized with Transcriptor high-fidelity cDNA synthesis kit (Roche) and RT-qPCR was performed using Luminaris HiGreen qPCR Master Mix (Thermo Scientific) in a CFX Connect Real-Time PCR Detection System (Bio-Rad). The 2−∆∆CT method was used for calculating Reln mRNA levels as normalized to Gapdh mRNA. The primer sequences were as follows: Reln-F (TTTACACTGAGCTGGGGAG); Reln-R (TGCCCACATCTGAACCTGAT); Gapdh-F (AATGTGCGTCGTGGATCTGACGTG); Gapdh-R (TTGCTGTGAAGTCGCAGGAGCAACC).

**Immunostaining.** An anti-REELIN antibody (Abcam, cat. no. ab78540) was used at a 1:1000 dilution for immunoblotting and 1:500 dilution for immunofluorescent staining. For immunoblotting, protein lysates were prepared using primary cortical cultures from E14.5 embryos and electroporated with the appropriate shRNA plasmid. At 5 DIV cells were lysed in ice-cold RIPA Buffer (0.05 M Tris-HCl pH 7.5, 0.15 M NaCl, 1% Triton-X 100, 1% Na-DOC, 0.1% SDS) containing freshly added protease inhibitors, incubated on ice for 15 min. and centrifuged for 20 min at 14,000 rpm at 4°C. Protein concentrations of lysates were measured by Bradford protein assay and 10 μg of total protein was loaded per well. Band intensities were quantified by ImageJ34. For immunofluorescent staining, embryonic brains were fixed at 4% PFA overnight, treated with 30% sucrose for one day, and sectioned to 50 μm with a Leica CM 1950 cryostat. Anti-REELIN antibody incubation was carried out in blocking buffer (3% BSA, 0.1% Triton X-100 in PBS, pH 7.4) overnight in 4°C. Images were captured using a Leica DMIB confocal microscope. Quantification of MZ, CP, and SVZ-IZ regions was defined based on DAPI staining, and quantification was performed with ImageJ34.

**In utero electroporation and image analysis.** Our in utero electroporation method was based upon a previously published protocol37. Briefly, 14.5 day pregnant mice were anesthetized by inhalation of isoflurane. 1 μl of DNA solution (composed of 500 ng of shRNA plasmid, 500 ng of pCAGGS-IRES-tdTomato and 0.1% Fast Green FCP) was injected unilaterally into the lateral ventricular of each embryo. 0.5 pulses at 30 V with 400 ms intervals were applied with a 5 mm tweezer electrode using an electroporator (BTX, Harvard Apparatus, ECM830). Following electroporation, embryos were placed back into the uterine cavity, and the pregnant female was sutured and placed into an incubation chamber for recovery. Embryos were retrieved at developmental age E17.5, followed by brain removal and placement into 4% PFA. Embryos were coronally sectioned to 50 μm with a cryostat (Leica CM1950). Confocal images of tdTomato-positive neurons were captured with Nikon Eclipse 90i (20X objective) or Leica DM18 (63X oil objective) and maximum intensity projection images were generated using Nikon NIS Elements AR or Leica LAS X Life Science software. A grid of ten equal bins was overlaid onto the cortical cross-section images, and the number of tdTomato-positive neurons in each bin was counted using the cell counting tool integrated into ImageJ34. These results were expressed as a percentage of total number of tdTomato-positive cells. In each experiment, littermate embryos from the same pregnant mouse were electroporated with non-silencing and shNeurod2-1, respectively.

**Animals.** All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Koç University. The study was approved by the Institutional Animal Care and Use Committee of Koç University with license number 2014–07. For in utero electroporations (CD-1 strain) and primary neuronal cultures (Balb/c strain), timed-pregnant mice were generated by The Koç University Experimental Animal Laboratory.

**Data availability.** RNA-Seq data can be downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE131494.

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G.G. carried out ChIP-qPCR and RNA-Seq experiments (Figs. 1, 3 and 4), and primary neuronal cultures, and Sholl analysis (Fig. 2). C.A. carried out all *in utero* electroporation experiments, confocal microscopy and data analysis, and D.A. assisted these experiments (Figs. 5 and 6). A.K., C.D.D. and N.O. contributed to data analysis. G.G. carried out ChIP-qPCR and RNA-Seq experiments (Figs. 1, 3 and 4), and primary neuronal cultures, and Sholl analysis (Fig. 2). C.A. carried out all *in utero* electroporation experiments, confocal microscopy and data analysis, and D.A. assisted these experiments (Figs. 5 and 6). A.K., C.D.D. and N.O. contributed to data analysis (Figs. 1, 3 and 4). G.I.D. conceived the hypothesis, designed research, analyzed data and wrote the paper. All authors discussed the results and reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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