Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex

João D. Pereira[a], Stephen N. Sansom[a], James Smith[a], Marc-Werner Dobenecker[b], Alexander Tarakhovsky[b], and Frederick J. Livesey[a,1]

[a]Gurdon Institute and Department of Biochemistry, University of Cambridge, Cambridge CB2 1QN, United Kingdom; and [b]Laboratory of Lymphocyte Signaling, The Rockefeller University, New York, NY 10021

Edited by Gerald R. Crabtree, Howard Hughes Medical Institute, Stanford, CA, and approved August 4, 2010 (received for review February 26, 2010)

Multipotent progenitor cells of the cerebral cortex balance self-renewal and differentiation to produce complex neural lineages in a fixed temporal order in a cell-autonomous manner. We studied the role of the polycomb epigenetic system, a chromatin-based repressive mechanism, in controlling cortical progenitor cell self-renewal and differentiation. We found that the histone methyltransferase of polycomb repressive complex 2 (PCR2), enhancer of Zeste homolog 2 (Ezh2), is essential for controlling the rate at which development progresses within cortical progenitor cell lineages. Loss of function of Ezh2 removes the repressive mark of trimethylated histone H3 at lysine 27 (H3K27me3) in cortical progenitor cells and also prevents its establishment in postmitotic neurons. Removal of this repressive chromatin modification results in marked up-regulation in gene expression, the consequence of which is a shift in the balance between self-renewal and differentiation toward differentiation, both directly to neurons and indirectly via basal progenitor cell genesis. Although the temporal order of neurogenesis and gliogenesis are broadly conserved under these conditions, the timing of neurogenesis, the relative numbers of different cell types, and the switch to gliogenesis are all altered, narrowing the neurogenic period for progenitor cells and reducing their neuronal output. As a consequence, the timing of cortical development is altered significantly after loss of PRC2 function.

development | epigenetics | stem cells | chromatin

Multipotent progenitor cells of the cerebral cortex generate complex neuronal and glial lineages in a fixed temporal order during embryonic development (1–3). The multipotency of neural progenitor cells is linked with the fundamental problem of maintaining the balance between progenitor cell self-renewal and neurogenesis. Several chromatin-modifying complexes regulate renewal and differentiation of a range of stem cell types, including ES cells and hematopoietic stem cells (4–6). The polycomb chromatin-modifying system (7) is of particular interest in this context. Studies in ES cells have found that loss of function of polycomb components compromises pluripotency and the ability of ES cells to generate differentiated progeny (8–10). Loss of polycomb function in the developing skin alters epithelial stem cell proliferation and accelerates the timing of skin development (11).

In vertebrates there are two distinct polycomb protein complexes with completely different constituents, polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (12). PRC2, containing the histone methyltransferase enhancer of Zeste homolog 2 (Ezh2), represses gene transcription by modifying the tail of histone H3 via methylation of lysine 27 (H3K27me3) (13, 14). PRC2 provides a substrate for PRC1 recruitment but also has repressive activities independent of PRC1 in several systems (12, 15). Both polycomb complexes repress expression of functionally diverse gene sets, including developmentally important transcription factors, several classes of cell-cycle regulators, and genes involved in mitochondrial function and the generation of reactive oxygen species (16–18).

Knockout of the Bmi1 subunit of PRC1 has little effect on progenitor cell self-renewal during development but is essential for neural stem cell maintenance in the adult CNS (19). However, acute deletion of Bmi1 by RNAi does compromise cortical progenitor cell self-renewal (20). Furthermore, removal of Ring1B, an ubiquitin ligase component of PRC1, from the developing cortex during neurogenesis lengthens the period of neurogenesis and delays the onset of gliogenesis (21). Deletion of Ezh2 at the same developmental stage appears to produce the same phenotype (21). We report here that deletion of the histone methyltransferase of PRC2, Ezh2, in mouse cortical progenitor cells before the onset of neurogenesis changes the balance between differentiation and self-renewal, significantly altering developmental timing in this system.

Results

Deletion of the Histone Methyltransferase Ezh2 Alters Cortical Neurogenesis. Ezh2 is highly expressed in progenitor cells of the cortex, with little protein expression in cortical neurons (Fig. 1*A* and Fig. S1). Notably, Ezh2 levels in cortical progenitor cells decrease over the period of neurogenesis, from high levels at the onset of neurogenesis to very low levels at the end of neurogenesis and onset of gliogenesis (Fig. S1). Ezh2 is absent from the Pax6-expressing progenitor cells in the remnant of the ventricular zone (VZ) at birth (Fig. S1). We deleted Ezh2 in cortical progenitor cells before the onset of neurogenesis using mice carrying conditional (floxed) alleles of Ezh2 (22) crossed with an Emx1-Cre line that drives cortex-specific Cre expression from embryonic day 9.5 (E9.5) (23). Cortex-specific deletion of Ezh2 resulted in the disappearance of Ezh2 protein by E12 (Fig. 1 *A*–*E*). Importantly, in the developing Ezh2-null cortex trimethylation of H3K27 (H3K27me3) was absent both from neural progenitor cells and from the neurons they generate (Fig. 1 *C* and *D*).

Cortex-specific Ezh2-null mice are born in expected numbers, are viable, survive to adulthood, mate successfully, and show no overt neurological or behavioral abnormalities. However, loss of Ezh2 function in cortical progenitor cells results in a pronounced cortical growth phenotype: At E12, the Ezh2-null cortex is indistinguishable from that of control littermates in overall thickness, in the amount of neuron-containing cortical plate present, and in the number of cells within the cortical plate (Fig. 1 *F*, *G*, *N*, and *O*). Strikingly, by E14 the Ezh2-null cortex is substantially thicker than in controls, and this thickness is attributable to a significantly enlarged number of neurons in the cortical plate in the Ezh2-null cortex (Fig. 1 *H*, *I*, *N*, and *O*). However, by E16 the control and Ezh2-null cortices are similar in size, and by birth the

Author contributions: J.D.P., S.N.S., and F.J.L. designed research; J.D.P., S.N.S., J.S., and F.J.L. performed research; M.-W.D. and A.T. contributed new reagents/analytic tools; J.D.P., S.N.S., and F.J.L. analyzed data; and J.D.P., S.N.S., and F.J.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: rick@gurdon.cam.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1002530107/-/DCSupplemental.

