

BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets

Luca Tiberi^{1,6}, Jelle van den Amele^{1,2,6}, Jordane Dimidschstein¹, Julie Piccirilli¹, David Gall³, Adèle Herpoel¹, Angéline Bilheu¹, Jerome Bonnefont¹, Michelina Iacovino⁴, Michael Kyba⁴, Tristan Bouschet¹ & Pierre Vanderhaeghen^{1,5}

During neurogenesis, neural stem/progenitor cells (NPCs) undergo an irreversible fate transition to become neurons. The Notch pathway is important for this process, and repression of Notch-dependent *Hes* genes is essential for triggering differentiation. However, Notch signaling often remains active throughout neuronal differentiation, implying a change in the transcriptional responsiveness to Notch during the neurogenic transition. We identified *Bcl6*, an oncogene, as encoding a proneurogenic factor that is required for proper neurogenesis of the mouse cerebral cortex. BCL6 promoted the neurogenic conversion by switching the composition of Notch-dependent transcriptional complexes at the *Hes5* promoter. BCL6 triggered exclusion of the co-activator Mastermind-like 1 and recruitment of the NAD⁺-dependent deacetylase Sirt1, which was required for BCL6-dependent neurogenesis. The resulting epigenetic silencing of *Hes5* led to neuronal differentiation despite active Notch signaling. Our findings suggest a role for BCL6 in neurogenesis and uncover Notch-BCL6-Sirt1 interactions that may affect other aspects of physiology and disease.

Neurogenesis is an important fate transition in neural development, during which relatively simple, mitotically active NPCs convert into postmitotic cells that differentiate further into fully mature neurons. The neurogenic conversion implies global changes in gene expression and epigenetic regulation, but the mechanisms by which these modifications affect single promoters or particular pathways remain poorly understood^{1–3}.

The Notch signaling pathway interacts with proneural factors to control the balance between self-renewal of NPCs and neuronal differentiation^{4–7}. Activation of the Notch receptor releases the Notch intracellular domain (NICD), which, after nuclear translocation, forms a complex with the CBF-1–SuH–LAG-1 (CSL) DNA binding protein and recruits co-activators such as Mastermind-like 1 (Maml1) to promote target gene transcription^{6,8}. Main Notch targets include basic helix loop helix *Hes* transcription factors that maintain NPC undifferentiated by repressing the expression of proneural factors^{5,9}. The neurogenic transition is thought to occur through stable down-regulation of Notch target genes and upregulation of proneural genes beyond a certain threshold, leading to neuronal differentiation^{10,11}. Several transcriptional co-repressors associate with CSL and may mediate repression of target genes in the absence of Notch signaling¹², but the precise mechanisms initiating and consolidating *Hes* gene repression remain unknown. Indeed, in many cases, Notch signaling remains active later on during neuronal differentiation, most notably in the mammalian forebrain^{13–16}. This implies a selective change in responsiveness to Notch during the neurogenic transition, with different sets of transcriptional targets being triggered following Notch activation, depending on the progression of differentiation.

In the cerebral cortex, most NPC are radial glial cells (RGCs)¹⁷, which produce pyramidal neurons, the major cortical neuron subtype, either directly through asymmetric self-renewing divisions or indirectly via intermediate progenitor cells (IPCs)^{18–20}. The rate of neuronal differentiation from RGCs and/or IPCs is regulated by various intrinsic and extrinsic cues, including Notch and proneural factors such as neurogenins, which collectively control the number and fate of cortical neurons^{21–23}.

We used an embryonic stem cell (ESC)-based model of cortical neurogenesis^{24,25} to screen for transcription factors involved in neurogenesis. We identified *Bcl6*, an oncogene in B lymphocytes that encodes a BTB/POZ zinc finger transcriptional repressor^{26,27}, as a proneurogenic gene in the mouse cerebral cortex. BCL6 was expressed during the transition from cortical NPCs to pyramidal neurons and was necessary for proper cortical neurogenesis *in vivo*. BCL6 acted by altering the composition of Notch-dependent transcriptional complexes at the level of the *Hes5* promoter, including the recruitment of the NAD⁺-dependent deacetylase Sirt1. The resulting epigenetic switch led to stable repression of *Hes5* despite ongoing Notch signaling, thereby enabling the neurogenic transition to proceed irreversibly.

RESULTS

An ESC screen identifies *Bcl6* as a proneurogenic gene

Using an *in vitro* model of cortical neurogenesis from mouse ESCs²⁴, we performed a gain of function screen for novel genes involved in this process (L.T., T.B. and P.V., unpublished data). About 20 transcription factors known to be expressed in the developing cortex, but without a well-characterized function, were overexpressed during

¹Université Libre de Bruxelles (ULB), Institute for Interdisciplinary Research, ULB Neuroscience Institute, Brussels, Belgium. ²Department of Neurology, Ghent University Hospital, Ghent, Belgium. ³Université Libre de Bruxelles, Laboratory of Neurophysiology and ULB Neuroscience Institute, Belgium. ⁴University of Minnesota, Lillehei Heart Institute, Minneapolis, Minnesota, USA. ⁵Welbio, Université Libre de Bruxelles, Brussels, Belgium. ⁶These authors contributed equally to this work. Correspondence should be addressed to P.V. (pierre.vanderhaeghen@ulb.ac.be).

Received 18 June; accepted 18 October; published online 18 November 2012; doi:10.1038/nn.3264

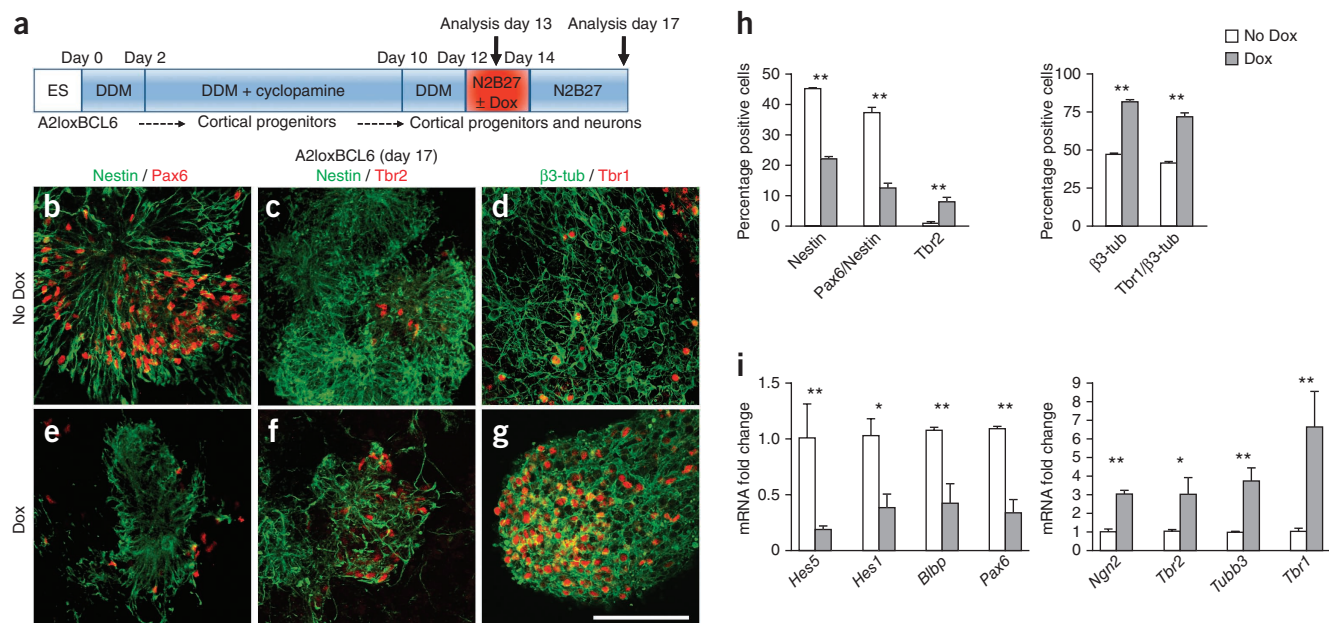


Figure 1 BCL6 triggers neuronal differentiation during ESC-derived cortical neurogenesis. **(a)** Schematic representation of the differentiation protocol from ESC. BCL6 expression was induced at day 12 of *in vitro* differentiation and cells were analyzed 1 or 5 d later. **(b–g)** Expression of the markers Nestin and Pax6 **(b,e)** or Tbr2 **(c,f)**, β3-tubulin and Tbr1 **(d,g)** at day 17. Scale bar represents 100 μm. **(h)** Histograms show the percentage of cells expressing a given marker among all Hoechst-positive cells at day 17. Data are presented as mean + s.e.m. ($n = 3$ differentiations; $t = 7.02$ (Nestin), $t = 5.02$ (Pax6), $t = -6.27$ (Tbr2), $t = -8.26$ (β3-tubulin), $t = -9.35$ (Tbr1)). **(i)** qRT-PCR analysis for *Hes5*, *Hes1*, *Blbp* (also known as *Fabp7*), *Pax6*, *Ngn2* (also known as *Neurog2*), *Tbr2* (also known as *Eomes*), *Tubb3* and *Tbr1* at day 17. Data are presented as fold change normalized to the mean of non-treated cells at day 17 + s.e.m. ($n = 4$ differentiations; $t = 4.6$ (*Hes5*), $t = 3.08$ (*Hes1*), $t = 3.82$ (*Blbp*), $t = 4.05$ (*Pax6*), $t = -3.91$ (*Ngn2*), $t = -2.88$ (*Tbr2*), $t = -4.06$ (*Tubb3*), $t = -4.12$ (*Tbr1*)). * $P < 0.05$, ** $P < 0.01$.

in vitro cortical neurogenesis, using recombinant ESC lines allowing inducible gene expression following doxycycline (Dox) addition^{28,29}. Among the genes tested, *Bcl6* (refs. 27,30) displayed a potent proneurogenic activity. When BCL6 was induced transiently at the onset of active neurogenesis, after 12 d in culture (**Fig. 1a** and **Supplementary Fig. 1a–c**), it displayed a potent neurogenic effect, characterized by a decrease in the proportion of Nestin⁺ Pax6⁺ RGCs and an increase in the number of Tbr2⁺ IPCs and β3-tubulin⁺ Tbr1⁺ NeuN⁺ neurons, as compared with non-induced control conditions (**Fig. 1b–h** and **Supplementary Fig. 1d,f,g**), which was confirmed by quantitative reverse transcription PCR (qRT-PCR) analyses (**Fig. 1i** and **Supplementary Fig. 1e**). We performed electrophysiological recordings at later stages of differentiation following BCL6 induction, which revealed, among most recorded neurons, normal functional features of pyramidal neurons (**Supplementary Fig. 1h,i**).

