RESEARCH ARTICLE

The polycomb component Ring1B regulates the timed termination of subcerebral projection neuron production during mouse neocortical development

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ABSTRACT

In the developing neocortex, neural precursor cells (NPCs) sequentially generate various neuronal subtypes in a defined order. Although the precise timing of the NPC fate switches is essential for determining the number of neurons of each subtype and for precisely generating the cortical layer structure, the molecular mechanisms underlying these switches are largely unknown. Here, we show that epigenetic regulation through Ring1B, an essential component of polycomb group (PcG) complex proteins, plays a key role in terminating NPC-mediated production of subcerebral projection neurons (SCPNs). The level of histone H3 residue K27 trimethylation at and Ring1B binding to the promoter of Fezf2, a fate determinant of SCPNs, increased in NPCs as Fezf2 expression decreased. Moreover, deletion of Ring1B in NPCs, but not in postmitotic neurons, prolonged the expression of Fezf2 and the generation of SCPNs that were positive for CTIP2. These results indicate that Ring1B mediates the timed termination of Fezf2 expression and thereby regulates the number of SCPNs.

KEY WORDS: Neocortical development, Fezf2, Polycomb, Neural stem cell, Neural precursor cell

INTRODUCTION

The mammalian neocortex contains various types of projection neurons, which are finely organized into a laminar structure. Projection neurons can be classified using their somal location, dendritic arborization and targets of axonal projection (Kwan et al., 2012; O’Leary and Koester, 1993). Intracortical projection neurons are located in both superficial and deep layers, whereas corticofugal projection neurons, such as subcerebral projection neurons (SCPNs), which form connections with subcerebral targets, are confined to deep cortical layers V and VI (Kwan et al., 2012; Molyneaux et al., 2007). SCPNs vary in number between cortical areas (Arlotta et al., 2005; Polleux et al., 1997), and the regulation of the number of SCPNs might be important in determining the function of some areas. For example, the primary motor area contains a relatively large number of SCPNs (such as those responsible for the pyramidal tract) that are essential for its motor-associated functions. Furthermore, the development of SCPNs is a focus of clinical interest because these neurons degenerate in response to spinal cord injury, and in some neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), they contribute to the malfunction of the motor system (Pasinelli and Brown, 2006).

The developing mouse neocortex contains multipotent neural precursor cells (NPCs) (McConnell and Kaznowski, 1991; Noctor et al., 2001; Guo et al., 2013; Franco et al., 2012), and these NPCs collectively produce various neuronal subtypes and then glial cells in a defined temporal order (Qian et al., 2000; Shen et al., 2006). The length of time for which NPCs produce each cell type is crucial for determining the cell composition (Polleux et al., 1997) – e.g. delayed termination of the production of a certain cell type would increase the number of cells of this type. Because newborn neurons migrate radially toward the pia and settle outside of earlier born neurons, the temporal order of the generation of neuronal subtypes from NPCs roughly corresponds to their spatial order within the cortical plate (Ayala et al., 2007; Kohwi and Doe, 2013; Kriegstein and Noctor, 2004). Deep cortical layers, including SCPNs, are thus generated early in neocortical development [from 12.5 to 14 days post coitum (dpc)] (Hevner et al., 2003; Molyneaux et al., 2007). Extrinsic signals can affect the fate transitions of NPCs, given that early-stage NPCs adopt a late-stage fate when grafted into a late-stage neocortex (McConnell and Kaznowski, 1991). Interestingly, late-stage NPC do not regain an early-stage fate when grafted into an early-stage neocortex, indicating a lack of the differentiation competence during the course of development [see the ‘progressive fate restriction’ model (Desai and McConnell, 2000)]. Together with previous reports showing that microcultures of neocortical NPCs exhibit the sequential fate switches in a similar order to those observed in vivo (Shen et al., 2006), these studies indicate that cell intrinsic mechanisms in NPCs, at least in part, govern the fate switches and maintain the limited competence of NPCs.

Although some of the molecules responsible for the generation of particular neuronal subtypes by NPCs have been identified, such as Foxg1 and COUP-TF (Hanashima et al., 2004; Naka et al., 2008), little is known about how the timing of the expression of these molecules is regulated to govern the fate switches during development. As for the specification of SCPNs, the transcription factor Fez family zinc-finger 2 (Fezf2) has been identified as a key player (Chen et al., 2005a,b; Molyneaux et al., 2005). The expression of Fezf2 is induced in early-stage NPCs when they produce SCPNs, and ablation of Fezf2 results in the loss of all SCPNs. When Fezf2 is misexpressed in late-stage NPCs or immature neurons that would normally generate superficial layer neurons, they ectopically produce neurons with molecular markers and axonal projections characteristic of SCPNs (Chen et al., 2005a,b; De la...
NPCs increases as Fezf2 expression is reduced. In mouse neocortical NPCs lacking Ring1B, Fezf2 expression was induced even in the late stage, resulting in the prolonged production of SCPNs. Our findings reveal that the timed termination of SCPN fate by PcG proteins is crucial for determining the number of neurons of this subtype.

