Lack of CUL4B leads to increased abundance of GFAP-positive cells that is mediated by PTGDS in mouse brain

Wei Zhao¹,†, Baichun Jiang¹,†, Huili Hu¹, Shuqian Zhang¹, Shuaishuai Lv¹, Jupeng Yuan¹, Yanyan Qian¹, Yongxin Zou¹, Xi Li¹, Hong Jiang², Fang Liu³, Changshun Shao¹,* and Yaoqin Gong¹,*

¹The Key Laboratory of Experimental Teratology, Ministry of Education and Institute of Molecular Medicine and Genetics, Shandong University School of Medicine, Jinan, Shandong 250012, China, ²Institute of Medical Psychology, Shandong University School of Medicine, Jinan, Shandong 250012, China and ³Department of Neuroscience, Centre for Addiction and Mental Health, Toronto, Ontario, Canada M5T 1R8

*To whom correspondence should be addressed at: The Key Laboratory of Experimental Teratology, Ministry of Education and Institute of Molecular Medicine and Genetics, Shandong University School of Medicine, 44 West Wenhua Road, Jinan, Shandong 250012, China. Tel: +0118653188380859; Fax: +0118653188382115; Email: yxg8@sdu.edu.cn (Y.G.); shaochangshun@sdu.edu.cn (C.S.)

Abstract

Astrocytes are the most abundant cell type in the mammalian brain and are important for the functions of the central nervous system. Glial fibrillary acidic protein (GFAP) is regarded as a hallmark of mature astrocytes, though some GFAP-positive cells may act as neural stem cells. Missense heterozygous mutations in GFAP cause Alexander disease that manifests leukodystrophy and intellectual disability. Here, we show that CUL4B, a scaffold protein that assembles E3 ubiquitin ligase, represses the expression of GFAP in neural progenitor cells (NPCs) during brain development. Lack of Cul4b in NPCs in cultures led to increased generation of astrocytes, marked by GFAP and S100β. The GFAP+ cells were also found to be more abundant in the brains of nervous system-specific Cul4b knockout mice in vivo. Moreover, we demonstrated that the increased generation of GFAP+ cells from Cul4b-null NPCs was mediated by an upregulation of prostaglandin D2 synthase PTGDS. We showed that the increased GFAP expression can be attenuated by pharmacological inhibition of the PTGDS enzymatic activity or by shRNA-mediated knockdown of Ptgsd. Importantly, exogenously added PTGDS could promote the generation of GFAP+ cells from wild-type NPCs. We further observed that Ptgsd is targeted and repressed by the CUL4B/PRC2 complex. Together, our results demonstrate CUL4B as a negative regulator of GFAP expression during neural development.

Introduction

Astrocytes are the most abundant cell type in the mammalian brain (1). As integral components of nervous system, astrocytes provide structural, metabolic and trophic support for neurons, contribute to the formation of the blood–brain barrier, recycle neurotransmitters and regulate synapse formation, maintenance and elimination (2–4). Mature astrocytes are usually identified by the expression of GFAP, an intermediate filament, though some GFAP-positive cells may also act as neural stem cells or progenitors (5,6). While Gfap knockout mice were reported to develop normally (7,8), heterozygous missense mutations (gain of function mutations) in GFAP can cause Alexander disease, a neurological disorder, in human (9–11). GFAP tends to be upregulated with aging and in response to oxidative stress (12). Transgenic mice engineered to overexpress human GFAP also exhibit stress response (13). However, the molecular mechanisms that regulate GFAP expression remain largely unclear.
CUL4B is a member of the cullin family, which are components of Cullin-RING ligase (CRL) complexes, the largest known class of ubiquitin ligases. The CRL complexes formed by CUL4B (CRL4B) recognize and target various substrates for proteosomal degradation (14–21), thereby regulating a broad variety of physiologically and developmentally controlled processes (22). Recently, we showed that CRL4 complex can also catalyze H2AK119 monoubiquitination and, by coordinating with PRC2 complex, promote H3K27 trimethylation, leading to the transcriptional repression of targeted genes (23). Mutations in human CUL4B gene led to syndromic X-linked mental retardation (XLMR) associated with short stature, abnormal gait, impaired speech and other abnormalities (24–29). Unlike in humans, constitutive knockout of Cul4b led to embryonic lethality (30,31). However, the mice that lack Cul4b only in epiblast but not in extraembryonic tissues (Cul4b<sup>lox<sub>ox</sub>-Cre<sub>+</sub>-null mice) were viable and showed impaired spatial learning, which potentially mimics mental retardation (32).