BCL6 induction did not change the rate of apoptosis, as assessed by fluorescence-activated cell sorting for cleaved caspase 3 (**Supplementary Fig. 1j**), indicating that BCL6 affects cortical neuron differentiation independently of survival, unlike in B cells or other types of neurons^{31–33}.

BCL6 is expressed during cortical neurogenesis

BCL6 expression was previously described in the developing cortex^{34–36}, but its role in neural development remains unknown. Given the proneurogenic effects of BCL6, we examined its pattern of expression during mouse corticogenesis.

During *in vitro* ESC-derived cortical neurogenesis, BCL6 endogenous expression increased steadily until day 17, the time of most active neurogenesis, consistent with it having a positive role on cortical neuron production in the system (**Supplementary Fig. 1k**).

In vivo, *Bcl6* mRNA and protein were detected in the cerebral cortex from embryonic day 12.5 (E12.5) until birth (**Fig. 2a–l** and **Supplementary Fig. 2**). BCL6 protein was expressed throughout the thickness of the cortex in a dynamic fashion, with expression being lowest in scattered cells in the ventricular and subventricular zones, and progressively increasing levels of expression in intermediate zone and cortical plate (**Fig. 2a–l** and **Supplementary Fig. 2**). BCL6 co-staining with Pax6 and Tbr2 at E12.5 revealed a partially overlapping pattern with NPCs in the ventricular zone and subventricular zone; 9.3% of Pax6⁺ ($n = 2$ embryos, s.e.m. = 1.2) and 39.2% of Tbr2⁺ ($n = 2$, s.e.m. = 2.2) cells colabeled with BCL6 (**Fig. 2a–l**), with lower levels of staining in double- than in single-labeled cells. A similar pattern was observed at E14.5 (data not shown). These data indicate that BCL6 is upregulated dynamically during the transition from NPC to differentiating neurons, either directly or indirectly through IPC generation. At later stages, *Bcl6* mRNA and protein were expressed at higher levels, but this expression was mostly confined to differentiated neurons in the cortical plate, as described previously^{34–36} (**Supplementary Fig. 2** and data not shown). Notably, at all of the embryonic and early postnatal stages tested, *Bcl6* mRNA and protein expression were highest in the anterior-most (frontal and parietal) parts of the neocortex, whereas expression was barely detectable in the most posterior (occipital) parts (**Supplementary Fig. 2**).

BCL6 promotes cortical neurogenesis *in vivo*

We further explored BCL6 function *in vivo* using *in utero* electroporation targeting the embryonic cortex. Overexpression of BCL6 resulted in a decrease in the proportion of Pax6⁺ RGCs and EdU⁺ cycling cells and an increase in the proportion of Tbr2⁺

cells after 24 h (Fig. 2m–s). After 48 h, this was followed by an increase in the proportion of cells in the intermediate zone and cortical plate and of $\beta 3$ -tubulin⁺ cells (Fig. 2t–w). The proportion of Tbr2 cells was decreased after 48 h, which could be a result of

the reduced number of RGCs and/or a direct effect of BCL6 on the conversion of IPCs into neurons (Fig. 2w).

At later stages, BCL6-electroporated neurons displayed an identity of early-born Tbr1⁺ deep layer cortical neurons, rather than later

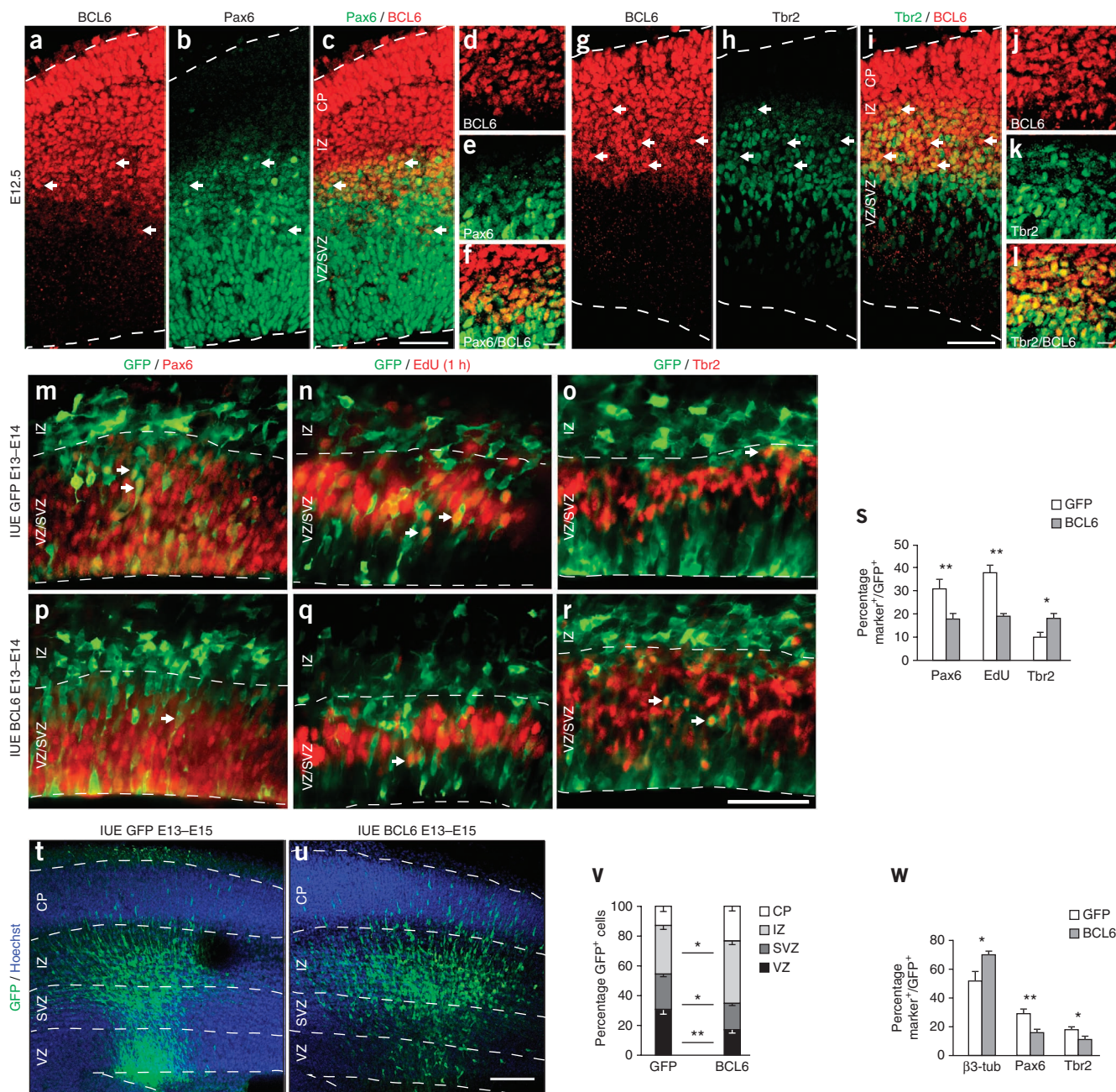


Figure 2 BCL6 induces precocious neuronal differentiation during *in vivo* corticogenesis. (a–l) Confocal immunofluorescence staining for BCL6 and Pax6 (a–f) or Tbr2 (g–l) of coronal sections of E12.5 wild-type cortex. Dashed lines indicate the pial and ventricular surfaces of the cortex. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zones. Arrows indicate BCL6 and either Tbr2 or Pax6 double-positive cells. (m–s) *In utero* electroporation (IUE) of pCIG either alone (m–o) or with pCAG-BCL6 (p–r) at E13.5. Immunofluorescence analysis of coronal sections stained for GFP and Pax6 (m,p), EdU (n,q) or Tbr2 (o,r) 1 d after electroporation. Dashed lines mark the basal and apical margins of the ventricular zone/SVZ. (s) Histogram showing the percentage of GFP⁺ cells expressing either Pax6, EdU or Tbr2. Data are presented as mean + s.e.m. ($n = 4$ embryos; $t = 3.97$ (Pax6), $t = 4.21$ (EdU), $t = -2.72$ (Tbr2)). (t,u) *In utero* electroporation of pCIG either alone (t) or with pCAG-BCL6 (u) at E13.5. Immunofluorescence analysis of coronal sections stained for Hoechst and GFP 2 d after electroporation. Dashed lines mark the basal and apical margins of the ventricular zone, SVZ, intermediate zone and cortical plate. (v) Histograms show the percentage of GFP⁺ cells in ventricular zone, SVZ, intermediate zone and cortical plate. Data are presented as mean + s.e.m. ($n = 9$ embryos; $t = 3.44$ (ventricular zone), $t = 2.63$ (SVZ), $t = -2.50$ (intermediate zone)). (w) Histogram showing the percentage of GFP⁺ cells expressing either $\beta 3$ -tubulin, Pax6 or Tbr2. Data are presented as mean + s.e.m. ($n = 6$ embryos, $t = -2.65$ ($\beta 3$ -tub), $t = 3.44$ (Pax6), $t = 2.37$ (Tbr2)). Scale bars represent 100 μ m (a–c,g–i,m–r,t–u) and 10 μ m (d–f,j–l). * $P < 0.05$, ** $P < 0.01$.