RESULTS
Deletion of Ring1B in NPCs increases the number of neurons that are CTIP2++ in the neocortex
The role of PcG complexes in the neurogenic-to-gliogenic transition of NPC fate in the perinatal neocortex prompted us to investigate their role in the neuronal subtype regulation of NPC fate during the neurogenic phase in the late embryonic neocortex. We thus conditionally deleted the Ring1B gene, a central component of PRC1, in NPCs in vivo by crossing Ring1B<sup>fl/fl</sup> mice with mice harboring a Nestin-CreERT2 transgene (NesCreERT2), which expresses the CreERT2 gene under the control of the NPC-specific Nestin enhancer (Imayoshi et al., 2006). We confirmed that a single intraperitoneal injection of tamoxifen into a pregnant mouse at 13 dpc induced genomic recombination of the Ring1B gene and reduced the amount of Ring1B protein to an undetectable level in neurons in layers II to V, which are generated after 13 dpc (Fig. 1A,B). At birth, Ring1B<sup>fl/fl</sup>;NesCreERT2 mice that had been injected with tamoxifen at 13 dpc exhibited six-layered cortices similar in size to control cortices. We first examined the composition of neuronal subtypes within the primary somatosensory (S1) area through immunohistochemistry using mice at 18.5 dpc or postnatal day 2 (P2). Ring1B deletion by using tamoxifen injection at 13 dpc did not markedly change the number of layer VI neurons that were positive for Tbr1, or that of neurons that were weakly positive for CTIP2 in the S1 area reproducibly exhibited more than a 30% increase in Ring1B-deficient mice compared with that of control mice.

![Image of Fig. 1. Deletion of Ring1B in NPCs increases the number of CTIP2++ cells in the neocortex and decreases the number of Cux1-positive cells. Control (A,C,E) or Ring1B<sup>fl/fl</sup>;NesCreERT2 (B,D,F) mice were treated with tamoxifen at 13.0 dpc. The pups’ brains were fixed at P2 (A,B,E,F) or 18.5 dpc (C,D) and subjected to immunohistochemistry with the antibodies indicated. The number of CTIP2++ cells or Cux1-positive (Cux1+) cells were determined. The S1 area is shown. Data are the means±s.e.m. of values of six corresponding areas of three control mice and eight corresponding areas of four knockout (KO) mice (G) and of eight corresponding areas of four hemispheres and 12 corresponding areas of six hemispheres (H). See also supplementary material Fig. S1. **P<0.01. CP, cortical plate; Hoechst, Hoechst 33342; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 50 μm (A), 100 μm (C,E).]
of superficial layer neurons that expressed Cux1 by 21% in P2 neocortex (Fig. 1E,F,H), and none of the increased number of CTIP2++ neurons in Ring1B knockout mice expressed Cux1 (supplementary material Fig. S1E-I). We further investigated whether Ring1B knockout affects the number of layer V callosal projection neurons (CPNs) that are produced during the same period as layer V SCPNs. We thus examined the number of cells that were positive for Satb2, a CPN marker (Alcami et al., 2008; Britanova et al., 2008; Leone et al., 2008), and located in layer V. We found that deletion of Ring1B did not significantly change the number of Satb2-positive cells located in layer V at stage E18 or P2, when Satb2-positive cells settle in layer V (supplementary material Figs S1C,D and S2A,D), suggesting that Ring1B does not largely affect the production of CPNs in layer V. These results indicate that Ring1B plays a role in controlling the composition of neuronal subtypes in the developing neocortex by reducing the number of CTIP2++ neurons.

A Ring1B-dependent developmental fate switch takes place in NPCs but not in postmitotic neurons