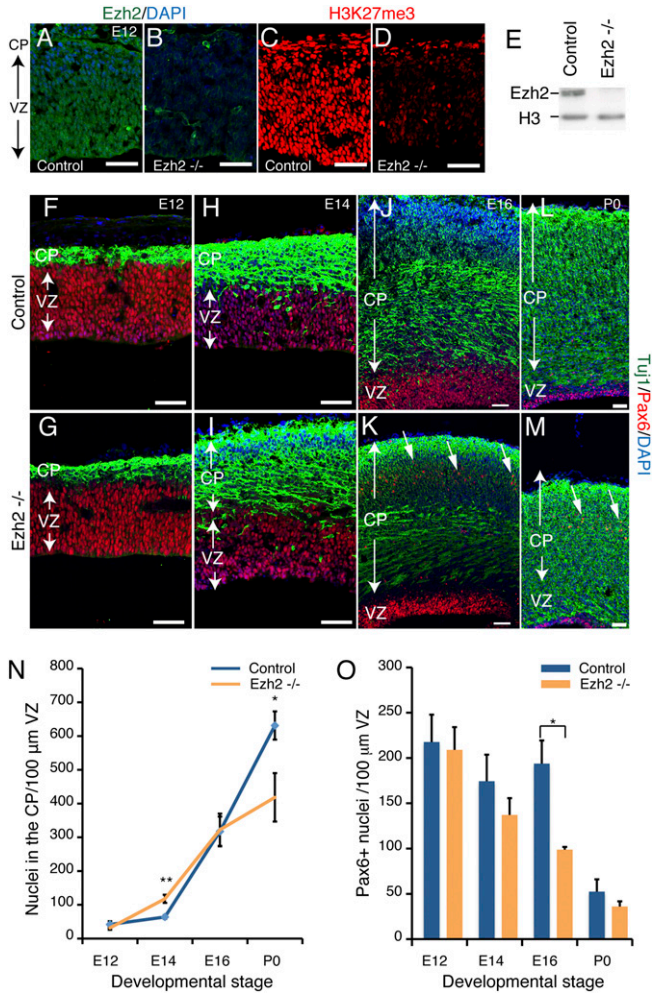

Fig. 1. Deletion of Ezh2 in cortical progenitor cells alters cortical growth. (*A–E*) Cortex-specific deletion of Ezh2 in vivo before the onset of neurogenesis results in undetectable levels of Ezh2 protein in the cerebral cortex at E12.5 (immunofluorescence in *A* and *B*; Western blot for Ezh2 and histone H3 in *E*). The repressive H3K27me3 chromatin modification is almost completely absent from progenitor cells and neurons at E12 (*C* and *D*). (*F–M*) The Ezh2-null cortex is of normal dimensions at E12 (*F*, *G*), but at E14 has a thicker cortical plate (CP) of postmitotic neurons (Tuj1) than seen in littermate controls (*H* and *I*). The cortical plate is of similar thickness in Ezh2-null and control cortices at E16 in the Ezh2-null cortex (*J* and *K*) but is considerably thinner in Ezh2-null cortex at P0 (*L* and *M*). The Pax6-expressing progenitor cell population in the VZ is of comparable size in Ezh2-nulls and controls at E12 and E14 (*F–I*) but is considerably smaller at E16 in the Ezh2-null cortex (*J*, *K*). In addition, ectopic Pax6 expression (arrows in *K* and *M*) is detected in the nuclei of neurons in the cortical plate at E16 and P0. Nuclei are visualized with DAPI (blue). (Scale bars: 50 μm *A–D* and *F–M*.) (*N*) The number of neurons in the cortical plate was analyzed by counting nuclei superficial to the VZ in three control and three Ezh2-null littermates at four developmental time points. At E14 the Ezh2-null cortex contain significantly more cells (nuclei in the cortical plate/100 μm tangential width of cortex) than the control cortex (**$P < 0.01$). At E16, Ezh2-null and control cortices have the same number of cells. At P0, Ezh2-null cortices have significantly fewer cells than control cortices (*$P < 0.05$). (*O*) At E16 the number of cortical progenitor cells per unit width of cerebral cortex, defined by nuclear Pax6 expression, is significantly lower in the Ezh2-null cortex than in the control cortex. At all other stages of cortical development, no significant difference in progenitor cell number was observed.

Ezh2-null cortex is substantially thinner than in littermate controls and has fewer total cells (Fig. 1). Therefore, Ezh2-null cortical progenitor cells appear to generate inappropriately more neurons than controls initially but later underproduce neurons to generate an overall smaller cortex. In parallel with neurogenesis, the apical Pax6-expressing progenitor cell population in the VZ is maintained in number from E12 to E14 but by E16 is significantly depleted in the Ezh2-null cortex (Fig. 1). However, at birth, there are similar numbers of Pax6-expressing cells in the remnant of the VZ in the control and Ezh2-null cortex. An additional notable finding in the Ezh2-null cortex is the ectopic expression of Pax6 in layer 5 CTIP2-expressing corticospinal motor neurons at E16 and postnatal day 0 (P0) (Fig. 1 *K* and *M* and Fig. S2).