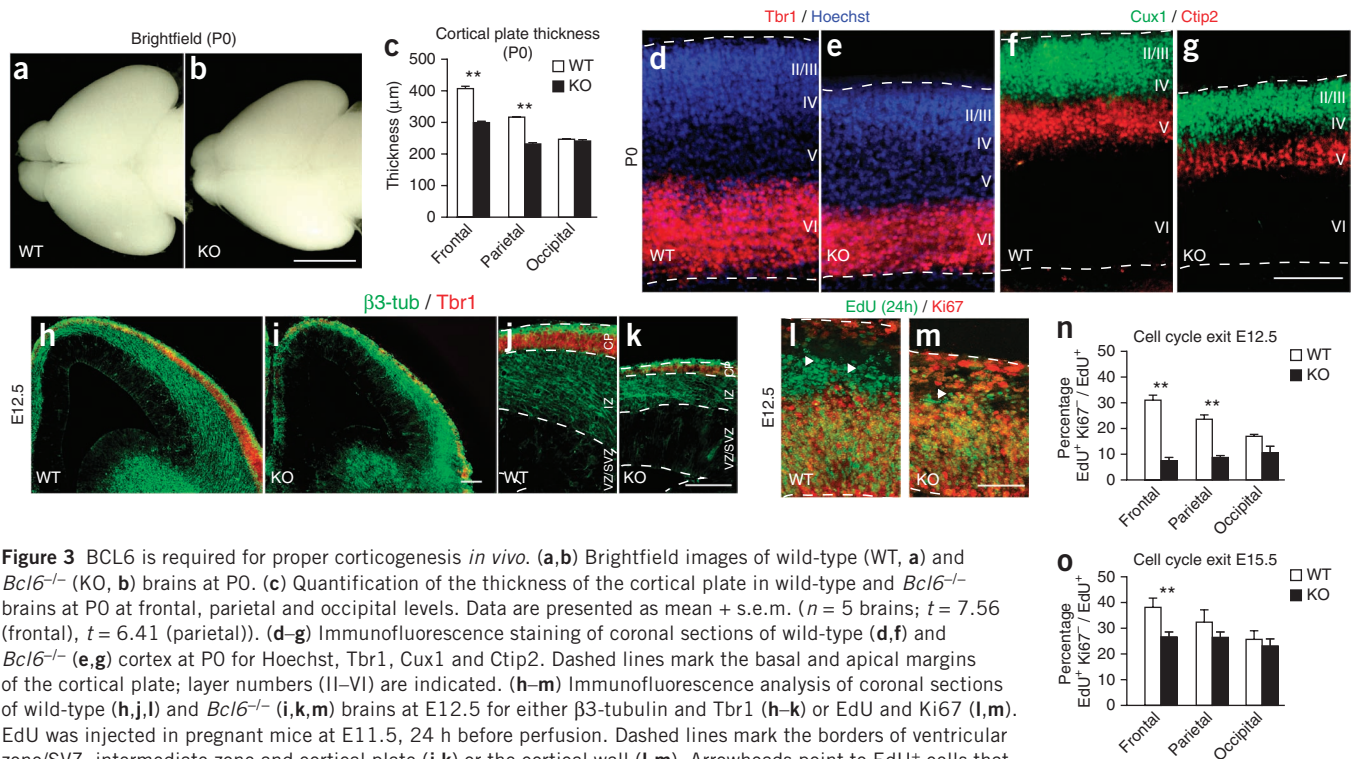


Figure 3 BCL6 is required for proper corticogenesis *in vivo*. (a,b) Brightfield images of wild-type (WT, a) and *Bcl6*^{-/-} (KO, b) brains at P0. (c) Quantification of the thickness of the cortical plate in wild-type and *Bcl6*^{-/-} brains at P0 at frontal, parietal and occipital levels. Data are presented as mean + s.e.m. ($n = 5$ brains; $t = 7.56$ (frontal), $t = 6.41$ (parietal)). (d–g) Immunofluorescence staining of coronal sections of wild-type (d,f) and *Bcl6*^{-/-} (e,g) cortex at P0 for Hoechst, Tbr1, Cux1 and Ctip2. Dashed lines mark the basal and apical margins of the cortical plate; layer numbers (II–VI) are indicated. (h–m) Immunofluorescence analysis of coronal sections of wild-type (h,j,l) and *Bcl6*^{-/-} (i,k,m) brains at E12.5 for either β 3-tubulin and Tbr1 (h–k) or EdU and Ki67 (l,m). EdU was injected in pregnant mice at E11.5, 24 h before perfusion. Dashed lines mark the borders of ventricular zone/SVZ, intermediate zone and cortical plate (j,k) or the cortical wall (l,m). Arrowheads point to EdU⁺ cells that are negative for Ki67 and thus have exited the cell cycle. (n,o) Quantification of cell cycle exit (EdU⁺ Ki67⁻ / EdU⁺) of BCL6 wild-type and *Bcl6*^{-/-} brains at E12.5 (n) and E15.5 (o), 24 h after injection of EdU (mean percentage + s.e.m.; $n = 4$ brains, $t = 4.50$ (E12.5 frontal), $t = 3.84$ (E12.5 parietal), $t = 4.01$ (E15.5 frontal)). Scale bars represent 2.5 mm (a,b) and 100 μ m (d–g,h–i,j–k,l–m). ** $P < 0.01$.

born Cux1⁺ upper layer neurons (Supplementary Fig. 3a–f), a fate change that is consistent with precocious differentiation. Analysis of dendritic and axonal growth revealed that the BCL6-induced neurons acquired mature properties of pyramidal projection neurons (Supplementary Fig. 3g–r).

BCL6 is required for proper neurogenesis of cortical neurons

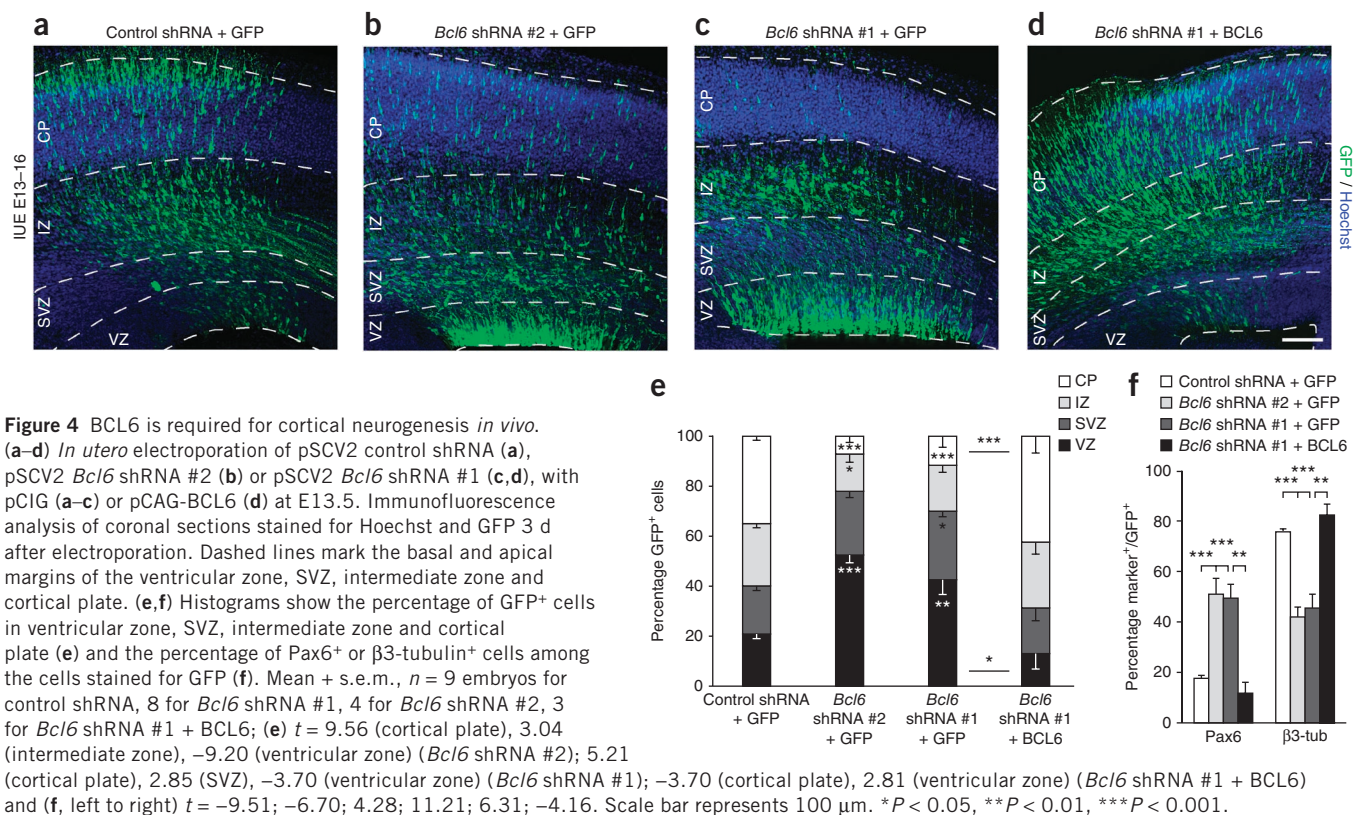
Given that these data suggest that BCL6 affects cortical neurogenesis, we tested whether BCL6 is required for corticogenesis using *Bcl6*^{-/-} mice³⁰. Inspection of gross brain morphology at birth revealed a reduced size of the cerebral hemispheres (Fig. 3a,b) and a reduced thickness of the cortical plate in *Bcl6*^{-/-} mice (Fig. 3c and Supplementary Fig. 4a–d), indicating that corticogenesis was defective. Cortical lamination appeared to be unaffected, but all of the cortical layers were reduced, as seen by a similar decrease in the thickness of Tbr1⁺ Ctip2⁺ deep layers and Cux1⁺ upper layers (Fig. 3d–g and Supplementary Fig. 4j–w). High mortality during the first postnatal weeks, presumably associated with immunodepression³⁰, precluded extensive analysis of the mutant mice at later stages, but examination of a few surviving *Bcl6*^{-/-} mice at postnatal day 21 (P21) revealed a similar decrease in cortical size (Supplementary Fig. 4x–ad), indicating that impairment of corticogenesis was long lasting and not merely a developmental delay. Notably, the defect was largely restricted to the frontal and parietal parts of the cortex, whereas occipital cortex was preserved overall (Fig. 3c and Supplementary Fig. 4), which correlates with the spatial pattern of expression of BCL6 (Supplementary Fig. 2).

When examining the cortex at earlier stages, cortical plate reduction was already detectable at E12.5 and E15.5 (Fig. 3h–k and Supplementary Fig. 4e–i), suggesting an early developmental defect that could result from disruption of neural cell proliferation, survival and/or differentiation. Apoptosis rates in cortical cells, as

determined by the number of cleaved caspase 3⁺ cells, were found to be similar in *Bcl6*^{-/-} mice and control littermates at E12.5 and E15.5 (Supplementary Fig. 5a–d). The proliferation of RGCs, which mostly occurs at the apical side of the ventricular zone, appeared to be largely unaffected in *Bcl6*^{-/-} cortex, as seen by comparable numbers of phospho-histone H3 (pH3)-positive mitotic figures in apical cells and Pax6⁺ RGC (Supplementary Fig. 5e–h,n).