Previous studies have indicated that the subtype specification of neurons during neocortical development can take place in both NPCs and postmitotic neurons (Bedogni et al., 2010; Fishell and Hanashima, 2008; Han et al., 2011; Joshi et al., 2008; Leone et al., 2008). Some of the molecules involved in neuronal subtype specification are expressed primarily in the postmitotic neurons, supporting that neuronal subtype determination occurs at a postmitotic stage. Moreover, a recent study has shown that postmitotic misexpression of Fez2, a fate determinant of layer V SCPNs, resulted in the reprogramming of upper layer neurons into SCPNs, suggesting that neuronal phenotypes can be reprogrammed even in postmitotic immature neurons (De la Rossa et al., 2010; Rouaux and Arlotta, 2013). However, it remains unclear whether the SCPN fate is normally determined within NPCs or neurons during neocortical development. We therefore asked whether the increased number of CTIP2++ neurons in Ring1B-deficient mice was due to the function of Ring1B in NPCs or that in neurons. To examine this, we deleted Ring1B in the neocortex by crossing Ring1Bflox/flox mice with NEX-Cre mice, which express Cre recombinase in differentiating neurons under the control of the Math2 (Neurod6 – Mouse Genome Informatics) promoter (Goebbels et al., 2006; Wu et al., 2005). In this mouse line (Ring1Bflox/flox;NEX-Cre), the amount of Ring1B protein compared with that in controls was substantially lower in postmitotic neurons but not in the ventricular zone (VZ), where NPCs reside (supplementary material Fig. S3A,B). However, we found that the numbers of CTIP2++ neurons and Cux1-positive neurons per unit width (per column) in Ring1Bflox/flox;NEX-Cre mice at P0 and P2 were similar to those in control mice (supplementary material Fig. S3C-K). Therefore, conditional deletion of Ring1B specifically in postmitotic neurons is not sufficient to increase the number of CTIP2++ neurons and decrease that of Cux1-positive neurons. This suggests that the increased number of CTIP2++ neurons in Ring1B-deficient mice resulted from the action of Ring1B in undifferentiated NPCs rather than in postmitotic neurons.

Deletion of Ring1B does not promote premature cell cycle exit and differentiation of NPCs

The increase in the number of CTIP2++ neurons in Ring1B-deficient mice could be explained by several possibilities, including the extended period of CTIP2++ production and the premature differentiation of NPCs during the period of CTIP2++ neuron production. Premature differentiation could also result in the exhaustion of NPCs and the reduction of late-born neurons, including Cux1-positive neurons. Indeed, a previous report has shown that an earlier (around 9.5 dpc) deletion of Ezh2 in NPCs or knockdown of another PRC1 component, Bmi1 (at around 11 dpc), suppresses the maintenance of NPCs (Fasano et al., 2007, 2009; Pereira et al., 2010). However, we found that Ring1B deletion at the later stages examined in this study (Ring1Bflox/flox;NesCreERT2 mice injected with tamoxifen at 13.0-13.5 dpc) did not reduce the number of Pax6-positive undifferentiated NPCs in the VZ at 15.5 dpc or P2 (Fig. 2A-D,K; supplementary material Fig. S4A-C), nor did it increase the number of cells that were positive for βIII tubulin (Tubb3 – Mouse Genome Informatics) (Fig. 2I,J), suggesting that Ring1B is not necessary for the maintenance of undifferentiated NPCs at this stage. We also asked whether Ring1B deletion affects the rate of cell cycle exit of NPCs. In this assay, 5-ethyl-2′-deoxyuridine (EdU) was injected at 14.5 dpc, and the rate of cell cycle exit during a 24 h period, judged by the fraction of cells that were negative for Ki67 (Mki67 – Mouse Genome Informatics) among EdU-positive cells, was examined at 15.5 dpc. We found that Ring1B deletion did not significantly affect the rate of cell cycle exit of proliferating cells within the VZ (Fig. 2N-P). Moreover, we found that Ring1B deletion did not affect the number of the cells that were positive for the M-phase marker phospho-histone H3 (pH3) or the intermediate progenitor marker Tbr2 (Fig. 2E-H,L,M). These results together suggest that Ring1B deletion at this late developmental stage does not substantially suppress the self-renewal of NPCs nor promote the neuronal differentiation that accompanies the cell cycle exit. Therefore, the increase in the number of CTIP2++ neurons in the Ring1B-deleted brain observed in this study does not appear to be due to the premature differentiation of NPCs.

Ring1B is necessary for the correct timing of the termination of CTIP2++ neuron production

Because we assumed that Ring1B regulates the timing of the restriction of the SCPN fate of NPCs, we performed a 5-bromo-2′-deoxyuridine (BrdU) birthdate analysis to determine whether Ring1B deletion extends the period of CTIP2++ neuron production. The production of CTIP2++ neurons in the S1 area normally ceases by 14 dpc (Hevner et al., 2003; Molyneaux et al., 2007), so when BrdU was injected at 14 dpc, few BrdU-labeled cells were found among the CTIP2++ neurons in the S1 area (Fig. 3B). However, when tamoxifen was injected intraperitoneally into pregnant Ring1Bflox/flox;NesCreERT2 mice at 13.0 dpc to delete the Ring1B gene and BrdU was injected at 14.0 dpc, the percentage of neurons that were double-positive for BrdU and CTIP2++ at 18.5 dpc was significantly greater in these mice than that in the control siblings (Fig. 3B-F). This result suggests that CTIP2++ cells were produced even at 14 dpc in Ring1B-deficient mice, indicating that Ring1B deletion extended the period of CTIP2++ neuron production. These results thus indicate that Ring1B plays a role in terminating CTIP2++ neuron production at the correct developmental time.