In this report, we show that CUL4B negatively regulate GFAP expression in neural progenitor cells (NPCs). We observed an increased abundance of GFAP+ cells in the brains of nervous system-specific Cul4b knockout mice in vivo. We also demonstrated that the increased generation of GFAP+ cells from Cul4b-deficient NPCs was mediated by an upregulation of prostaglandin D2 synthase PTGDS.

Results

CUL4B was highly expressed in developing and adult mouse brain

We examined the expression pattern of CUL4B in the developing and adult mouse brain by immunostaining with a CUL4B-specific antibody. At E17.5, CUL4B expression was detected in most brain regions, though showing the highest level in cortex (Fig. 1A). In newborn pups, Cul4b is highly expressed in most brain regions, including cortex (Fig. 1B) and hippocampus (Fig. 1C). Importantly, high level of CUL4B expression was also detected in NPCs in subventricular zone (Fig. 1D and Supplementary Material, Fig. S1A and B). The expression level of CUL4B remains high in cortex and hippocampus of 2-week (Fig. 1E) and 1-month-old (Fig. 1F) mice. Western blot analysis of CUL4B in cortex and hippocampus at multiple time points showed a similar trend (Fig. 1G). Co-immunostaining of CUL4B and NeuN showed that Cul4b expression was postmitotic neurons (Fig. 1H). CUL4B expression was also detected in astrocytes (Fig. 1I and Supplementary Material, Fig. S1C) and oligodendrocytes (Fig. 1J). In summary, CUL4B protein is highly expressed in developing and adult mouse brain, and in all types of neural cells, including NPCs, neurons, astrocytes and oligodendrocytes, suggesting that CUL4B may function in multiple neural lineages and across different developmental stages of neural development.

Cul4b-null NPCs were skewed toward differentiation into GFAP+ radial glia-like cells in vitro

To determine whether CUL4B regulates NPC differentiation, we generated nervous system-specific Cul4b knockout mice (Cul4b<sup>lox<sub>ox</sub>-NeuN-Cre<sub>+</sub>-null mice) by crossing Cul4b floxed mice with Nestin-Cre transgenic mice, and isolated NPCs from E15.5 Cul4b<sup>lox<sub>ox</sub>-NeuN-Cre<sub>+</sub>-null and littermate control forebrains for in vitro studies. The proliferation and apoptosis assays revealed no significant difference between Cul4b-null and wild-type NPCs (Supplementary Material, Fig. S2), suggesting that CUL4B is probably dispensable for the expansion of NPCs. To induce the differentiation of NPCs, NPC cultures were cultured in the absence of BFGF and EGF for 4–8 days and the neural derivatives were characterized by immunostaining using cell lineage-specific markers, GFAP and S100β for astrocytes, and TUJ1 for neurons. Both control and Cul4b-null NPCs could be induced to differentiate into neurons and astrocytes by those markers; however, Cul4b-null NPCs resulted in increased generation of GFAP-positive radial glia-like (RGL) cells compared with control NPCs (Fig. 2A, P < 0.001, and Supplementary Material, Fig. S3). Similar results were obtained with immunostaining of S100β, another marker for astrocytes (Fig. 2B, P < 0.01). On the other hand, Cul4b-null NPCs showed reduced neuronal differentiation, as shown by TUJ1-positive cells, when compared with control NPCs (Fig. 2C, P < 0.01). Immunostaining using oligodendrocytes lineage-specific antibodies CNPase showed that lack of Cul4b did not affect the differentiation of oligodendrocytes (data not shown). Edu incorporation assay showed that lack of Cul4b did not affect the proliferation of astrocytes or neurons (Supplementary Material, Fig. S4), suggesting that the altered representation of the two types of neural derivatives was not due to an altered proliferation capacity. We next expressed CUL4B in Cul4b-null and control NPCs to test whether the ectopic expression of CUL4B can rescue the increased generation of GFAP+ cells from Cul4b-null NPCs. As shown in Figure 2D, overexpression of CUL4B resulted in a reduced generation of GFAP+ cells from Cul4b-null NPCs, suggesting that CUL4B functions to suppress GFAP expression.