Loss of PRC2 Function Results in Up-Regulated Gene Expression with Little Down-Regulation. H3K27me3 is a chromatin modification associated with the repression of gene expression (14); thus loss of Ezh2 function would be expected to result in marked up-regulation of gene expression. Expression profiling of the Ezh2-null cortex during the early stages of cortical neurogenesis (E12.5), found that 1,080 transcripts showed up-regulated expression, and only 12 were down-regulated (Fig. 2*A* and Dataset S1). Comparison of the intersection between the 1,080 genes up-regulated following deletion of Ezh2 before the onset of neurogenesis and previously reported data on 305 genes up-regulated 2-fold or more in E18 cortical progenitor cells cultured for 3 d after deletion of Ring1B (21) found a set of 50 genes commonly up-regulated in both datasets, with the majority of changes unique to each mutation. Gene Ontology (GO) analysis of the up-regulated transcripts found significant enrichments for a wide range of functional classes, including genes involved in neurogenesis and neuronal differentiation (Fig. 2*B* and Dataset S2).

We used the GenePaint mouse in situ hybridization database (24) to examine the expression of the top 100 up-regulated genes. Of the 77 of these genes for which data are available, 45 had cortical expression at E14.5. (A detailed breakdown is given in Dataset S3.) Notable among the genes significantly up-regulated at E12.5 in the Ezh2-null cortex are a large set of transcription factors and other genes specifically expressed in differentiating cortical neurons (Fig. 2*C*), such as Neurod6, Mef2c, Myt1l, and Bcl11b (expressed in all cortical neurons) and genes specifically expressed in either early-born or later-born cortical neuron types, including corticothalamic and callosal projection neurons (25): Reelin (marginal zone neurons); Moxd1 and Tmem163 (subplate neurons); Tbr1 and Foxp2 (layer 6 neurons), Foxo1 and Pou3f1/SCIP (layer 5 neurons); Lmo4 and Foxp1 (callosal projection neurons of layers 3–5); Bhlhb5/Bhlhe22 (neurons in layers 2–5); Ror-beta (layer 4 neurons); and Satb2 (neurons in layers 2–4) (25–31). The increase in layer-specific neuronal gene expression at this early point in cortical development suggested that cortical development may be more advanced in the Ezh2-null cortex than in control littermates.

To confirm the direct regulation of genes up-regulated in the Ezh2-null cortex, we carried out ChIP for H3K27me3, the repressive mark deposited by PRC2, followed by quantitative, gene-specific PCR for a set of six genes up-regulated in the Ezh2-null cortex. To do so, we focused our analysis on genes that have been described as PRC2-bound in ES cells (32). That analysis confirmed that all six genes are enriched in H3K27me3 on their promoters in the wild-type E12.5 neocortex (Fig. 2).

Loss of polycomb function in a variety of cell types leads to up-regulated transcription of the negative regulators of the cell cycle encoded by the Ink4/Arf locus (Cdkn2a/Cdkn2b) (11, 33, 34). Cdkn2a and Cdkn2b are not normally expressed in the developing cerebral cortex (35–37), and we found the H3K27me3 modification present on the Cdkn2a and Cdkn2b promoters in the wild-type E12 cortex (Fig. 2). However, Cdkn2a and Cdkn2b mRNAs were detected at very low levels in the Ezh2-null cortex (Fig. 2 and Dataset S1). In contrast, the related Ink4 family members Ink4c/Cdkn2c and Ink4d/Cdkn2d normally are expressed in the developing cortex from E13.5, Cdkn2c in cortical progenitor cells and Cdkn2d in differentiating neurons (36). Cdkn2c expression was increased 2-fold in the Ezh2-null cortex, with no change in Cdkn2d expression (Dataset S1).