In contrast, EdU pulse-chase experiments combined with Ki67 staining, enabling precise measurements of cell cycle exit rates, revealed a marked reduction of cell-cycle exit at E12.5 and E15.5, selectively in the frontal and parietal cortex of *Bcl6*^{-/-} embryos, indicating defective transition from NPCs to postmitotic neurons (Fig. 3l–o). In addition, the number of Tbr2⁺ IPCs, which divide mostly at the basal side of the ventricular and subventricular zone, was somewhat decreased in the *Bcl6*^{-/-} cortex at E15.5 (Supplementary Fig. 5i–l), whereas their proliferation appeared to be similar (Supplementary Fig. 5m), suggesting a defect in their generation.

To confirm the specific requirement of BCL6 during the neurogenic conversion of cortical neurons, we performed acute knockdown by *in utero* electroporation of BCL6-specific short hairpin RNAs (shRNAs; Supplementary Fig. 5o,p). This resulted in a decrease of β 3-tubulin⁺ neurons and cells located in the cortical plate after 72 h, and a converse increase in the numbers of Pax6⁺ cells and of cells located in the ventricular zone (Fig. 4 and Supplementary Fig. 5q–t). These cells also retained a typical radial glia-like morphology with well-structured glial fibers (Fig. 4b,c). The proportion of Tbr2⁺ cells remained unchanged after 72 h, probably reflecting a balance between an increase in the number of Pax6⁺ RGCs and a decrease in the rate of their conversion to IPCs (Supplementary Fig. 5t). Similar results were obtained with two independent *Bcl6* shRNA targeting sequences, which was fully rescued by coexpression of a *Bcl6*



cDNA construct insensitive to one of the shRNA constructs (Fig. 4d–f and Supplementary Fig. 5o). These data indicate that BCL6 is required in the embryonic cortex for the proper neurogenic transition from NPCs.

BCL6 directly represses the *Hes5* gene

To gain insight into the molecular mechanisms underlying the neurogenic function of BCL6, we profiled a panel of genes related to neurogenesis by qRT-PCR in wild-type and *Bcl6*^{–/–} cortex (Fig. 5a and Supplementary Fig. 6a). Although this analysis confirmed the downregulation of genes that are normally expressed in differentiating neurons,

as well as upregulation of some NPC markers, the only gene normally expressed in NPCs that was consistently upregulated was *Hes5*.

Consistent with this observation, *Hes5* was strongly repressed 6 h after BCL6 induction during *in vitro* ESC-cortical neurogenesis, whereas all of the other markers of cell fate that we examined were unchanged (Fig. 5b). Similarly, *ex utero* electroporation of BCL6 in E13.5 embryonic cortex resulted in a selective downregulation of *Hes5* after 24 h (Fig. 5c).

To test whether *Hes5* repression was linked causally to the neurogenic effects of BCL6, we examined functional interactions

Figure 5 BCL6 induces neurogenesis through repression of *Hes5*. (a,b) qRT-PCR analysis for *Hes5*, *Hes1*, *Pax6*, *Blbp*, *Tbr2*, *Tbr1* and *Tubb3* of wild-type and *Bcl6*^{–/–} dissected cortex at E15.5 (a) or 6 h after BCL6 induction at day 12 of *in vitro* differentiation of A2loxBCL6 cells (b). Data are presented as fold change normalized to the mean of wild-type or nontreated cells + s.e.m., *n* = 4 brains or differentiations (a, *t* = –4.42 (*Hes5*), *t* = 3.3 (*Tbr2*), *t* = 4.92 (*Tbr1*), *t* = 4.51 (*Tubb3*); b, *t* = 4.15 (*Hes5*)). (c) qRT-PCR analysis for *Bcl6*, *Hes5*, *Pax6*, *Hes1*, *Tbr2* and *Tubb3* of embryonic cortex 24 h following *ex utero* electroporation at E13.5 of pCIG either alone or with pCAG-BCL6. Data are presented as fold change normalized to the mean of GFP + s.e.m., *n* = 5 embryos for GFP and 4 for BCL6 (*t* = –9.48 (*Bcl6*), *t* = 2.82 (*Hes5*)). (d–g) Immunofluorescence analysis of coronal sections stained for Hoechst and GFP 2 d after *in utero* electroporation (E13.5–15.5) of pCIG either alone (d) or with pCAG-BCL6 (e), and of pCIG-*Hes5* either alone (f) or together with pCAG-BCL6 (g). (h) Quantification of cells stained for β3-tubulin, Pax6 and Tbr2 among the GFP⁺ cells. Mean + s.e.m., *n* = 11 embryos for GFP and BCL6, 7 for *Hes5* and 9 for BCL6 + *Hes5*; (left to right) *t* = –3.00, 8.22, 5.79, –5.08, 2.38, –4.65. Scale bar represents 100 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

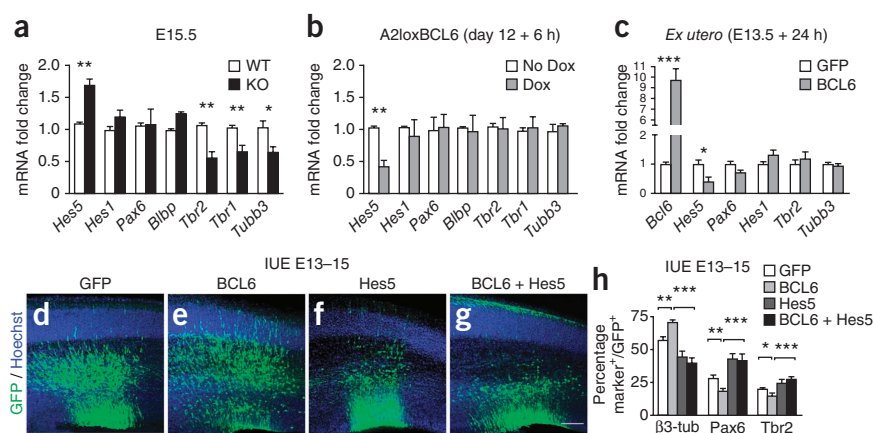
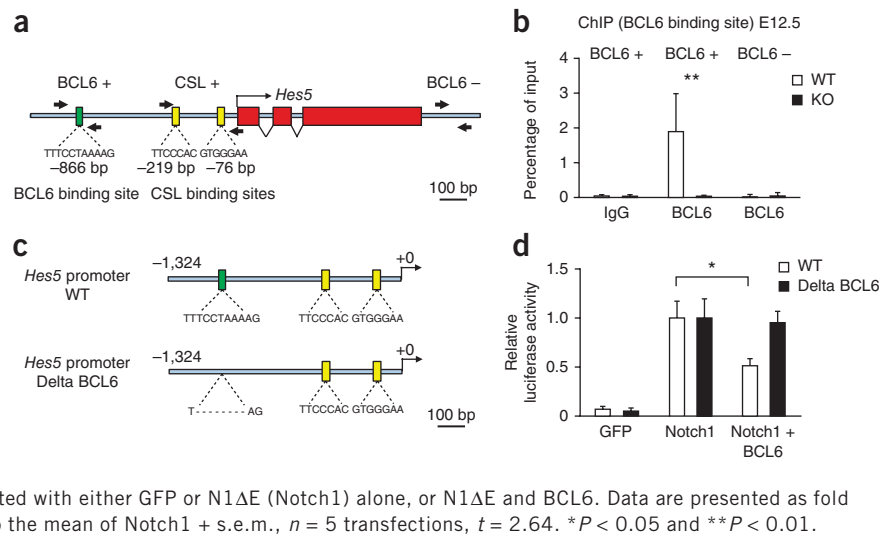


Figure 6 BCL6 directly represses the transcription of the Notch target *Hes5*. (a) Schematic representation of the genomic region of *Hes5*, showing the exons (red) and the BCL6 (green) and CSL (yellow) binding sites. The primers used to measure the enrichment following ChIP are presented by thick arrows. (b) ChIP analysis of the putative BCL6-binding site and a negative control site in the *Hes5* promoter in wild-type and *Bcl6*^{-/-} cortex at E12.5 with control IgG (IgG) or antibody to BCL6. Mean + s.e.m., *n* = 3 embryos, *t* = 3.90 (BCL6⁺). (c) Schematic representation of the genomic region of the *Hes5* promoter used for the luciferase assay showing the BCL6 (green) and CSL (yellow) binding sites and the deletion mutant. (d) Luciferase assay of P19 cells transfected with either GFP or N1ΔE (Notch1) alone, or N1ΔE and BCL6. Data are presented as fold change in relative luciferase activity normalized to the mean of Notch1 + s.e.m., *n* = 5 transfections, *t* = 2.64. **P* < 0.05 and ***P* < 0.01.



between BCL6 and *Hes5* *in vivo* by *in utero* electroporation. As expected⁹, *Hes5* gain of function resulted in a strong decrease in neurogenesis, whereas BCL6 overexpression had the opposite effect (Fig. 5d–h). Notably, coexpression of *Hes5* and BCL6 resulted in a complete block of BCL6-dependent neurogenesis (Fig. 5h), thereby directly linking *Hes5* repression to the proneurogenic effect of BCL6 *in vivo*.

In silico inspection of the *Hes5* promoter revealed the presence of a bona fide BCL6 binding site³¹ located <1 kb upstream of the transcriptional starting point (Fig. 6a), suggesting that BCL6 could directly bind to the *Hes5* promoter. Chromatin immunoprecipitation (ChIP) experiments in wild-type and *Bcl6*^{-/-} E12.5 embryonic cortex with an antibody to BCL6 revealed a specific enrichment directly on this site (Fig. 6b and Supplementary Fig. 8a–f), which was confirmed using an electrophoretic mobility shift assay (EMSA; Supplementary Fig. 6b,c).

We next tested whether BCL6 could repress the *Hes5* promoter directly, using a *Hes5* reporter construct containing the BCL6 binding site and two well-described CSL binding sites (Fig. 6c). The reporter was potentially transactivated following Notch overexpression (Notch1ΔE)³⁷, but, notably, this was repressed following BCL6 expression (Fig. 6d), despite strong expression of active Notch (Supplementary Fig. 6d). The repressive effect of BCL6 was fully dependent on the presence of its binding site in the *Hes5* promoter,

as a mutant promoter devoid of this site (Delta-BCL6; Fig. 6c) was no longer sensitive to BCL6 repression (Fig. 6d). Together, these data indicate that BCL6 induces neurogenesis through direct repression of the *Hes5* promoter.