Lack of CUL4B led to increased abundance of GFAP-positive cells in vivo

We then examined the roles of CUL4B in GFAP expression in vivo. Consistent with the in vitro result, the numbers of GFAP+ cells were significantly increased in multiple brain regions of 2-month-old Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice, including cortex, hippocampus, thalamus, hypothalamus and striatum, when compared with those in littermate controls (Fig. 3A and B; Supplementary Material, Fig. S5). The same trend was observed in 2-week-old mice (Supplementary Material, Fig. S6). The GFAP+ cells in Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice were like radial glia in morphology (Fig. 3C). However, staining of other astrocyte markers, ALDH1L1, AldoC and GS, revealed no numerical differences between the control and Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice (Fig. 4). Therefore, the increase in radial glia-like cells in Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice may only apply to GFAP+ cells. In other words, lack of CUL4B converted more GFAP- cells into GFAP+ cells that are not necessarily functional astrocytes.

In contrast to the reduced generation of neurons from Cul4b-null NPCs in vitro, the numbers and distributions of neurons (NeuN-positive) in the forebrains of Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice did not appear different from those in littermate controls (Supplementary Material, Fig. S7). Furthermore, no remarkable difference was noted in oligodendrocytes (CNPase-positive) between Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice and controls (Supplementary Material, Fig. S8).

Lack of Cul4b led to up-regulation of PTGDS

CUL4B, as a core component of an E3 ubiquitin ligase complex, plays an important role in ubiquitin-dependent protein degradation. To reveal the molecular mechanism underlying the upregulation of GFAP when Cul4b is lacking, we determined the expression levels of known CUL4B substrates in the cortex and hippocampus of Cul4b<sup>NeuN-Cre<sub>+</sub>-null mice at different developmental stages by western blotting. The levels of known CUL4B substrates,
including β-catenin, Cyclin E, WDR5 and PRDX3 did not show notable differences between Cul4b^Nestin-Cre^ and wild-type controls (Supplementary Material, Fig. S9 and data not shown).