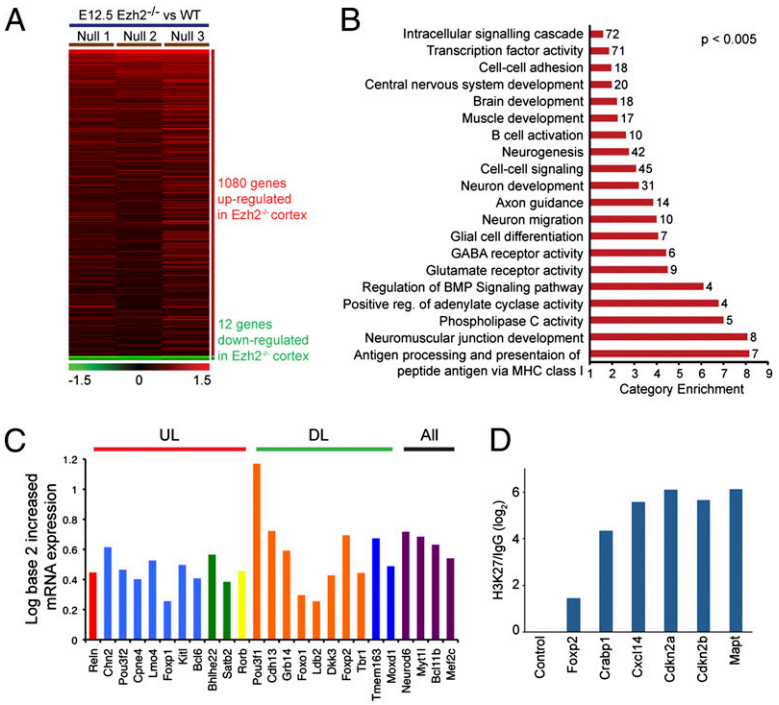

Fig. 2. Loss of PRC2 function results in up-regulation of gene expression with little down-regulation. (*A*) Predominantly up-regulated progenitor cell gene expression following PRC2 loss of function in the E12 cortex. Cluster diagram of genes showing statistically significant changes in gene expression (significance analysis of microarrays, false-discovery rate < 5%) in three E12 Ezh2-null littermates: 1,080 genes are up-regulated, and 12 genes are down-regulated. (*B*) GO analysis of the set of genes up-regulated in the E12 cortex following Ezh2 deletion. Statistically significantly ($P < 0.005$, Benjamini–Hichberg corrected testing) enriched functional groups are shown, compared with the occurrence of each class in the genome, together with the number of genes in each class found in the up-regulated gene dataset. (*C*) Up-regulated expression of many genes expressed specifically in different cortical layers in the E12 Ezh2-null cortex (see text for details). All, genes enriched in expression in all cortical projection neurons; DL, genes enriched in expression in deep-layer neurons; UL, genes enriched in expression in upper-layer neurons. Colors denote layer-specific expression: red, marginal zone; light blue, neurons primarily of layer 2/3 or layers 2–4; green, layers 2–5; yellow, layer 4; orange, layers 5 and 6; dark blue, subplate; purple, all cortical neurons. (*D*) ChIP for H3K27me3 followed by gene-specific PCR confirmed PRC2 binding to the promoter of a test set of six genes in the E12 cortex in vivo. Data were normalized to the background enrichment detected for a control region without H3K27me3 (*SI Materials and Methods*).

Loss of PRC2 Function in Cortical Progenitor Cells Alters Rates of Neurogenesis in Vivo. At E14, approximately halfway through the period of cortical neurogenesis, Ezh2-null cortical progenitor cells have generated twice as many layer 5 and layer 6 neurons as control littermates (Fig. 3 *A*–*E*). At E16, near the end of the neurogenic period, Ezh2-null and control cortices have equal numbers of layer 5 and layer 6 neurons, with slightly fewer upper-layer, Brn2-expressing neurons in the Ezh2-null cortex (Fig. 3 *F*–*J*). By birth (P0), there is a 2-fold reduction in the number of Brn2-expressing later-born neurons in the Ezh2-null cortex (layers 2–4)

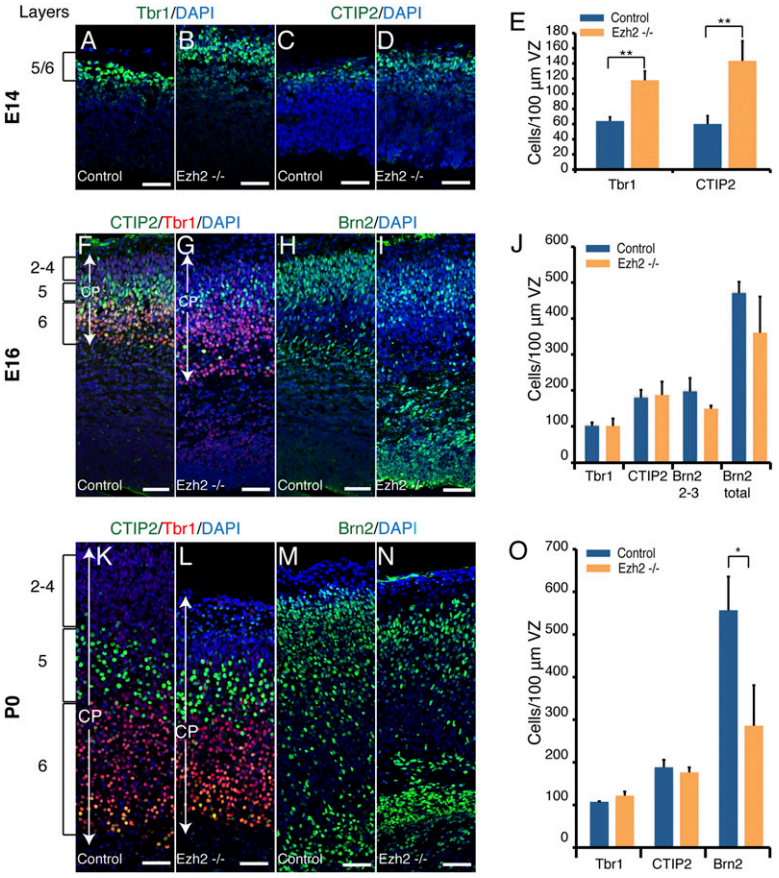

Fig. 3. Loss of Ezh2 function in cortical progenitor cells alters rates of neurogenesis in vivo. (*A*–*E*) Progenitor cells lacking Ezh2 overproduce the first classes of neurons generated in the cortical lineage at an inappropriately early stage of development. (*E*) At E14, at which stage control progenitor cells have generated a set of layer 5/6 neurons three to four cells thick (Tbr1/CTIP2-expressing cells), Ezh2-null cortical progenitor cells have generated approximately twice as many layer 5/6 neurons (**$P < 0.01$). (*F*–*J*) At E16, the numbers of neurons in each cortical layer do not differ significantly in the Ezh2-null and control littermate cortices (*J*). Layer 2/3 Brn2-expressing neurons show aberrant locations near the VZ as well as within the cortical plate (*I*). (*K*–*O*) By the end of neurogenesis (P0), Ezh2-null progenitor cells have generated the correct number of early-generated layer 5/6 neurons (*K*, *L*, and *O*) but have generated less than half as many later-generated layer 2/3 (Brn2-expressing) neurons as wild-type controls (*M*, *N*, and *O*; *$P < 0.05$). Also, many of the later-generated neurons in Ezh2-nulls remain deep in the cortex near the VZ (*N*). Nuclei are visualized with DAPI (blue). (Scale bars, 50 µm.)