Excessively produced CTIP2++ neurons project normally into the pons in Ring1B-deficient mice

Although CTIP2 is a marker for SCPNs (Arlotta et al., 2005), the extended expression of CTIP2 through Ring1B deletion might not necessarily cause an extended production phase of SCPNs. We therefore examined whether excessively produced CTIP2++ neurons in Ring1B-deleted mice indeed project into the subcerebral areas.
terminating the production of CTIP2++ SCPNs in layer V. Ring1B restricts the expression of Fezf2 at the time of terminating the production of CTIP2++ neurons

Our results suggest that Ring1B mediates the termination of CTIP2++ neuron production in NPCs rather than in postmitotic neurons. If this is the case, Ring1B should regulate its target gene(s) expressed within the VZ and subventricular zone (SVZ) (Guo et al., 2011) (Fig. 3H-O). Importantly, substantial parts of the neurons produced in the ‘extended period’ of CTIP2++ neuron production in Ring1B-deleted mice, marked by EdU that had been injected at 14.0 dpc, were also labeled in a retrograde manner with CTB555 (Fig. 3I). The percentage of CTB555-labeled cells among Edu-positive CTIP2++ neurons in the Ring1B-deficient mice was similar to that among EdU-negative CTIP2++ neurons (Fig. 3I), suggesting that the CTIP2++ neurons produced during the ‘extended period’ in the mutant mice project into the pons normally. This result strongly supports the notion that Ring1B plays an essential role in terminating the production of CTIP2++ SCPNs in layer V.

Ring1B restricts the expression of Fezf2 at the time of terminating the production of CTIP2++ neurons

Our results suggest that Ring1B mediates the termination of CTIP2++ neuron production in NPCs rather than in postmitotic neurons. If this is the case, Ring1B should regulate its target gene(s) expressed within the VZ and subventricular zone (SVZ) (Guo et al., 2011; Hirata et al., 2004), we tested whether Ring1B and/or other PcG proteins mediate the suppression of Fezf2 at the time of terminating SCPN production in vivo. We directly isolated undifferentiated NPCs (CD133[high], Pax6[high], CD133 is also known as Prom1 – Mouse Genome Informatics) and differentiating NPCs (CD133[med], Pax6[med]) from the neocortex by using fluorescence-activated cell sorting (FACS) and CD133 immunoreactivity and found that Fezf2 expression in the CD133[med] population was higher than that in CD133[high] cells at 14 dpc and reduced at 16 dpc (Fig. 4A), consistent with the time of its reported reduction (Hirata et al., 2004). We then deleted Ring1B in these NPCs by introducing tamoxifen at 13 dpc into Ring1Bflox/flox;NesCreERT2 mice (Fig. 4B,C) and found that this deletion significantly increased the level of Fezf2 mRNA in CD133[med] NPCs (Fig. 4D). We then asked whether the Fezf2 locus is a direct target of Ring1B proteins in NPCs. Because the recruitment of the Ring1B-containing complex (PRC1) to its target genomic loci is largely dependent upon the preceding deposition of the histone modification H3K27me3, we examined whether the levels of H3K27me3 and Ring1B increase at the Fezf2 locus at the end of SCPN production, i.e. between 14 and 16 dpc. We found that the levels of both H3K27me3 and Ring1B at the Fezf2 locus in CD133[high] NPCs were greater at 16 dpc than at 14 dpc, whereas those at other control regions, such as the Gapdh locus did not significantly change (Fig. 4E,F). The increased levels of H3K27me3 and Ring1B at the Fezf2 locus without apparent expression of its mRNA in CD133[high] NPCs suggest that Ring1B proteins have already restricted the transcriptional competence (permissiveness) of the Fezf2 locus in undifferentiated CD133[high] NPCs at 16 dpc so that the induction of Fezf2 expression is suppressed in differentiating cells (supplementary material Fig. S8). The level of Ring1B mRNA did not increase in CD133[high] NPCs between 14 and 16 dpc (supplementary material Fig. S6), suggesting that the accumulation of Ring1B at the Fezf2 promoter was not due to
the increase of Ring1B transcription but was due to the preceding deposition of H3K27me3.

Because dissociation of neocortical NPCs and their isolation by using FACS might affect the chromatin state and gene expression patterns, we also analyzed the cells in the neocortical VZ and SVZ region immediately after manual dissection without the dissociation process. We also confirmed that the expression of Fezf2 in the VZ and SVZ was reduced (data not shown) and that the level of H3K27me3 was increased at the Fezf2 locus between 14 and 16 dpc (supplementary material Fig. S7). These results are consistent with those obtained using FACS-isolated NPCs.