BCL6 regulates Notch-dependent transcriptional complexes

The ability of BCL6 to repress directly *Hes5* expression is intriguing, as BCL6 was recently shown to repress a selective set of Notch-dependent genes during left-right asymmetry patterning in *Xenopus* through direct binding competition between NICD, BCL6 and Maml1 (ref. 38). Co-immunoprecipitation (Co-IP) experiments revealed that BCL6 bound to the Notch1 intracellular domain (N1ICD) in the cortex *in vivo* (Fig. 7a), confirming results obtained during early *Xenopus* development³⁸. Using ChIP, we next examined N1ICD and Maml1 recruitment to the *Hes5* promoter in the cortex of control and *Bcl6*^{-/-} embryos. Recruitment of N1ICD and Maml1 at the *Hes5* promoter was increased in *Bcl6*^{-/-} cortex (Fig. 7b–e and Supplementary Fig. 8g–i), whereas the total level of N1ICD appeared to be unaffected (Supplementary Fig. 7a).

To test whether these differences in the *Hes5* promoter transcriptional complex reflected a direct effect of BCL6, rather than a change in cortical cell composition (that is, NPC versus neurons), we performed ChIP during *in vitro* ESC-derived cortical neurogenesis, shortly (6 h) after addition of Dox and induction of BCL6, before

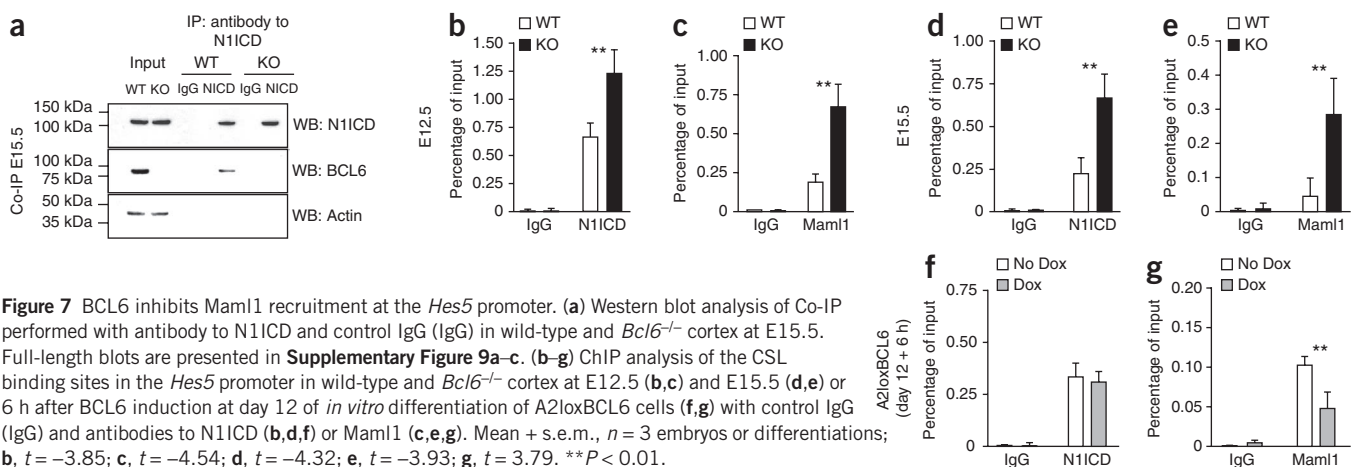


Figure 7 BCL6 inhibits Maml1 recruitment at the *Hes5* promoter. (a) Western blot analysis of Co-IP performed with antibody to N1ICD and control IgG (IgG) in wild-type and *Bcl6*^{-/-} cortex at E15.5. Full-length blots are presented in Supplementary Figure 9a–c. (b–g) ChIP analysis of the CSL binding sites in the *Hes5* promoter in wild-type and *Bcl6*^{-/-} cortex at E12.5 (b,c) and E15.5 (d,e) or 6 h after BCL6 induction at day 12 of *in vitro* differentiation of A2lox:BCL6 cells (f,g) with control IgG (IgG) and antibodies to N1ICD (b,d,f) or Maml1 (c,e,g). Mean + s.e.m., *n* = 3 embryos or differentiations; b, *t* = -3.85; c, *t* = -4.54; d, *t* = -4.32; e, *t* = -3.93; f, *t* = 3.79. ***P* < 0.01.

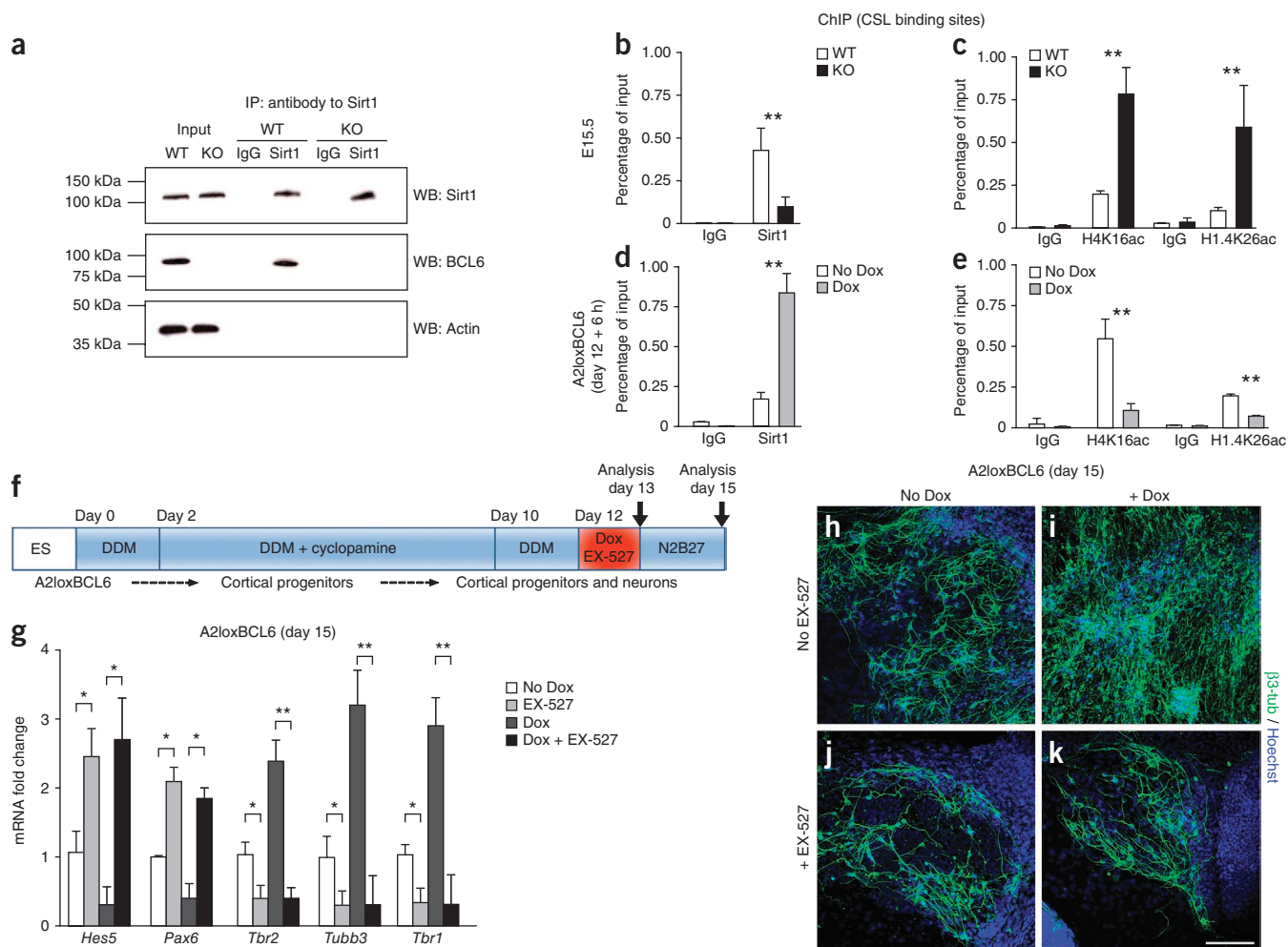


Figure 8 BCL6 neurogenic effect requires Sirt1 recruitment at the *Hes5* promoter to modulate histone acetylation. (a) Western blot analysis of Co-IP performed with antibody to Sirt1 and control IgG (IgG) in wild-type and *Bcl6*^{-/-} cortex at E15.5. Full-length blots are presented in **Supplementary Figure 9d,e**. (b–e) ChIP analysis of the CSL binding sites in the *Hes5* promoter in wild-type and *Bcl6*^{-/-} cortex at E15.5 (b,c) or 6 h after BCL6 induction at day 12 of *in vitro* differentiation of A2loxBCL6 cells (d,e) with control IgG (IgG) and antibody to Sirt1 (b,d), H4K16ac (c,e) or H1.4K26ac (c,e). Mean + s.e.m., *n* = 3 embryos or differentiations; b, *t* = 4.76; c, *t* = -4.23, *t* = -3.88; d, *t* = -4.76; e, *t* = 4.23, *t* = 3.91. (f) Schematic representation of differentiation from ESCs and treatment with doxycycline and/or with a Sirt1 specific inhibitor (EX-527) for 24 h. (g–k) BCL6 expression was induced at day 12 of *in vitro* differentiation and cells were treated with a Sirt1-specific inhibitor (EX-527) on the same day for 24 h, followed by qRT-PCR analysis for *Hes5*, *Pax6*, *Tbr2*, *Tubb3* and *Tbr1* (g) or immunofluorescence for β 3-tubulin counterstained with Hoechst (h–k) at day 15. Data are presented as fold change normalized to the mean of nontreated cells at day 15 + s.e.m., *n* = 3 differentiations; (g from left to right) *t* = -2.71, -3.20, -2.98, -2.50, 3.17, 4.76, 3.23, 4.31, 3.24, 4.36. Scale bar represents 100 μ m. **P* < 0.05, ***P* < 0.01.

any cell fate change was detectable (Fig. 5b). We observed a strong decrease of Mam1 recruitment at the *Hes5* promoter, whereas no difference could be detected in N1ICD recruitment (Fig. 7f,g and Supplementary Fig. 8m–r).