NEUROSCIENCE

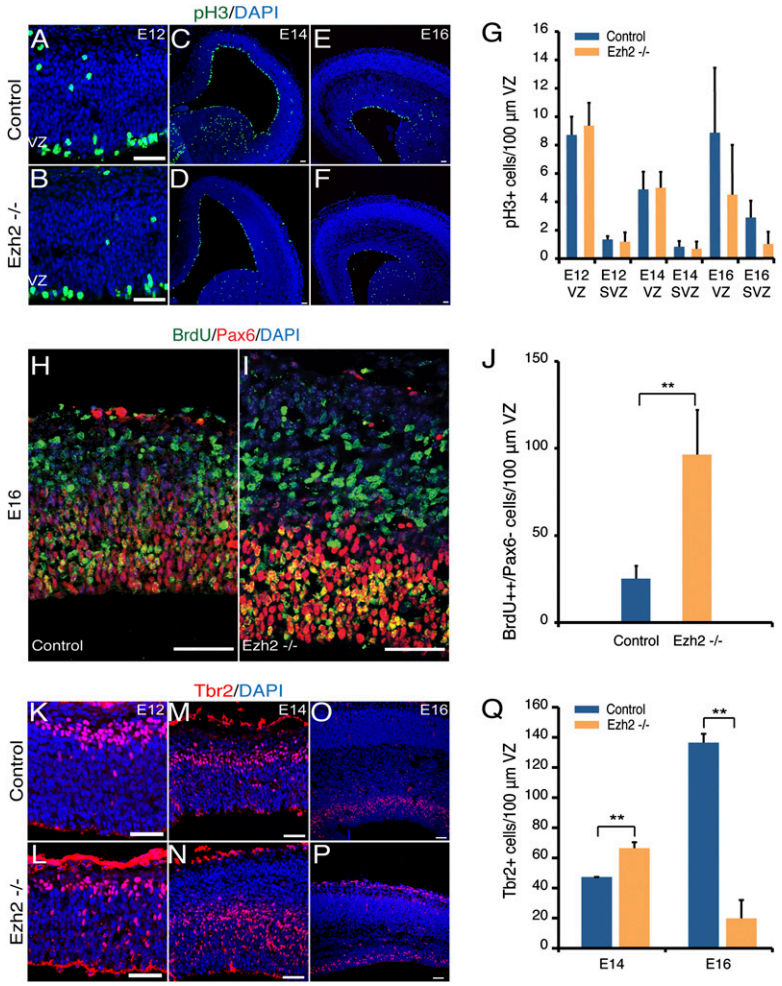

Fig. 4. Cortical progenitor cells lacking Ezh2 overproduce basal progenitor cells and neurons at the expense of self-renewal early in cortical development. (*A–G*) Ezh2-null cortical progenitor cells do not show statistically significant differences in the number of mitotic progenitor cells in the VZ or subventricular zone (SVZ) at E12, E14, or E16. pH3, phosphohistone-H3. A large but variable difference in the number of mitotic cells in the VZ and SVZ of the Ezh2-null cortex was observed at E16. (*H–J*) The neuronal output from cortical progenitor cells lacking PRC2 is significantly increased compared with littermate controls at E13 (**$P < 0.01$). Pulse labeling of neuronal output from cortical progenitor cells over 24 h by in vivo BrdU administration demonstrated that Ezh2-null cells generate more than three times as many $BrdU^+$, $Pax6^-$ neurons than do controls. (*K–Q*) Cortical apical progenitor cells lacking Ezh2 overproduce basal progenitor cells early in cortical development, so that there are 50% more Tbr2-expressing basal progenitor cells in the Ezh2-null cortex than in control cortex at E14 (*M*, *N*, and *Q*). However, by E16 the Ezh2-null cortex contains fewer than 15% of the number of basal progenitor cells observed in control cortex (**$P < 0.01$). Nuclei are visualized with DAPI (blue). (Scale bars, 50 µm.)

(Fig. 3 *K–O*) and in the number of Satb2-expressing outer-layer neurons (Fig. S3). However, at P0 there are equal numbers of deep-layer, early-born neurons in the Ezh2-null and control cortex (Fig. 3 *K–O*). Therefore, following loss of PRC2, cortical progenitor cells produce early-born, layer 5 and layer six neurons in the appropriate numbers but on an earlier timescale and subsequently generate significantly fewer late-born neurons. The reduction in outer-layer neurons is not the result of cell death, because there is only a small increase in apoptosis in the Ezh2-null cortex at all ages studied (Fig. S4).

In addition to the altered production of classes of cortical neurons, there are alterations in terminal differentiation of specific subsets of neurons. As described above, layer 5 CTIP2-expressing corticospinal motor neurons ectopically express Pax6 late in development. The Cux1 transcription factor, normally expressed in layers 2–4, is weakly expressed in the Ezh2-null cortex and is almost undetectable in the cortical plate at birth (Fig. S3). It is likely, therefore, that loss of the repressive H3K27me3 modification in neurons leads to cell-specific patterns of abnormal gene expression in postmitotic neurons.