**Developmental stage-dependent and Ring1B-mediated repression of the Fezf2 locus in neocortical NPCs**

Because previous reports have suggested that the developmental fate switch of NPCs largely depends on cell-intrinsic programs, we wished to examine whether Ring1B also mediates developmental stage-dependent fate switch in isolated NPCs in vitro (Eiraku et al., 2008; Gaspard et al., 2008; Shen et al., 2006). NPCs were isolated by culturing neocortical cells from mouse embryos at 11 dpc [0 days in vitro (DIV)] in suspension with fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) for 3 or 6 DIV (referred to as 3- and 6-DIV cultures, respectively). The fraction of CTIP2-positive neurons that was induced by an additional 5 days of FGF2 deprivation was reduced between 3 DIV and 6 DIV cultures (Fig. 5A), suggesting that the production of deep layer neurons and SCPNs is terminated between these time points. We found that the levels of H3K27me3 and Ring1B at the Fezf2 locus were increased in the 6-DIV culture compared with the 3-DIV culture (Fig. 5B,C) and that the expression of Fezf2 mRNA after 2 h of induction (FGF2 deprivation) was reduced between 3 and 6 DIV (Fig. 5D). Importantly, when Ring1B was conditionally deleted in NPCs isolated from Ring1Bflox/flox;NesCreERT2 Cre mice by treating them with 4-hydroxy-tamoxifen (4-OHT) for 3 days (Fig. 6A), the level of Fezf2 mRNA in 6-DIV cultures that had been treated with 4-OHT was significantly higher than that of control cultures that had not been treated with 4-OHT (Fig. 6B), although the treatment with 4-OHT did not change the levels of Pax6 and βIII-tubulin mRNA or the proliferation rate (Fig. 6C-E). Consistent with the derepression of Fezf2 mRNA, the fraction of CTIP2-positive neurons that was induced upon neuronal differentiation was also greater when Ring1B was conditionally deleted in the 6-DIV culture (Fig. 6F,G). These results support the idea that the Fezf2 locus is directly repressed by Ring1B proteins within NPCs in a developmental time-dependent manner, even when cultured in isolation.
Fezf2 expression mediates the increase in CTIP2-positive neurons upon Ring1B deletion

Finally, we asked whether the increased expression of Fezf2 accounts for the increased production of CTIP2-positive neurons upon Ring1B deletion. We thus performed knockdown of Fezf2 with two independent siRNA oligonucleotide pairs (siFezf2 #1, siFezf2 #2) in cells isolated at 14 dpc and then cultured for 3 DIV. The increase in the percentage of CTIP2-positive neurons upon Ring1B deletion in cells that had been transfected with the control siRNA (siControl) was partially suppressed upon Fezf2 knockdown (Fig. 6H,I). This result suggests that the suppression of Fezf2 is responsible for the termination of CTIP2-positive neuron production through Ring1B, at least in part, although we cannot rule out that other factor(s) regulated by Ring1B are also involved in the fate specification of SCPNs.

PRC2 component Ezh2 also represses Fezf2 expression and CTIP2-positive neuron production

Recent reports (Gao et al., 2012; Leeb et al., 2010; Luis et al., 2012; Schoeftner et al., 2006; Tavares et al., 2012) have shown that Ring1B plays PRC1-independent roles. Therefore, we examined whether the deletion of the Ezh2 gene, which is a component of PRC2 and is required for the trimethylation of H3K27 in neocortical NPCs (Hirabayashi et al., 2009), affects the expression of Fezf2 and production of CTIP2-positive neurons in vitro, although we could not analyze the effect of Ezh2 deletion on the timed fate switch of NPCs in vivo owing to the premature differentiation of NPCs (Pereira et al., 2010). We isolated neocortical NPCs from Ezh2ΔSETfloxed;NesCreERT2-Cre mice at 12.5 dpc. Treating these NPCs with 4-OHT effectively reduced the amount of intact Ezh2 mRNA (Fig. 7A). The level of Fezf2 mRNA and the ratio of CTIP2-positive neurons among total neurons in 6-DIV cultures, isolated at 12.5 dpc, that had been treated with 4-OHT were higher than those of controls (Fig. 7B,D,E). Because the incorporation of BrdU and the ratio of neuronal differentiation in 6-DIV cultures did not significantly change (Fig. 7C,F), these results suggest that deletion of the SET domain of Ezh2 derepresses Fezf2 expression but does not result in premature neuronal differentiation under this condition. These results suggest that Ezh2 is required for the suppression of Fezf2 in late-stage NPCs.