We recently demonstrated that CUL4B-Ring E3 ligase complex (CRL4B) can catalyze H2AK119 monoubiquitination and, in coordination with PRC2, repress the transcription of various target
To determine whether CUL4B functions as a transcriptional co-repressor during brain development, we examined the level of trimethylation at H3K27 (H3K27me3), a PRC2-mediated chromatin modification mark, in the brains of the newborn mice. Immunostaining revealed a significantly decreased level of H3K27me3 in the cortex of Cul4bNestin-Cre mice compared with that of littermate controls (Fig. 5A). This result was confirmed by western blotting (Fig. 5B). Because H3K27me3 is associated with transcriptional repression, the lack of CUL4B function would be expected to result in an up-regulation of its target genes. Therefore, we next carried out a microarray transcriptome analysis to identify the genes that are deregulated in the brains of Cul4bNestin-Cre mice. Gene-expression profiles of wild-type and Cul4b-null brains at P0 were compared. As expected, more genes were found to be upregulated than those that were downregulated in Cul4b-null brains. A total of 178 genes were found to be upregulated at least 1.5-fold. In contrast, only 77 genes were downregulated at least 1.5-fold (Supplementary Material, Table S1). Moreover, while 19 genes were up-regulated more than 2-folds in Cul4b-null brains, only one gene was downregulated more than 2-folds (Fig. 5C). Expression changes of representative genes were verified by quantitative real-time RT-PCR. Strikingly, the expression of Ptgds was greatly elevated in Cul4bNestin-Cre brains compared with that in littermate controls (Fig. 5D). In addition, the expressions of Zic1 and Igf2 were also significantly increased in Cul4bNestin-Cre mouse brains (Fig. 5D). Western blotting using extracts from neonatal cortex confirmed the greatly increased level of PTGDS in Cul4bNestin-Cre mice.
(Fig. 5E). Immunostaining further confirmed the upregulation of PTGDS in Cul4bNestin-Cre mice (Fig. 5F). The expression of PTGDS was also upregulated in Cul4b-null NPCs in vitro, compared with that in control NPCs (Fig. 5G). These results indicate that lack of Cul4b results in an up-regulation of PTGDS.

Increased GFAP expression in the absence of Cul4b was mediated by PTGDS

The greatly elevated expression of PTGDS in Cul4b-null brains and NPCs promoted us to investigate whether PTGDS mediates the upregulation of GFAP. We first examined the effect of AT56, an inhibitor of PTGDS activity (33), on the expression of GFAP during astrocytic differentiation of Cul4b-null NPCs. AT56 treatment efficiently attenuated the expression of GFAP, as shown by immunofluorescence staining (Fig. 6A and B). Western blotting again showed that GFAP level was increased in Cul4b-null cells compared with that in control cells, and this increase can be attenuated by the addition of AT56 (Fig. 6C). Moreover, knockdown of Ptgds expression in Cul4b-null NPCs by lentiviral particles containing shRNA could also attenuate GFAP expression (Fig. 6D). Strikingly, addition of exogenous PTGDS protein to the cultures of wild-type NPCs greatly increased the percentages of GFAP+ cells (Fig. 6E). Together, these data suggested that the increased GFAP expression in the absence of Cul4b was mediated by PTGDS.

CUL4B directly binds to and represses Ptgds

Because CRL4B complex can coordinate with PRC2 to transcriptionally repress target genes, we next performed chromatin
Figure 4. No numerical differences of astrocytes in the forebrains between Cul4b\textsuperscript{Nestin-Cre}\textsuperscript{+} and littermate control mice. The forebrain slices from 2-month-old Cul4b\textsuperscript{Nestin-Cre}\textsuperscript{+} and littermate control mice were immunostained for ALDH1L1 (A), AldoC (B) and GS (C), markers of astrocytes.

immunoprecipitation (ChIP) assay to examine whether Ptgds gene is repressed by CRL4B complex. As expected, ChIP assay showed that CUL4B could directly bind to the promoter of Ptgds (Fig. 7A). Notably, H3K27me3, a PRC2-dependent chromatin modification mark, was also enriched in the same region. To explore the functional relationship between CUL4B, EZH2 and WDR5 in the regulation of Ptgds expression, we performed ChIP assay in wild-type and Cul4b-null brain tissues with antibodies against CUL4B, EZH2, WDR5, H3K27me3 as well as H3K4me3. As shown in Figure 7B, CUL4B, EZH2 as well as H3K27me3 were all enriched at the promoter of Ptgds in wild-type brain cells. Importantly, loss of CUL4B nearly abolished the binding of EZH2 to Ptgds, indicating that the binding of PRC2 complex to Ptgds in the brain depends on CRL4B complex. Furthermore, loss of CUL4B resulted in a significant decrease of H3K27 trimethylation, providing further support that CUL4B is required for PRC2-mediated H3K27 trimethylation at Ptgds. Concomitantly, there was an increased binding of WDR5 to Ptgds in Cul4b\textsuperscript{Nestin-Cre}\textsuperscript{+} brain tissue compared with littermate controls, although the total protein level of WDR5 was not changed in cortex and hippocampus of Cul4b\textsuperscript{Nestin-Cre}\textsuperscript{+} mice compared with that in littermate controls (Supplementary Material, Fig. S9). ChIP assay using NPCs also confirmed decreased H3K27me3, as well as increased H3K4me3, in the promoter of Ptgds in Cul4b-null NPCs compared with those in control NPCs (Fig. 7C and D). Taken together, these results demonstrated that CRL4B/PRC2 complexes can repress Ptgds by promoting H3K27 trimethylation.