Cortical Progenitor Cells Lacking PRC2 Function Overproduce Basal Progenitor Cells and Neurons at the Expense of Self-Renewal. Shortening of the neural progenitor cell cycle would increase the number of cells exiting cycle in a given time interval. However, at E12 and E14 we did not observe a difference in the number of M-phase cells in the Ezh2-null cortex compared with littermate controls (Fig. 4 *A–G*), whereas at E16 there was a large but variable reduction in the number of M-phase progenitor cells in the Ezh2-null cortex (Fig. 4 *A–G*). To investigate whether an increased fraction of progenitor cells exited the cell cycle following Ezh2 deletion, we analyzed the neuronal output from cortical progenitor cells at E13 in vivo by BrdU pulse labeling. Twenty-four hours after BrdU administration there was a 3-fold increase in the number of heavily BrdU-labeled neurons in the cortical plate, demonstrating that Ezh2-null progenitor cells overproduce neurons at the expense of self-renewal (Fig. 4 *H–J*). There are two routes to neurogenesis in the cerebral cortex: directly from cortical progenitor cells and indirectly via the basal progenitor cell population (38). Neurogenesis is increased through both these routes following loss of Ezh2 function at the peak of neurogenesis: The size of the basal progenitor cell population is increased by one third in the Ezh2-null cortex at E14 (Fig. 4 *M*, *N*, and *Q*), demonstrating that at this stage cortical progenitor cells overproduce basal progenitor cells as well as neurons. However, by E16 the Tbr2-expressing basal progenitor population is almost completely absent (Fig. 4 *O*, *P*, and *Q*), and the VZ, the Pax6-expressing pool of apical progenitor cells, is greatly reduced, as described above. Therefore, Ezh2-null cortical progenitor cells alter the balance between self-renewal and differentiation toward differentiation both directly to neurons and indirectly via basal progenitor cells, depleting both populations of progenitor cells by E16. However, it is noteworthy that a Pax6-expressing population persists in the VZ of the Ezh2-null cortex and is approximately equal in number to the population in the control cortex at P0.

To study the effects of loss of PRC2 function on progenitor cell self-renewal at single-cell resolution, we cultured primary cortical progenitor cells isolated from E12.5 cortex at clonal density, under which conditions they generate complex lineages

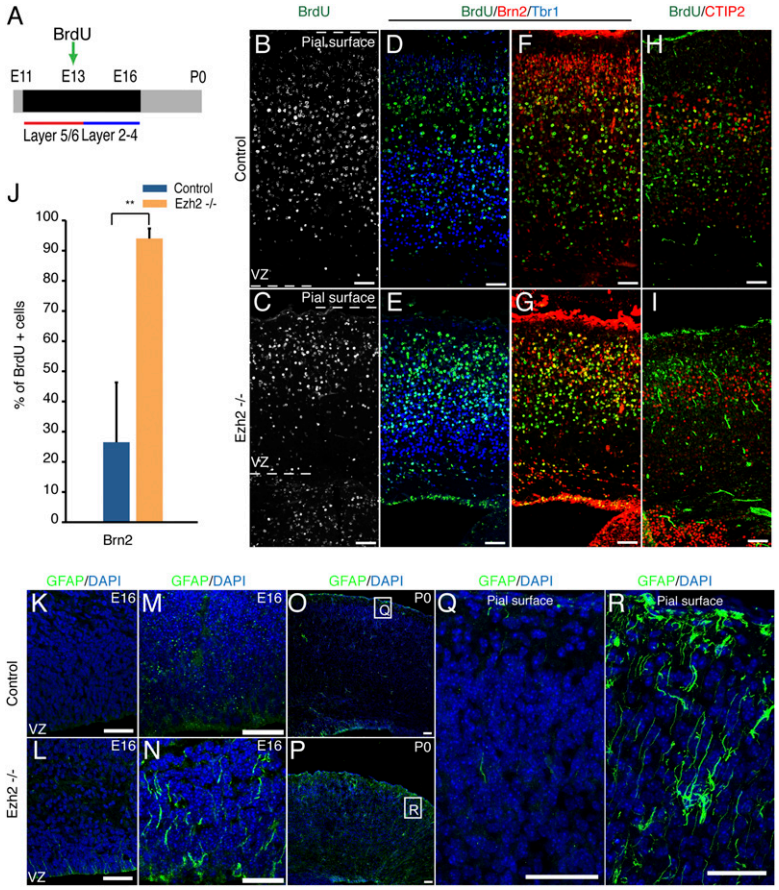

Fig. 5. Changes in developmental timing in cortical progenitor cells following loss of PRC2 function. (*A*) Neuronal birthdating experimental design. BrdU is administered at E13 in vivo and is incorporated by cycling progenitor cells that subsequently generate strongly BrdU$^+$ postmitotic neurons. Continued progenitor cell cycling dilutes the label so that later-generated neurons are weakly BrdU labeled. At this stage in cortical development, progenitor cells predominantly generate a mixture of layer 5 and layer 6 neurons. (*B–I*) Cells born at E13 in control mice populate the middle of the P0 cerebral cortex (*B*), superficial to the Tbr1-expressing layer 6 neurons (*D*) and deep to the Brn2-expressing layers 2 and 3 (*F*). Neurons born at E13 in the Ezh2-null cortex are displaced to the outer half of the cortex (*C*), superficial to both the Tbr1-expressing layer 6 neurons (*E*) and CTIP2-expressing layer 5 neurons (*I*), but colocalize with the Brn2-expressing layer 2/3 neurons (*G*). (*J*) In control mice, fewer than 30% of neurons born at E13 express the layer 2/3-enriched transcription factor Brn2. In contrast, more than 90% of neurons born at E13 by Ezh2-null progenitor cells express Brn2 (**$P < 0.01$). (*K–R*) Astrocytes, defined by GFAP expression, are not found in the cortex of control E16 mice (*K* and *M*) but are found in the VZ of Ezh2-null mice at this stage (*L* and *N*). Similarly, mature GFAP-expressing astrocytes are not found in the cortical plate of control mice at birth but already are present in the progenitor cell-containing VZ (*O* and *Q*). In contrast, many mature GFAP-expressing astrocytes are found in the cortical plate of the Ezh2-null cortex (*P* and *R*). Boxed regions in *O* and *P* correspond to the higher-power images in *Q* and *R*, as indicated. Nuclei are visualized with DAPI (blue). (Scale bars: 50 μm in *A–L* and *O–R*; 25 μm in *M* and *N*.)

in a manner similar to that in vivo (39, 40). After 3 d in culture (approximately six cell cycles), three classes of clones were observed: clones composed of progenitor cells only, of neurons only, and of mixed neuron/progenitor cell clones (Fig. S5 *A–C*). For clones of three or more cells, the relative proportions of each clone type differed in control and Ezh2-null progenitor cells, with Ezh2-null progenitor cells producing proportionally more neuron-only clones (Fig. S5 shows data from three individual mice of each genotype: 301 clones from control littermates and 163 Ezh2-null clones). Following loss of Ezh2 function, cortical progenitor cells generate smaller neuron-only clones compared with control cells and also generate fewer large, complex clones.