DISCUSSION

Neocortical NPCs sequentially generate various neuronal subtypes in a defined order within a limited period during development (Molyneaux et al., 2007). Several molecules involved in the subtype specification have been identified, but the mechanisms underlying the NPC transitions from producing one subtype to another have remained largely unclear. Here, we report that the PcG component Ring1B plays an essential role in terminating SCPN production at the correct time during neocortical development. Our results suggest that the termination of SCPN production is mediated by Ring1B-dependent suppression of Fezf2, a molecular determinant of SCPN

Fig. 4. Ring1B restricted Fezf2 mRNA expression at the late stage of the neurogenic phase, and H3K27me3 and Ring1B accumulate at the Fezf2 promoter concurrently with the termination of Fezf2 mRNA expression. (A) Cells isolated from mouse neocortices at 14 dpc and 16 dpc were stained with an antibody against CD133 and CD133high and CD133mid cells were obtained using FACS (see also supplementary material Fig. S5A,B). The amount of Fezf2 mRNA was measured by using RT-qPCR. Data are means+s.d. of values from four experiments. (B-D) CD133high and CD133mid cells were obtained from cells isolated from control embryos at 17 dpc and Ring1Bflox/flox;NesCreER (Ring1B KO) embryos treated with tamoxifen at 13.0 dpc in the same manner as shown in Fig. 5A (supplementary material Fig. S5C-E). The amount of Ring1B (B), Pax6 (C) and Fezf2 (D) mRNA was measured by using RT-qPCR. Data are means+s.e.m. of values from four control embryos and four KO embryos. (E,F) The chromatin complex was immunoprecipitated, using antibodies against Ring1B and H3K27me3, from CD133high NPCs obtained from each developmental stage using FACS (supplementary material Fig. S5A,B). The amount of H3K27me3 and Ring1B at the Fezf2 promoter and Gapdh promoter was assessed by using qPCR. See also supplementary material Fig. S7. Data are means+s.e.m. of eight experiments (E) and three experiments (F). In F, a one-tailed Student’s t-test was used. *P<0.05 for comparison between CD133mid and CD133high, **P<0.01 for comparison between 14 dpc and 16 dpc.
The different functions of PcG proteins at different developmental stages illustrated above might lend support to the previously proposed ‘progressive fate restriction’ model. Interestingly, a recent report (Touma et al., 2012) has also shown a similar role of PcG proteins in Drosophila development. Therefore, PcG proteins might commonly regulate the developmental fate restriction of competence in neural (tissue) stem cells in various organisms and developmental contexts.

Besides SCPNs, layer V contains CPNs (Leone et al., 2008; Molyneaux et al., 2007). We found that the numbers of cells expressing Satb2, a fate determinant of CPNs (Alcamo et al., 2008; Britanova et al., 2008), and their distribution within the cortical plate were roughly the same between control and Ring1B-deleted neocortices (supplementary material Fig. S2A). Also, Ring1B deletion did not increase the expression of lipoprotein lipase (Lpl), another CPN marker in layer V (data not shown). These results indicate that PcG proteins do not terminate layer V CPN production, which seems to make sense because CPNs continue to be produced in layers IV and II and/or III. More markers to distinguish layer-specific CPN are necessary to understand how PcG complexes affect the number of CPN subtypes at each layer.

Although a previous report has shown that the ablation of the Ezh2 gene compromised the maintenance of undifferentiated NPCs and induced premature differentiation of CTIP2-positive neurons (Pereira et al., 2010; Testa, 2011), Ring1B deletion in the developing neocortex did not cause overt premature differentiation and preserved the undifferentiated Pax6-positive population (supplementary material Fig. S4D-I). This difference might be due to a redundant function of Ring1A (Endoh et al., 2008). We therefore assume that Ring1B is dispensable for the function of Ezh2 in maintaining NPCs.
but is indispensable for its function in promoting the developmental changes of NPC fate.

An issue that has been questioned with regard to the subtype specification of neocortical neurons is whether these subtypes are specified within NPCs or at postmitotic stages. Microculture (Shen et al., 2006) and heterochronic transplant experiments (McConnell and Kaznowski, 1991) have suggested that neocortical neuronal subtypes are, at least in part, determined within NPCs in the VZ (independent of extracellular signaling). Our findings presented here are consistent with this notion, as Ring1B complexes modulate the SCPN identity within NPCs: H3K27me3 and Ring1B accumulated at the Fezf2 locus in a developmental stage-dependent manner in NPCs, and Ring1B deletion derepressed the Fezf2 locus in the late-stage NPCs. Importantly, Ring1B deletion using the Nestin-Cre driver in NPCs prolonged the production of CTIP2+++ neurons, but deletion in postmitotic neurons, using the NEX-Cre driver, did not increase the number of CTIP2+++ neurons. Therefore, Ring1B does not seem to play a role in this subtype specification in postmitotic neurons. Although our results support a model in which NPC-intrinsic mechanisms are important for subtype specification, it is possible that further