BCL6 recruits Sirt1 deacetylase to repress *Hes5*

These data indicate that BCL6 inhibits the formation of the NICD–Mam1 co-activator complex on the *Hes5* promoter, suggesting a molecular mechanism for BCL6-dependent *Hes5* repression. However, the transition from NPCs to neurons is irreversible, regardless of ongoing Notch signaling^{13–15}. This would imply stable, likely epigenetic, silencing of *Hes5*. In search of such a mechanism, we turned to the NAD⁺-dependent deacetylase Sirt1³⁹, which has been implicated in neurogenesis⁴⁰ and was recently shown to repress Notch targets⁴¹.

Using Co-IP, we detected BCL6 in the same complex as Sirt1 in the developing cortex *in vivo* (Fig. 8a and Supplementary Fig. 7b).

ChIP revealed that Sirt1 was enriched at the CSL binding site of the *Hes5* promoter and that this enrichment was markedly reduced in *Bcl6*^{-/-} cortex (Fig. 8b and Supplementary Fig. 7c), whereas the total level of Sirt1 appeared to be similar to the wild type (Supplementary Fig. 7a). These *in vivo* data suggest that the specific recruitment of Sirt1 to the *Hes5* promoter is dependent on BCL6.

Sirt1 has been shown to induce repressive chromatin remodeling and facilitate heterochromatin formation by deacetylation of histone H4 lysine 16 (H4K16) and histone H1.4 lysine 26 (H1.4K26) in other contexts^{41,42}. To determine whether BCL6 can influence chromatin acetylation patterns at the *Hes5* promoter, we performed similar *in vivo* ChIP experiments using antibodies to acetylated H4K16Ac and H1.4K26Ac, which revealed strong enrichment on the *Hes5* promoter in *Bcl6*^{-/-} embryonic cortex compared with wild type (Fig. 8c). A direct link between BCL6, Sirt1 and histone acetylation patterns on the *Hes5* promoter was further established during ESC–cortical

neurogenesis *in vitro*, where a 6-h induction of BCL6 expression was sufficient to trigger recruitment of Sirt1 to the *Hes5* promoter (Fig. 8d) and a converse decrease in acetylated H4K16Ac and H1.4K26Ac (Fig. 8e). Overall, these results strongly suggest that BCL6 modulates Notch signaling by recruiting Sirt1 to the *Hes5* promoter, thereby influencing the acetylation state of the chromatin, leading to a stable epigenetic repression of *Hes5* during the neurogenic transition.

To test for the functional links between Sirt1 and the neurogenic effects of BCL6, we examined the effect of a Sirt1 inhibitor (EX-527)⁴³ during *in vitro* cortical neurogenesis (Fig. 8f). Sirt1 inhibition resulted in a complete block of BCL6-induced neurogenesis (Fig. 8g–k and Supplementary Fig. 7d–h) and *Hes5* repression (Fig. 8g and Supplementary Fig. 7d), indicating that Sirt1 function is required for the biological and molecular effects of BCL6 during cortical neurogenesis.

DISCUSSION

Important and irreversible changes in gene expression occur during neurogenesis, which requires extensive chromatin remodeling^{1–3}, but it remains unclear how these modifications affect selective and relevant sets of genes. Similarly, given the almost universal use of Notch in cell fate renewal and transition, a central question in developmental biology is how specific parts of the Notch-dependent transcriptional program are used selectively, depending on the cellular context^{6,8,44}.

We identified *Bcl6*, a widely studied lymphoma oncogene, as a proneurogenic factor and selective regulator of Notch-dependent transcription during cortical neurogenesis (Supplementary Fig. 7i). BCL6 acts by modifying the composition of transcriptional complexes and chromatin structure at the promoter of the Notch target gene *Hes5*. By selective binding to the *Hes5* promoter, BCL6 excludes Maml1 from the Notch transcriptional complex, preventing transcriptional activation, and recruits the NAD⁺-dependent deacetylase Sirt1, which results in histone deacetylation and consequent epigenetic silencing of *Hes5*. BCL6 therefore acts as an essential trigger for the neurogenic conversion of NPCs into neurons.

In the cerebral cortex, the Notch pathway promotes self-renewal of RGCs, but it is also active later on during the neurogenic transition¹³, as well as in differentiated neurons^{14–16}. This raises the question of how cells switch their competence to respond to Notch signaling during cortical neurogenesis in a selective way. Although various negative regulators of Notch signaling have been identified during neurogenesis^{45,46}, most of them appear to inhibit generic components of the Notch pathway rather than specific transcriptional targets. Similarly, various transcriptional co-repressors have been identified that can repress Notch target gene expression, including Sirt1 (ref. 41), but are thought to act mainly in the absence of active Notch signaling, and it remains unknown how they are able to repress differentially specific NICD/CSL targets¹².

In contrast, our data point to a role for BCL6 in selective regulation of Notch signaling, as it appears to act as a site-specific repressor of defined Notch transcriptional targets such as *Hes5*. Similarly, BCL6 was shown to repress a selected set of Notch target genes during the establishment of left-right asymmetry in *Xenopus laevis*, where it appears to act through the exclusion of Maml1 (ref. 38). This suggests that BCL6 may contribute to target-specific restriction of Notch signals in a wide variety of cellular contexts. On the other hand, the Hamlet transcription factor was recently reported to modulate *Drosophila* olfactory neuron specification through selective repression of Notch-dependent targets in a cell context-dependent fashion, reminiscent of our observations with BCL6 (ref. 47).

Although our data suggest that *Hes5* repression is an important mechanism of action of BCL6 during neurogenesis, other target genes are likely to be involved. Future work should investigate the transcriptional targets of BCL6 at the genome-wide level and determine which of these are actually targeted by the Notch-CSL complex and/or involve the recruitment of Sirt1, not only during cortical neurogenesis, but also during later aspects of cortical development, given the prominent expression of BCL6 in postmitotic pyramidal neurons. Conversely, it will be important to determine the upstream factors that trigger the upregulation of BCL6 during the neurogenic transition. The importance of regional expression of BCL6 in distinct areas of the cortex, with the highest levels of expression occurring in frontal/parietal regions, also remains to be investigated, as it may reveal a link between the regulation of neurogenesis and cortical areal patterning²¹.

Finally, our findings uncover for the first time, to the best of our knowledge, a direct molecular link between BCL6 and Sirt1. Sirt1 deacetylase activity has been associated with a wide variety of protein substrates and cellular processes, from metabolic control to oncogenesis³⁹. These processes include neurogenesis and gliogenesis, in which Sirt1 has been proposed to act as a sensor linking cell fate and metabolic state^{40,48}. Although our findings suggest that histones are the most critical substrates of Sirt1 in the context of *Hes5* repression, consistent with a recent study linking Notch target repression and Sirt1 in *Drosophila*⁴¹, other proteins present at the *Hes5* locus may be targeted by Sirt1 deacetylation, including NICD itself⁴². Our data raise the possibility that BCL6-dependent Sirt1 recruitment to specific promoters may represent a more general mechanism in other processes in which BCL6 is involved. Conversely, BCL6 could be involved in other Sirt1-dependent processes in physiology and disease, including synaptic plasticity⁴⁹ and neurodegeneration⁵⁰. Future work should reveal which other processes are regulated by the BCL6-Notch-Sirt1 *ménage à trois*.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the [online version of the paper](#).

ACKNOWLEDGMENTS

We thank G. Vassart for continuous support and interest, D. Christophe and members of the Vanderhaeghen lab and Institute for Interdisciplinary Research for helpful discussions and advice, F. Bollet-Quivogne (Fonds National de la Recherche Scientifique (FNRS) Logistic Scientist) of the Light Microscopy Facility for his support with imaging, R. Dalla-Favera (Columbia University) for generously sharing *Bcl6*^{−/−} mice, F. Guillemot (National Institute of Medical Research) for kindly providing P19 cells, and G. Del Sal ((Laboratorio Nazionale CIB) Trieste) for the pCS2-NΔE construct, and B. Hassan, A. Soldano and K. De Backer for critically reading the manuscript. This work was funded by grants from the Belgian Queen Elizabeth Medical Foundation, the Fondations Pierre Clerdent and Roger de Spoelberch, the Action de Recherches Concertées Programs, the Interuniversity Attraction Poles Program, Belgian State, Federal Office for Scientific, Technical and Cultural Affairs, the Belgian FNRS and Fonds pour la Recherche Scientifique Médicale, and the Welbio and Programme d'Excellence CIBLES of the Walloon Region (to P.V.), as well as an EMBO Long-Term Fellowship (to L.T.) and a Marie Curie Fellowship (to T.B.). P.V. is Research Director, L.T. Postdoctoral Fellow, and J.v.d.A. and J.P. Research Fellows of the FNRS.

AUTHOR CONTRIBUTIONS

L.T., J.v.d.A., J.D., J.P., D.G., A.H., A.B. and J.B. performed all experiments. T.B., M.I. and M.K. provided crucial cell reagents. L.T., J.v.d.A. and P.V. designed and analyzed all experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/doi/10.1038/nn.3264>.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