Developmental Timing Is Advanced in Cortical Progenitor Cells Following Loss of Ezh2. To test whether overall developmental timing is changed following loss of Ezh2 function, the output of Ezh2-null and control cortical progenitor cells at E13 in vivo was studied by BrdU birthdating (Fig. 5; details are given in *SI Materials and Methods*), at which time the majority of control cortical progenitor cells generate neurons destined for layers 5 and 6 (41). Birthdating of E13 progenitor cells showed that, whereas control cells generate cells destined for layers 5 and 6 (Fig. 5*B*), Ezh2-null progenitor cells generate cells that populate the upper layers of the null cortex (Fig. 5*C*). However, as described above, layers 2–4 in Ezh2-null cortex are reduced to approximately half the size observed in the control cortex, raising the possibility that the change in laminar positioning of E13-born neurons may reflect the absence of later-born neuron types or altered migration to the cortical plate. To test these possibilities, expression of the layer 2/3 transcription factor Brn2 by all BrdU$^+$ cortical plate neurons was scored, regardless of laminar position. Although 20% of control neurons born at E13 express Brn2, ≈90% of E13-born cells from Ezh2-null progenitor cells are layer 2/3, Brn2-expressing neurons (Fig. 5*J*). We conclude, therefore, that by E13 Ezh2-null cortical progenitor cells already have shifted to producing upper-layer neurons destined for layers 2/3 at E13.

Given the acceleration in developmental timing that occurs in the Ezh2-null cortex, we also investigated the timing of the appearance of astrocyte differentiation in the Ezh2-null cortex. At E16, in the later stages of the neurogenic period, GFAP-expressing astrocytes are not normally found in the mouse cerebral cortex. Unusually, GFAP-expressing cells are found in the VZ of the E16 Ezh2-null cortex (Fig. 5 *K–N*). By birth (P0), GFAP-expressing glial cells can be found in the VZ of the control wild-type cortex, with few if any GFAP-expressing, mature astrocytes within the cortical plate (Fig. 5 *O* and *Q*). In contrast, mature, GFAP-expressing glial cells are abundant within the Ezh2-null cortical plate at birth (Fig. 5 *P* and *R*). Therefore, gliogenesis and glial differentiation occur on an accelerated timescale from Ezh2-null cortical progenitor cells.

Discussion

We found that PRC2 is essential for controlling the rate at which development progresses within cortical progenitor cell lineages. To do so, PRC2 regulates the self-renewal and multipotency of cortical progenitor cells as well as their neurogenic potential. Loss of Ezh2 function removes the H3K27me3 repressive mark in cortical progenitor cells and also prevents its establishment in postmitotic neurons. Removal of this repressive chromatin modification results in a marked up-regulation in gene expression, the consequence of which is a shift in the balance between self-renewal and differentiation toward differentiation, both directly to neurons and indirectly via basal progenitor cell genesis. Although the temporal order of neurogenesis and gliogenesis are broadly conserved under these conditions, the timing of neurogenesis, the relative numbers of different cell types, and the switch to gliogenesis are all altered, narrowing the neurogenic period for progenitor cells and reducing their neuronal output.

Mechanistically, the changes in gene expression we observed by expression analysis of the E12 Ezh2-null cortex suggest several different pathways that may be responsible for the different aspects of the altered progenitor cell behavior. Loss of PRC2 function in ES cells compromises their pluripotency and ability to differentiate, a phenotype that has been attributed to the loss of repression of expression of key developmental regulators (8, 9, 42). In the Ezh2-null cortex at E12, there is a significant up-regulation in expression of a large set of transcription factors expressed in differentiating cortical neurons, including Neurod6, Bhlhb5, Tbr1, and Foxp2 (28, 43), and at least one of these transcription factors, Foxp2, carries the repressive H3K27me3 chromatin modification on its promoter in vivo. A key question is whether the lack of repressive chromatin on the promoters of this class of transcription factors and their increased transcription biases cortical progenitor cells toward neurogenesis and away from self-renewal.

The findings reported here contrast with a recent report of the consequences of deletion of Ring1B (PRC1) and of Ezh2 in the E12 cortex during the neurogenic period (21). Deletion of Ring1B at that stage results in a phenotype opposite that reported here: The neurogenic period is extended, and gliogenesis is delayed (21). From the brief description provided in that work, it appears that a similar phenotype was observed when Ezh2 was deleted at the same developmental stage (21). We find that deletion of Ezh2 before the onset of neurogenesis results in the opposite phenotype, with accelerated neurogenesis and an early onset of gliogenesis. This difference points to a potential role for polycomb in regulating major developmental transitions in cortical progenitor cells: expanding early neuroepithelial cells to neurogenic radial glia cells; changing the competence of cortical progenitor cells to generate neurons of different laminar fates; and switching from neurogenesis to gliogenesis. Deletion of Ezh2 before the switch to neurogenesis alters the balance between self-renewal and differentiation in radial glial cells. It is possible that the altered timing of neurogenesis and accelerated onset of gliogenesis that occur are secondary to this primary function of PRC2 in cortical progenitor cells.