Discussion

We studied the effect of Cul4b deficiency on neural differentiation using Nestin-Cre conditional knockout mice and found that lack of Cul4b could lead to increased generation of radial glia-like cells that are GFAP-positive. We further observed that Ptgds was upregulated due to loss of transcriptional repression by CUL4B/PRC2 complex. Importantly, we demonstrated that the increased expression of GFAP during astrocytic differentiation of Cul4b-null NPCs was mediated by PTGDS. Thus, these findings demonstrated a critical role of CUL4B in regulating the expression of GFAP, a gene that encodes the major intermediate filaments in astrocytes and other neural cells.

Dysfunction of astrocytes has been reported to be associated with numerous neurodevelopmental diseases, such as Alexander disease (9–11), Rett syndrome (34–36), fragile X mental retardation (37–39) and Down syndrome (40,41). Alexander disease, which is characterized by cytoplasmic protein aggregates known as Rosenthal fibers within astrocytes, is caused by gain of function mutations in GFAP (9–11). Overexpression of human wild-type GFAP in transgenic mice also led to the development of encephalopathy and Rosenthal fibers (13). A knock-in mouse model for Alexander disease was shown to manifest deficits in adult neurogenesis, contextual fear conditioning and spatial learning (42). These phenotypes associated with overproduction or gain of function of GFAP, which are in contrast to absence of obvious phenotypes in Gfap knockout mice, suggest that while GFAP is not essential for neural development, its overexpression or gain-of-function mutation may have deleterious consequences. Thus, its expression needs to be properly controlled. Importantly, upregulation of GFAP also marks reactive astrogliosis, which occurs in response to trauma, ischemia and other pathological conditions (43). It is therefore possible that the increased abundance of GFAP+ cells in Cul4b knockout mice may reflect or resemble reactive astrogliosis.

Our study showed that regulation of GFAP expression by Cul4b is mediated by PTGDS. PTGDS, the only enzyme within the lipocalin family of proteins, acts as a PGD2-synthesizing enzyme as well as an extracellular transporter of various lipophilic small molecules (44), such as retinal, retinoic acid (45), biliverdin, bilirubin (46), gangliosides (47), as well as amyloid β peptides (48,49). Although PTGDS is one of the most abundant proteins in the cerebrospinal fluid (CSF) (50,51), little is known about its function in the central nervous system (CNS). Nevertheless, a number of neurological disorders are associated with abnormal expression of PTGDS. The expression of PTGDS was found to be increased in attention deficit-hyperactivity disorder (52) and in mouse models of globoid cell leukodystrophy and lysosomal...
storage disorders (47). Levels of PTGDS in CSF were reduced in Alzheimer's disease and frontotemporal dementia (49). In patients with multiple sclerosis, although the CSF level of PTGDS was not changed, PTGDS was increased in the white matter of the patients, especially in the oligodendrocytes within the shadow plaques and in hypertrophied astrocytes within the chronic plaques (53). In addition, a recent study showed that PTGDS induces glial cell migration and morphological changes independent of the PGD2 products (44). Our results indicate that Ptgds is tightly regulated by CUL4B during perinatal neurodevelopment. While an upregulation of PTGDS was detected in the brains of newborn Cul4bNestin-Cre mice when compared with control mice, the upregulation was not detectable in older mice. This narrow window in which Ptgds is repressed by CUL4B is also the period critical for astrogenesis. Future studies may answer whether or not the various neurological disorders associated with abnormal PTGDS expression are due to dysregulated expression of GFAP.