Fig. 6. Knockout of the Ring1B gene increases the amount of Fezf2 mRNA and the percentage of CTIP2-positive neurons in vitro. (A–D) Ring1B is necessary for the repression of Fezf2 expression in vitro. NPCs isolated from Ring1Bflox/flox;ERT2-Cre mice at 12.5 dpc were cultured in suspension for 3 days. Then cells were cultured for 3 days with or without 4-hydroxytamoxifen (4-OHT). The amount of the mRNA indicated was measured by using RT-qPCR. Data are means+s.d. of values from five experiments. (E) Knockout (KO) of the Ring1B gene did not affect cell proliferation in vitro. NPCs isolated from Ring1Bflox/flox;ERT2-Cre mice at 12.5 dpc were treated as in A. Cells were then treated with BrdU for 30 min, and the percentage of BrdU-positive (BrdU+) cells was determined. Data are the means+s.e.m. of values from three samples. (F,G) Ring1B regulates the production of CTIP2+++ neurons in vitro. NPCs isolated from Ring1Bflox/flox;ERT2-Cre mice at 12.5 dpc were treated as in A and cultured for 5 days without FGF2 or EGF. The percentage of CTIP2-positive (CTIP2+) cells (F) and CTIP2-positive cells among βIII-tubulin-positive (βIII-tubulin+) cells (G) were determined. Data are means+s.e.m. of values from six samples. *P<0.05, **P<0.01. (H,I) Fezf2 derepression in Ring1B KO cells is responsible for the increase in the percentage of CTIP2-positive cells in vitro. NPCs isolated from 14.5 dpc Ring1Bflox/flox (control) or Ring1Bflox/flox;ERT2-Cre (Ring1B KO) mice were cultured in suspension for 3 days with 4-OHT and transfected with control siRNA oligonucleotide and two siRNA oligonucleotides against Fezf2 (siFezf2 #1 and siFezf2 #2). Cells were then cultured for an additional 3 days without FGF2 or EGF. The level of Fezf2 mRNA was determined (H). The percentage of CTIP2-positive neurons among βIII-tubulin-positive cells was determined (I). Data are means+s.e.m. of values from 12 samples. **P<0.01 (control versus Ring1B KO mice), *P<0.05, **P<0.01 (control siRNA versus Fezf2 siRNA oligo), n.s., not significant.
specification occurs at postmitotic stages, perhaps through extrinsic signaling. Previous studies have indicated that the laminar and areal identities of some neocortical neuronal subtypes, including those determining axonal and/or dendritic patterns, are also affected after neuronal differentiation through interactions with various cues in the cortical plate. Indeed, some transcription factors (e.g. SOX5, Tbr1 and Bhlhb5) that are expressed only in postmitotic neurons have been shown to modulate the laminar and areal identities of neuronal subtypes (Bedogni et al., 2010; Fishell and Hanashima, 2008; Han et al., 2011; Joshi et al., 2008; Lai et al., 2008). It is therefore plausible that neuronal subtype identities are specified at multiple steps before and after exiting the cell cycle.

MATERIALS AND METHODS

Animals

A Ring1Bflox/fox mouse (Cales et al., 2008; Endoh et al., 2008) was crossed with either a ROSA26::ERT2-Cre mouse (ERT2-Cre driven by the endogenous ROSA26 promoter), a NesCreERT2 mouse (ERT2-Cre driven by the Nestin enhancer) (Imayoshi et al., 2006), a NEX-Cre mouse (Goebels et al., 2006) or an Emx1-CreERT2 mouse (Kessaris et al., 2006). An Ezh2ΔSETflox/fox mouse (Hirabayashi et al., 2009) was crossed with a ROSA26::ERT2-Cre mouse.

Tamoxifen (Sigma) was dissolved in sunflower oil (Nacalai) at a concentration of 10 mg/ml. To induce ERT2-Cre activity, pregnant mice were injected intraperitoneally with 150 µl of tamoxifen solution. All mice were maintained according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

FACS

Cell suspensions were obtained from dissociated neocortices and stained with a Phycocerythrin (PE)-conjugated antibody against CD133 (BioLegend). Cell sorting was performed using a FACS Aria cell sorter (Becton Dickinson). Analysis was performed using the FACS Canto flow cytometer (Becton Dickinson).

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described (Hirabayashi et al., 2004). Briefly, brains were fixed for 2 hours in 4% PFA in PBS, incubated overnight at 4°C with 30% (w/v) sucrose in PBS, embedded in OCT compound (Sakura Finetek) and cut with a cryostat to yield 10 or 12 µm-thick sections. Sections were blocked in 0.1% Triton X-100 and 2% donkey serum in Tris-buffered saline for 1 hour at room temperature. Primary antibodies diluted in blocking solution were added before an overnight incubation at 4°C. Then, sections were incubated with secondary antibodies diluted in blocking solution for 40 minutes at room temperature, and mounted in Mowiol (Calbiochem). Images were analyzed using a laser scanning confocal microscope (LSM510, Carl Zeiss; TSC-SP5, Leica) and processed with Photoshop CS software (Adobe). For the primary antibodies and dilutions, see supplementary materials.