Although the requirement reported here for PRC2, via Ezh2, in regulating neurogenesis and differentiation appears initially to be in line with its proposed role in maintaining stem cell populations such as adult hematopoietic stem cells (18, 44), it is clear that the role of PRC2 is more complex in this cell type. The cortical progenitor cell population is not simply exhausted upon the removal of PRC2 and the disappearance of H3K27me3, because a Pax6-expressing progenitor cell population persists in the mutant cortex until after birth. Thus PRC2 not only acts to promote self-renewal but also controls fate choices within this multipotent lineage, so that in its absence neural progenitor cells alter their decision making to generate too many deep-layer neurons and basal progenitor cells early in their lineage, underproduce later upper-layer neurons, and then switch to making glial cells inappropriately early. Identifying the targets of Ezh2 responsible for these altered progenitor cell behaviors will provide critical insights into the cellular control of neural progenitor cell multipotency and fate choices.

Materials and Methods

Mice with cortex-specific deletion of Ezh2 were generated as described in *SI Materials and Methods* under local and national UK ethical and legal regulations. Details of BrdU birthdating, immunohistochemistry, confocal microscopy, microarray analysis, and ChIP studies are provided in *SI Materials and Methods*.

1. Mountcastle VB (1998) *The Cerebral Cortex* (Harvard Univ Press, Cambridge, MA.).
2. Rakic P (2007) The radial edifice of cortical architecture: From neuronal silhouettes to genetic engineering. *Brain Res Brain Res Rev* 55:204–219.
3. McConnell SK, Kaznowski CE (1991) Cell cycle dependence of laminar determination in developing neocortex. *Science* 254:282–285.
4. Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. *Cell* 128:747–762.
5. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. *Nat Rev Genet* 8:263–271.
6. Majewski IJ, et al. (2008) Polycomb repressive complex 2 (PRC2) restricts hematopoietic stem cell activity. *PLoS Biol* 6:e93.
7. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G (2007) Genome regulation by polycomb and trithorax proteins. *Cell* 128:735–745.
8. Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K (2007) The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol Cell Biol* 27: 3769–3779.
9. Boyer LA, et al. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441:349–353.
10. O'Carroll D, et al. (2001) The polycomb-group gene Ezh2 is required for early mouse development. *Mol Cell Biol* 21:4330–4336.
11. Ezhkova E, et al. (2009) Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell* 136:1122–1135.
12. Simon JA, Kingston RE (2009) Mechanisms of Polycomb gene silencing: Knowns and unknowns. *Nat Rev Mol Cell Biol* 10:697–708.
13. Cao R, Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* 14:155–164.
14. Cao R, et al. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298:1039–1043.
15. Leeb M, et al. (2010) Polycomb complexes act redundantly to repress genomic repeats and genes. *Genes Dev* 24:265–276.
16. Liu J, et al. (2009) Bmi1 regulates mitochondrial function and the DNA damage response pathway. *Nature* 459:387–392.
17. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M (1999) The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 397:164–168.
18. Pietersen AM, van Lohuizen M (2008) Stem cell regulation by polycomb repressors: Postponing commitment. *Curr Opin Cell Biol* 20:201–207.
19. Molofsky AV, et al. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 425:962–967.
20. Fasano CA, et al. (2007) shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. *Cell Stem Cell* 1:87–99.
21. Hirabayashi Y, et al. (2009) Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. *Neuron* 63:600–613.
22. Su IH, et al. (2003) Ezh2 controls B cell development through histone H3 methylation and IgH rearrangement. *Nat Immunol* 4:124–131.
23. Gorski JA, et al. (2002) Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci* 22:6309–6314.
24. Visel A, Thaller C, Eichele G (2004) GenePaint.org: An atlas of gene expression patterns in the mouse embryo. *Nucleic Acids Res* 32(Database issue):D552–D556.
25. Molyneaux BJ, et al. (2009) Novel subtype-specific genes identify distinct subpopulations of callosal projection neurons. *J Neurosci* 29:12343–12354.
26. Alcamo EA, et al. (2008) Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57:364–377.
27. Britanova O, et al. (2008) Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron* 57:378–392.
28. Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. *Nat Rev Neurosci* 8:427–437.
29. Hoerder-Suabedissen A, et al. (2009) Novel markers reveal subpopulations of subplate neurons in the murine cerebral cortex. *Cereb Cortex* 19:1738–1750.
30. Hevner RF, et al. (2001) Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* 29:353–366.
31. Ferland RJ, Cherry TJ, Preware PO, Morrisey EE, Walsh CA (2003) Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. *J Comp Neurol* 460:266–279.
32. Ku M, et al. (2008) Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genet* 4:e1000242.
33. Chen H, et al. (2009) Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev* 23:975–985.
34. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R (2005) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev* 19:1432–1437.
35. Zindy F, Quelle DE, Roussel MF, Sherr CJ (1997) Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* 15:203–211.
36. Zindy F, et al. (1997) Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development. *Cell Growth Differ* 8:1139–1150.
37. Nishino J, Kim I, Chada K, Morrison SJ (2008) Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. *Cell* 135:227–239.
38. Pontious A, Kowalczyk T, Englund C, Hevner RF (2008) Role of intermediate progenitor cells in cerebral cortex development. *Dev Neurosci* 30:24–32.
39. Shen Q, et al. (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat Neurosci* 9:743–751.
40. Qian X, et al. (2000) Timing of CNS cell generation: A programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28:69–80.
41. Hevner RF, et al. (2003) Beyond laminar fate: Toward a molecular classification of cortical projection/pyramidal neurons. *Dev Neurosci* 25:139–151.
42. Shen X, et al. (2008) EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol Cell* 32:491–502.
43. Joshi PS, et al. (2008) Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. *Neuron* 60:258–272.
44. Kamminga LM, et al. (2006) The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* 107:2170–2179.