Our study also showed that CRL4B/PRC2 complexes can occupy the promoter of Ptgds and repress its transcription. We propose that under physiological conditions, the CRL4B/PRC2 complexes may limit the expression of PTGDS to restrain GFAP expression. In the absence of CUL4B, Ptgds becomes derepressed, leading to excessive GFAP expression (Fig. 7E, model). Polycomb repressive complexes, PRC1 and PRC2, are known to play crucial
roles in governing the balance between proliferation and differentiation (54,55). Several recent studies demonstrated their roles in regulating self-renewal and differentiation of neural precursor cells. For instance, deletion of Ring1B or Ezh2 in the developing cortex can prolong the period of neurogenesis and delay the onset of gliogenesis (56). However, deletion of Ezh2 in mouse cortical progenitor cells before the onset of neurogenesis can change the balance between differentiation and self-renewal, significantly altering cortical developmental timing (57).

We demonstrated that PTGDS is transcriptionally repressed by CRL4B/PRC2 complexes, further illustrating the important role of histone modification in the regulation of neural development.

As a member of the E3 ubiquitin ligase family, CUL4B plays important roles in protein degradation. The substrates of CRL4B complexes include estrogen receptor α (14), Cyclin E (15), topoisomerase I (Topo I) (16), peroxiredoxin III (PRDX3) (17), WDR5 (18), TSC2 (19), JAB1/CSNS5 (20) and p53 (21). Therefore, we expected CUL4B to play a role in regulating the levels of key proteins involved in neural development. However, the levels of all tested CUL4B substrates were not found to change in cortex and hippocampus of Cul4bNestin-Cre mice at different developmental stages, which is consistent with the results made with Cul4bSox2-Cre mice (32). However, it cannot be ruled out that dysregulation of other yet to be identified CUL4B substrates may contribute to the impaired neural development associated with CUL4B dysfunction.

In summary, our results showed that CUL4B plays critical role in the regulation of GFAP. PTGDS is positive regulator of GFAP. By promoting H3K27 trimethylation at the promoter of Ptgsd, CRL4B restricts Ptgsd expression, leading to the inhibition of GFAP...
expression during neural differentiation. Lack of CUL4B leads to the upregulation of PTGDS and GFAP. Future studies may reveal whether the CUL4B-PTGDS-GFAP cascade is involved in the pathogenesis of neurological disorders.

Materials and Methods

Mice

Cul4b floxed mice were generated in Model Animal Research Center of Nanjing University as previously reported (30). To produce conditional knockout mice in which Cul4b was specifically deleted in nervous system, Cul4b floxed mice were crossed to Nestin-Cre transgenic mice (58), in which Cre recombinase was under the control of the promoter and enhancer of rat nestin. The resulting Cul4b-/-Nestin-Cre mice (Cul4bNestin-Cre) were crossed to fragment b of Pdgfs fl/fl mice. NPCs were derived from the cell aggregates of ES cells transfected with retroviral vector encoding Cul4bNestin-Cre. Tumor formation assay was performed by ECL PLUS kit (Amersham Pharmacia Biotech). GAPDH was used as a loading control (Sigma, 1:1000).

Culture of NPCs

Mouse forebrains were dissected and the cells were mechanically dissociated by pipetting. Samples were resuspended in growth medium (neurobasal medium and B27 supplement, Invitrogen) containing 20 ng/ml bFGF and EGF (PeproTech) and were cultured at 250 000–500 000 cells per 60 mm dish at 37°C with 5% CO2. For NPCs differentiation, neurospheres were dissociated into single cells by TrypLE (Invitrogen) digestion and pipetting, then the cells were plated at 100 000 cells per well in 24-well plates with cover glass in differentiation medium (neurobasal medium and B27 supplement, Invitrogen) containing 1% FBS without bFGF and EGF. Forty-two-four well plates and the cover glass were coated with laminin and polylysine before use. Cells were fixed using 4% paraformaldehyde in PBS and immunostained 4–8 days after induction.