1. Hsieh, J. & Gage, F.H. Chromatin remodeling in neural development and plasticity. *Curr. Opin. Cell Biol.* **17**, 664–671 (2005).
2. Yoo, A.S. & Crabtree, G.R. ATP-dependent chromatin remodeling in neural development. *Curr. Opin. Neurobiol.* **19**, 120–126 (2009).
3. Hirabayashi, Y. & Gotoh, Y. Epigenetic control of neural precursor cell fate during development. *Nat. Rev. Neurosci.* **11**, 377–388 (2010).
4. Bertrand, N., Castro, D.S. & Guillemot, F. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517–530 (2002).
5. Kageyama, R., Ohtsuka, T., Shimojo, H. & Imayoshi, I. Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. *Nat. Neurosci.* **11**, 1247–1251 (2008).
6. Louvi, A. & Artavanis-Tsakonas, S. Notch signaling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93–102 (2006).
7. Pierfelice, T., Alberi, L. & Gaiano, N. Notch in the vertebrate nervous system: an old dog with new tricks. *Neuron* **69**, 840–855 (2011).
8. Kopan, R. & Ilagan, M.X. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216–233 (2009).
9. Ohtsuka, T., Sakamoto, M., Guillemot, F. & Kageyama, R. Roles of the basic helix-loop-helix genes *Hes1* and *Hes5* in expansion of neural stem cells of the developing brain. *J. Biol. Chem.* **276**, 30467–30474 (2001).
10. Shimojo, H., Ohtsuka, T. & Kageyama, R. Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* **58**, 52–64 (2008).
11. Kawaguchi, D., Yoshimatsu, T., Hozumi, K. & Gotoh, Y. Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon. *Development* **135**, 3849–3858 (2008).
12. Borggrefe, T. & Liefke, R. Fine-tuning of the intracellular canonical Notch signaling pathway. *Cell Cycle* **11**, 264–276 (2012).
13. Mizutani, K., Yoon, K., Dang, L., Tokunaga, A. & Gaiano, N. Differential Notch signaling distinguishes neural stem cells from intermediate progenitors. *Nature* **449**, 351–355 (2007).
14. Redmond, L., Oh, S.R., Hicks, C., Weinmaster, G. & Ghosh, A. Nuclear Notch1 signaling and the regulation of dendritic development. *Nat. Neurosci.* **3**, 30–40 (2000).
15. Sestan, N., Artavanis-Tsakonas, S. & Rakic, P. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741–746 (1999).
16. Hashimoto-Torii, K. *et al.* Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex. *Neuron* **60**, 273–284 (2008).
17. Kriegstein, A. & Alvarez-Buylla, A. The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* **32**, 149–184 (2009).
18. Noctor, S.C., Martinez-Cerdeno, V., Ivic, L. & Kriegstein, A.R. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* **7**, 136–144 (2004).
19. Miyata, T. *et al.* Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* **131**, 3133–3145 (2004).
20. Malatesta, P., Hartfuss, E. & Gotz, M. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253–5263 (2000).
21. Tiberi, L., Vanderhaeghen, P. & van den Aemeele, J. Cortical neurogenesis and morphogens: diversity of cues, sources and functions. *Curr. Opin. Cell Biol.* **24**, 269–276 (2012).
22. Götz, M. & Huttner, W.B. The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* **6**, 777–788 (2005).
23. Okano, H. & Temple, S. Cell types to order: temporal specification of CNS stem cells. *Curr. Opin. Neurobiol.* **19**, 112–119 (2009).
24. Gaspard, N. *et al.* An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* **455**, 351–357 (2008).
25. Gaspard, N. & Vanderhaeghen, P. Mechanisms of neural specification from embryonic stem cells. *Curr. Opin. Neurobiol.* **20**, 37–43 (2010).
26. Chang, C.C., Ye, B.H., Chaganti, R.S. & Dalla-Favera, R. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *Proc. Natl. Acad. Sci. USA* **93**, 6947–6952 (1996).
27. Ye, B.H. *et al.* Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. *Science* **262**, 747–750 (1993).
28. Iacovino, M. *et al.* Inducible cassette exchange: a rapid and efficient system enabling conditional gene expression in embryonic stem and primary cells. *Stem Cells* **29**, 1580–1588 (2011).
29. van den Aemeele, J. *et al.* Eomesodermin induces *Mesp1* expression and cardiac differentiation from embryonic stem cells in the absence of Activin. *EMBO Rep.* **13**, 355–362 (2012).
30. Ye, B.H. *et al.* The *Bcl6* proto-oncogene controls germinal-centre formation and T_H2 -type inflammation. *Nat. Genet.* **16**, 161–170 (1997).
31. Phan, R.T. & Dalla-Favera, R. The *Bcl6* proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**, 635–639 (2004).
32. Otaki, J.M., Hatano, M., Matayoshi, R., Tokuhisa, T. & Yamamoto, H. The proto-oncogene *Bcl6* promotes survival of olfactory sensory neurons. *Dev. Neurobiol.* **70**, 424–435 (2010).
33. Zhang, S.J. *et al.* Decoding NMDA receptor signaling: identification of genomic programs specifying neuronal survival and death. *Neuron* **53**, 549–562 (2007).
34. Bedogni, F. *et al.* *Tbr1* regulates regional and laminar identity of postmitotic neurons in developing neocortex. *Proc. Natl. Acad. Sci. USA* **107**, 13129–13134 (2010).
35. Funatsu, N., Inoue, T. & Nakamura, S. Gene expression analysis of the late embryonic mouse cerebral cortex using DNA microarray: identification of several region- and layer-specific genes. *Cereb. Cortex* **14**, 1031–1044 (2004).
36. Leamey, C.A. *et al.* Differential gene expression between sensory neocortical areas: potential roles for *Ten_m3* and *Bcl6* in patterning visual and somatosensory pathways. *Cereb. Cortex* **18**, 53–66 (2008).
37. Ong, C.T. *et al.* Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation probability. *J. Biol. Chem.* **281**, 5106–5119 (2006).
38. Sakano, D. *et al.* BCL6 canalizes Notch-dependent transcription, excluding Mastermind-like1 from selected target genes during left-right patterning. *Dev. Cell* **18**, 450–462 (2010).
39. Herranz, D. & Serrano, M. SIRT1: recent lessons from mouse models. *Nat. Rev. Cancer* **10**, 819–823 (2010).
40. Hisahara, S. *et al.* Histone deacetylase SIRT1 modulates neuronal differentiation by its nuclear translocation. *Proc. Natl. Acad. Sci. USA* **105**, 15599–15604 (2008).
41. Mulligan, P. *et al.* A SIRT1-LSD1 co-repressor complex regulates Notch target gene expression and development. *Mol. Cell* **42**, 689–699 (2011).
42. Guarani, V. *et al.* Acetylation-dependent regulation of endothelial Notch signaling by the SIRT1 deacetylase. *Nature* **473**, 234–238 (2011).
43. Min, S.W. *et al.* Acetylation of tau inhibits its degradation and contributes to tauopathy. *Neuron* **67**, 953–966 (2010).
44. Bray, S. & Bernard, F. Notch targets and their regulation. *Curr. Top. Dev. Biol.* **92**, 253–275 (2010).
45. Hoeck, J.D. *et al.* Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. *Nat. Neurosci.* **13**, 1365–1372 (2010).
46. Sabharwal, P., Lee, C., Park, S., Rao, M. & Sockanathan, S. GDE2 regulates subtype-specific motor neuron generation through inhibition of Notch signaling. *Neuron* **71**, 1058–1070 (2011).
47. Endo, K. *et al.* Chromatin modification of Notch targets in olfactory receptor neuron diversification. *Nat. Neurosci.* **15**, 224–233 (2012).
48. Prozorovski, T. *et al.* Sirt1 contributes critically to the redox-dependent fate of neural progenitors. *Nat. Cell Biol.* **10**, 385–394 (2008).
49. Gao, J. *et al.* A novel pathway regulates memory and plasticity via SIRT1 and miR-134. *Nature* **466**, 1105–1109 (2010).
50. Donmez, G., Wang, D., Cohen, D.E. & Guarente, L. SIRT1 suppresses beta-amyloid production by activating the alpha-secretase gene ADAM10. *Cell* **142**, 320–332 (2010).

ONLINE METHODS

Cell culture and differentiation. ICE (A2lox.Cre) mouse ESCs²⁸ were routinely propagated on irradiated mouse embryonic fibroblasts in DMEM (Invitrogen) supplemented with 15% ESC-certified fetal bovine serum (vol/vol, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), 50 U ml⁻¹ penicillin/streptomycin and 10³ U ml⁻¹ leukemia inhibitor factor (ESGRO). For differentiation, ESC were plated at low density (20 $\times$ 10³ ml⁻¹) on gelatin-coated coverslips and cultured as previously described⁵¹. P19 cells (a gift from F. Guillemot) were routinely cultured as a monolayer in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine (vol/vol) and 1% penicillin/streptomycin (vol/vol).

Plasmids. The coding sequence of BCL6 was amplified by PCR from cDNA with an N-terminal Myc tag, sequence verified and cloned into the p2lox plasmid²⁸ and pCAG. The coding sequence of Hes5 was amplified by PCR from cDNA and cloned into pCAG-IRES-GFP (pCIG). pCS2-N1ΔE was a kind gift from G. Del Sal (LNCIB Trieste). Double-stranded oligonucleotides coding for *Bcl6* shRNA #1 (target sequence, 5'-gacacggatctgagaatc-3')³¹, *Bcl6* shRNA #2 (target sequence, 5'-tgatgttcttcaacctaa-3')³³ and control shRNA (target sequence, 5'-actaccgtgttatagtg-3') were cloned downstream of the *U6* promoter into the pSilencer2.1-CAG-Venus (pSCV2)-plasmid (a kind gift from F. Polleux, the Scripps Research Institute) according to the pSilencer instructions from Ambion. A fragment of the *Hes5* promoter (from -1,324 to 0 bp) was amplified by PCR from genomic DNA, sequence verified and cloned into the pGL3 basic vector (Promega). We deleted 8 bp (-875 to -868 bp) of the BCL6 binding site by PCR-mediated ligation (primers: forward, 5'-ggcagccatggTAGccacgcggagtagtgatc-3'; reverse, 5'-tcccgctggcTAccatgctgcccttaactcc-3').

Generation of a tetracyclin-inducible MycBCL6 ESC line. A tetracyclin-inducible MycBCL6 ESC line was generated as described previously^{28,29}. Briefly, 5 $\times$ 10⁶ ICE (A2lox.Cre) ESCs were electroporated with the p2lox-MycBCL6 vector, allowing unidirectional recombination of the transgene in the *Hprt* locus. Clones were screened by immunofluorescence against BCL6 after 24 h in the absence or presence of 1 μ g ml⁻¹ doxycyclin to verify transgene expression. Results were confirmed in three independent clones.

Transactivation assay. Transactivation assays were performed in P19 cells using a 1,324-bp *Hes5* promoter upstream of Luciferase⁵². For transfection, P19 cells were seeded at 25 $\times$ 10³ cells per 24 wells and transfected 24 h later using Lipofectamin2000 reagent⁵² with 10 ng of pRL-TK and 200 ng of pHes5-pGL3, with or without 200 ng of pCS2-N1ΔE and 200 ng of pCAG-BCL6. pCIG was used as control vector up to a final DNA amount of 800 ng. Renilla and firefly luciferase activities were measured after 48 h using a Dual Luciferase kit (Promega). Data from five independent experiments are presented as firefly luciferase activity relative to renilla luciferase activity and subsequently normalized to the mean of the control conditions.

RNA isolation and qRT-PCR. For total RNA preparation, cells were lysed in RNeasy lysis buffer + 1% β -mercaptoethanol (vol/vol), and RNA was isolated using RNeasy RNA preparation minikit (Qiagen) according to the manufacturer's instructions. Reverse transcription was done using Superscript II kit and protocol (Invitrogen). Quantitative PCR (qPCR) was performed in duplicate using Power SybrGreen Mix and a 7500 Real-Time PCR System (Applied Biosystems). Results are presented as linearized Ct values normalized to the housekeeping gene *Tbp* and the indicated reference value (2^{-ΔΔCt}). The results of qRT-PCR following *ex utero* electroporation were normalized to the mean value of all GFP electroporations for each litter separately. The primers used are summarized in **Supplementary Table 1**.