EdU or BrdU birthdating

BrdU (100 mg/kg; Sigma) or EdU (50 mg/kg; Invitrogen) was injected into pregnant mice at 14.5 dpc (1 dpc was defined as 12 h after detection of the vaginal plug). Pups were collected at 18 dpc or P2, and BrdU- and EdU-positive cells were determined by immunohistochemistry. EdU was detected using Clik-it kit (Invitrogen).

Reverse transcription quantitative PCR

For reverse transcription quantitative PCR (RT-qPCR), total RNA was obtained from NPCs using RNAiso (Takara) following the instructions of the manufacturer. Reverse transcription (RT) was performed using 2-5 µg of total RNA, oligo(dT)12-18 (Invitrogen) primers and ReverTra Ace (TOYOBO). The resulting cDNA was subjected to real-time PCR in a Roche LightCycler with SYBR Premix Ex Taq (Takara; Roche). The primers used are given in the supplementary materials.

Chromatin immunoprecipitation assay

For H3K27me3 chromatin immunoprecipitation (ChIP), cells were first fixed in 0.5% PFA, placed in a lysis buffer, and then sonicated to shear genomic chromatin into DNA fragments. The lysate was incubated with
Dynabeads Protein A (Invitrogen), after which the beads were removed and the lysate was incubated with antibody and protein A beads. The beads were then isolated and washed with Wash buffer and with TE buffer. After elution of immune complexes, the proteins were then eliminated by digestion with proteinase K.

For Ring1B ChIP, cells were first fixed in 1% formaldehyde and then placed in a swelling buffer. After removing the swelling buffer, they were suspended in RIPA buffer and sonicated to shear genomic chromatin into DNA fragments. The lysate was incubated with Dynabeads Protein A (Invitrogen), after which the beads were removed and the lysate was incubated with antibody and protein A beads. The beads were then isolated and rinsed with RIPA buffer, then washed with RIPA buffer, with RIPA + 500 mM NaCl buffer, with LiCl wash buffer and with TE buffer. After elution of immune complexes, the proteins were eliminated by digestion with proteinase K.

The DNA was then extracted with PCI (phenol/chlorophorm/isooamylalcohol) and EtOH, after which it was rinsed with 70% EtOH and suspended in water. The eluted DNA was subjected to real-time PCR in a Roche Light-cycler using Thunderbird qPCR mix (TOYOBO). Details of the antibodies, primers and buffer compositions are given in the supplementary material methods.

**Retrograde labeling**
SCPNs were labeled in a retrograde manner at P1 by injection of CTB555 (5 mg/ml in PBS, Invitrogen) into the pons-midbrain junction of control (Ring1Blox/llox) or Ring1Blox/llox, NesCreERT2 pups. At 1.5 days after the injection, perfusion was performed.

**Primary NPC culture**
Primary NPCs were prepared from the dorsal cerebral cortex of ICR mouse embryos at 11 dpc or Ring1Blox/llox,ROSACreERT mouse embryos as previously described (Hirabayashi et al., 2009).

**BrdU proliferation assay**
Cells were labeled with BrdU (20 μg/ml) for 30 min. BrdU-positive cells were quantified using immunocytochemistry.

**Immunocytochemistry**
Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in 3% BSA in PBS for 30 min, incubated with primary antibodies diluted in 3% BSA in PBS overnight at 4°C and then with secondary antibodies for 40 min at room temperature and mounted in Mowiol (Calbiochem). For the primary antibodies and dilutions, see supplementary materials.

**siRNA**
siRNAs were introduced into 3-DIV NPCs collected from Ring1Blox/llox, ROSACreERT mice at 14 dpc by using Amaxa (Lonza). For the siRNA previously described (Hirabayashi et al., 2009).

**Statistical analysis**
All data are representative of results obtained from at least three independent experiments. Statistical significance was determined by two-tailed Student’s t-test, unless otherwise indicated.

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**Competing interests**
The authors declare no competing financial interests.

**Author contributions**
N.M. designed and performed experiments and data analysis and prepared the manuscript with Y.H. and Y.G. Y.H. conceived and supervised the entire project with Y.G. K.T. performed experiments and edited the manuscript. J.S. and H.K. generated the Ezh2 dSET mice. M.V. and H.K generated the Ring1Blox/llox mice.

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**Supplementary material**
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.112276/-/DC1

**References**