Immunostaining

Immunohistochemistry and Immunofluorescence were performed as described previously (30). Briefly, brain specimens were dissected and fixed in 4% paraformaldehyde at 4°C overnight, followed by cryo-section. Primary antibodies used include: anti-CUL4B (Sigma, 1:1000), anti-NeuN (Millipore, 1:200), anti-PTGDS (Sigma, 1:200), anti-GFAP (Millipore, 1:200), anti-CNPase (Sigma, 1:200), anti-SOX2 (Millipore, 1:100) and anti-Ki67 (Abcam, 1:200). Secondary antibodies include: goat anti-mouse (or rabbit) horseradish peroxidase (HRP) (Jackson ImmunoResearch; 1:10 000). Chemiluminescence detection was performed by ECL PLUS kit (Amersham Pharmacia Biotech). GAPDH was used as a loading control (Sigma, 1:1000).

Gene-expression microarray

Total RNA from each sample was isolated by Trizol (Invitrogen) and purified by Rneasy Mini Kit and RNase-free DNase Set.
(Qiagen), according to the manufacturer’s protocols. cRNA for each sample was synthesized by using 3′ IVT EXPRESS KIT (Affymetrix) according to the manufacturer’s protocols. The purified cRNA was fragmented by incubation in fragmentation buffer at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to GeneChip Mouse Genome 430 2.0 Array (Affymetrix) and hybridized in Genechip hybridization oven 640 (Affymetrix) at 45°C for 18 h. After washing and staining in Genechip fluids station 450 (Affymetrix), the arrays were scanned by using Genechip scanner 3000 (Affymetrix). The gene expression levels of samples were normalized and compared by using Partek GS 6.5 (Partek).

Reverse transcription and real-time RT-PCR
Total RNA from the brain tissues of newborn mice of different genotypes was isolated using Trizol reagent (Invitrogen), and treated with RQ1 RNase-Free DNase (Promega) to eliminate genomic DNA contamination. Freshly isolated RNA was reversely transcribed to generate cDNA using Super Script first-strand synthesis system (Invitrogen) following the manufacturer’s recommendations. The mRNA levels were measured by LightCycler 480 SYBR Green I Master (Roche) using the LightCycler 480 instrument (Roche). The sequences of the primers were listed in Supplementary Material, Table S2. Four independent measurements per sample were performed. The quantified individual RNA expression levels were normalized to Gapdh.

ChIP assay
ChIPs were performed as described previously (23). Briefly, brain cells or NPCs were mechanically dissociated by pipetting and 1 x 10^6 cells were cross-linked with 1% formaldehyde, sonicated, pre-cleaned and incubated with 5 μg of antibody per reaction. Complexes were washed with low- and high-salt buffers, and the DNA was extracted and precipitated. The enrichment of the DNA template was analyzed by conventional PCR or quantitative real-time PCR using primers specific to Cpgs promoter. The sources of the antibodies were: anti-CUL4B (Sigma-Aldrich); anti-H3K4me3 and anti-H3K27me3 (Millipore); anti-EZH2 (BD biosciences); anti-WDR5 (Abcam). Protein A/G Sepharose CL-4B beads were from Amersham Biosciences, and protease inhibitor mixture cocktail was from Roche Applied Science. The primers used were listed in Supplementary Material, Table S3.

Statistical analysis
Data were expressed as the mean ± SEM. Data from two groups were evaluated statistically by the two-tailed unpaired t-test for any significant differences. Data were evaluated statistically by ANOVA to test for any differences among multiple groups. If significant differences were found by ANOVA, the Bonferroni method of multiple comparisons was used to determine which groups were significantly different from each other. A P-value of <0.05 is considered statistically significant.

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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