Western blot, Co-IP, ChIP, ChIP qPCR and EMSA. Western blot and Co-IP were performed as previously described on dissected E10.5, E12.5, E14.5, E15.5, E16.5 and E18.5 cortex⁵³. ChIP was performed as described previously⁵³ on differentiating ESCs or dissected E12.5 or 15.5 cortex. qPCR analysis was performed in duplicate using primers amplifying genomic DNA encompassing consensus BCL6 or CSL binding sites. Primers are summarized in **Supplementary Table 1**.

Results were analyzed comparing the particular antibody to the input and presented as a percentage of the input. EMSA was performed as described⁵⁴ on P19 cells transfected with pCIG or pCAG-MycBCL6. Briefly, 50 fM DIG-labeled probe and 1 μ g of nuclear proteins were used. The antibody detection reaction was performed with a CDP-Star (Roche cat. no.1685627). Competitive EMSA was performed by adding a 5- or 50-fold molar excess of unlabeled double-stranded oligonucleotide (competitor) to the mixture. The wild-type oligonucleotide (sense, aaggcagccatgggttctctaaagccacg; antisense, aacgtggctttaggaaacatgctgccc) corresponded to the endogenous sequence of the *Hes5* promoter containing the BCL6 binding site, whereas the mutant oligonucleotides (sense, aaggcagccatgggtgcccacagccacg; antisense, aacgtggctgtgggaacatgctgccc) contained a mutation in the BCL6 binding site.

Immunofluorescence staining and immunohistochemistry. Cells were fixed in 4% paraformaldehyde (wt/vol) for 30 min and washed three times in phosphate-buffered saline (PBS). Embryos and P0 pups were fixed by perfusion with 4% paraformaldehyde and where appropriate cryoprotected in 30% sucrose (wt/vol, Merck). Immunofluorescence stainings were performed on coverslips, 20- μ m-thick cryosections or 60–100- μ m vibratome sections. Blocking solution consisted of PBS supplemented with 5% horse serum (Invitrogen), 0.3% Triton X-100 (Sigma) and 3% bovine serum albumin (BSA, vol/vol, Sigma). Antibody solution consisted of PBS supplemented with 1% horse serum, 0.1% Triton X-100 and 3% BSA. Primary antibodies were incubated overnight at 4 °C and secondary during 2 h at 15–25 °C. Nuclei were stained with bisbenzimidazole (Hoechst#33258, Sigma). Sections and coverslips were mounted with glycerol (DAKO). EdU revelation was performed with a solution containing 100 mM Tris, 1 mM CuSO₄, 100 mM ascorbic acid and fluorescent azide (Alexa Fluor 488 Azide, Invitrogen).

Immunohistochemistry was performed in 4- μ m-thick paraffin sections³⁰. Briefly the slides were deparaffinized with toluene, rehydrated and antigen retrieval was performed by incubating slices for 30 min in retrieval solution in a water bath at 98 °C. Retrieval solution consisted of 10 mM EDTA and 0.2% Tween-20 (vol/vol) at pH 7.5. BCL6 primary antibody (Santa Cruz N3 sc-858) diluted (1:25) in PBS with 3% horse serum and 0.1% Triton X-100 was incubated overnight at 4 °C and afterwards for 1 h at 15–25 °C with a biotin-conjugated antibody to rabbit (Jackson ImmunoResearch 711-065-152) diluted (1:250) in PBS with 3% horse serum with 0.1% Triton X-100. ABC solution was used 2 h at 15–25 °C (Vectastain Elite ABC Kit Standard PK-6100). The sections were incubated with the substrate at 15–25 °C until suitable staining was observed (DAB Peroxidase Substrate Kit, SK-4100).

For BCL6-Tbr2 and BCL6-Pax6 double labeling, paraffin sections were first processed for BCL6 immunofluorescence with tyramide signal amplification (TSA kit T30954, Invitrogen), and then for Pax6 or Tbr2 IF. Double labeling was confirmed using confocal immunofluorescence (single optical section, thickness = 1 μ m in **Fig. 2a–c,g–i** and 0.38 μ m in **Fig. 2d–f,j–l**). All BCL6 immunostainings were controlled negatively with sections of *Bcl6*^{-/-} mice, which did not display any substantial staining (**Supplementary Fig. 2** and data not shown).

Antibodies. For primary antibodies, we used antibodies to β 3-tubulin (TuJ1, MMS435P, Covance), Tbr2 (ab23345, Abcam), Cux1 (sc-13024, Santa Cruz Biotechnology), BCL6 (sc-858, Santa Cruz Biotechnology), Ki67 (ab15580, Abcam), GFP (ab13970, Abcam), Nestin (ab6142, Abcam), pH3 (10543, Abcam), Ctip2 (ab18465, Abcam), Tbr1 (Eurogentec), Pax6 (PRB278P, Covance), cleaved caspase 3 (MAB835, R&D), N1ICD (2421, Cell Signaling), Sirt1 (Co-IP, sc-15404, Santa Cruz Biotechnology), Sirt1 (ChIP, 07-131, Millipore), H1.4K26Ac (H7789, Sigma), H4K16Ac (07-329, Millipore) and control-IgG (sc-805, Santa Cruz Biotechnology). For secondary antibodies (1:400), we used donkey antibodies to mouse (715-166-150), goat (705-165-147) and rabbit (711-165-152, 711-175-152) coupled to cyanin-3 or cyanin-5 (Jackson ImmunoResearch) or donkey antibodies to mouse (A21202), goat (A11055) and rabbit (A21206) coupled to Alexa Fluor 488 (Molecular Probes).

Mice, in utero and ex utero electroporation. All mouse experiments were performed with the approval of the Université Libre de Bruxelles Committee for animal welfare. The *Bcl6*^{+/-} mice (mixed C57BL/6 and CD1 background), in which the null allele lacked exons 4–10, were a generous gift from R. Dalla-Favera (Columbia University)³⁰. For EdU labeling, timed-pregnant female mice were

injected intraperitoneally with a single pulse (50 mg per kg of body weight) of EdU and killed after 1 or 24 h. *In utero* surgery and electroporation were performed as described⁵⁵. Briefly, timed pregnant CD1 mice at E13.5 or E12.5 were anesthetized and their uterus was exposed. A solution of pCIG, pCAG-MycBCL6 or pCIG-Hes5 (total concentration of 1 $\mu\text{g } \mu\text{l}^{-1}$), or pSCV2-Bcl6 shRNA or pSCV2-control shRNA (0.5 $\mu\text{g } \mu\text{l}^{-1}$) together with pCIG-GFP or pCAG-MycBCL6 (1 $\mu\text{g } \mu\text{l}^{-1}$) was injected into one lateral ventricle of *in utero* embryos or both ventricles of *ex utero* embryos, and 5–8 electric pulses at 30 V at E13.5 or 25 V at E12.5, were delivered using forceps-type electrodes. After *ex utero* electroporation, brains were dissected and 300- μm -thick vibratome sections were cultured for 24 h (ref. 56) after which the GFP-expressing regions were microdissected under a fluorescent binocular microscope and further processed for RNA extraction.

Statistical analysis. Unless stated otherwise, data are presented as mean + s.e.m. of at least three biologically independent *in vitro* experiments, or of 3–13 embryos from two to five different litters. For quantification of cell numbers *in vitro*, at least 200 cells were counted in five different fields from at least three biologically independent experiments; for *in vivo* cell counting, all positive cells were counted in a 100- or 200- μm -wide area of the cortex, where appropriate at three rostro-caudal levels, carefully matched between animals. For quantification of pH3, all positive cortical progenitors were counted per brain section. Whenever possible, quantifications were performed blind to genotype or condition, and no animals or data points were excluded during the analysis. Two-sided Student's *t* test was used for calculating significance values.

In situ RNA hybridization. *In situ* RNA hybridization using DIG-labeled RNA probes on brain cryosections was performed as described⁵⁷. All hybridization results obtained with antisense probes were compared with control sense probes. *In situ* hybridization probes for *Bcl6*: bp 1,088–1,828 (**Supplementary Fig. 2a–l**), bp 102–331 and bp 928–1,576 (data not shown) of the mRNA (NCBI reference sequence: NM_009744.3).

Imaging. Pictures of the *in situ* RNA hybridization, immunohistochemistry and immunofluorescence staining were acquired with an Axioplan2 Zeiss microscope and a Spot RT camera, or with a LSM510 Zeiss Confocal.

Electrophysiology. Whole-cell patch-clamp recordings were performed on ESC-derived individual neurons identified as previously described²⁴. All recordings were made with a Dual EPC10 amplifier and acquired using Pulse software (HEKA). Signals were filtered at 4 kHz using the built-in filter of EPC10 and digitally sampled at 20 kHz. Data were analyzed with Igor Pro software (WaveMetrics). The presence of voltage-gated sodium current was assessed by submitting the cells to a depolarizing step to 0 mV from a holding potential of –70 mV. Intrinsic excitability was investigated in current clamp by setting membrane potential at –70 mV and injecting 1-s step current pulses with 10-pA increments. Spontaneous excitatory postsynaptic currents were recorded at a holding potential of –70 mV. To further check the nature of spontaneous postsynaptic currents, we used 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM) and D-(-)-2-amino-5-phosphonopentanoic acid (10 μM) to block AMPA and NMDA receptors, respectively.

51. Gaspard, N. *et al.* Generation of cortical neurons from mouse embryonic stem cells. *Nat. Protoc.* **4**, 1454–1463 (2009).
52. Heng, J.I. *et al.* Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. *Nature* **455**, 114–118 (2008).
53. Rustighi, A. *et al.* The prolyl-isomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. *Nat. Cell Biol.* **11**, 133–142 (2009).
54. Takeda, N. *et al.* Bcl6 is a transcriptional repressor for the IL-18 gene. *J. Immunol.* **171**, 426–431 (2003).
55. Saito, T. & Nakatsuji, N. Efficient gene transfer into the embryonic mouse brain using *in vivo* electroporation. *Dev. Biol.* **240**, 237–246 (2001).
56. Dufour, A. *et al.* Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. *Neuron* **39**, 453–465 (2003).
57. Depaepe, V. *et al.* Ephrin signalling controls brain size by regulating apoptosis of neural progenitors. *Nature* **435**, 1244–1250 (2005).