Prdm16 is required for the maintenance of neural stem cells in the postnatal forebrain and their differentiation into ependymal cells

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We and others showed previously that PR domain-containing 16 (Prdm16) is a transcriptional regulator required for stem cell function in multiple fetal and neonatal tissues, including the nervous system. However, Prdm16 germline knockout mice died neonatally, preventing us from testing whether Prdm16 is also required for adult stem cell function. Here we demonstrate that Prdm16 is required for neural stem cell maintenance and neurogenesis in the adult lateral ventricle subventricular zone and dentate gyrus. We also discovered that Prdm16 is required for the formation of ciliated ependymal cells in the lateral ventricle. Conditional Prdm16 deletion during fetal development using Nestin-Cre prevented the formation of ependymal cells, disrupting cerebrospinal fluid flow and causing hydrocephalus. Postnatal Prdm16 deletion using Nestin-CreERT2 did not cause hydrocephalus or prevent the formation of ciliated ependymal cells but caused defects in their differentiation. Prdm16 was required in neural stem/progenitor cells for the expression of Foxj1, a transcription factor that promotes ependymal cell differentiation. These studies show that Prdm16 is required for adult neural stem cell maintenance and neurogenesis as well as the formation of ependymal cells.

[Keywords: Prdm16; ependymal cell; hydrocephalus; neural stem cell]

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Neural stem cells give rise to the cerebral cortex during fetal development [Lui et al. 2011] and persist throughout adult life in the forebrain [Alvarez-Buylla et al. 2008]. In the adult forebrain, neural stem cells reside in the lateral wall of the lateral ventricle, where they give rise to transient amplifying progenitors, which differentiate into neuroblasts that migrate to the olfactory bulb and form interneurons [Doetsch et al. 1999; Alvarez-Buylla et al. 2008], as well as in the subgranular layer of the dentate gyrus, where they form granule neurons [Aimone et al. 2014]. In the lateral ventricle subventricular zone (SVZ), neural stem cells are highly quiescent Glast+GFAP+Sox2+ cells [Doetsch et al. 1999, Ferri et al. 2004, Codega et al. 2014; Mich et al. 2014]. These cells give rise to mitotically active, but multipotent, neurosphere-initiating cells, which express lower levels of GFAP and Glast and are relatively short-lived in the brain [Codega et al. 2014; Mich et al. 2014]. These multipotent progenitors in turn give rise to neuroblasts and differentiated neurons [Lim and Alvarez-Buylla 2014]. The SVZ also contains astrocytes, endothelial cells, and ependymal cells, each of which regulates neural stem cell function and neurogenesis [Lim et al. 2000; Mirzadeh et al. 2008; Porlan et al. 2014]. Ependymal cells are multiciliated cells that line the walls of the ventricles in the central nervous system (CNS) and promote the directional flow of cerebrospinal fluid (CSF) [Sawamoto et al. 2006; Mirzadeh et al. 2010b; Faubel et al. 2016]. CSF contains cytokines that are critical for the maintenance and proliferation of neural stem cells, and CSF flow regulates neuroblast migration [Sawamoto et al. 2006]. Depletion of ciliated ependymal cells disrupts the flow of CSF, resulting in the accumulation of CSF and the swelling of ventricles, a condition known as hydrocephalus [Jacquet et al. 2009; Del Bigio 2010; Tissir et al. 2010; Ohata et al. 2014]. Hydrocephalus affects 0.1% of infants and is associated with disruption of CNS architecture and intellectual developmental delays [Fliegauf et al. 2007; Tully and Dobyns 2014]. Hydrocephalus can be caused by blockage of CSF flow, overproduction of CSF by the choroid plexus, reduced absorption of CSF at
arachnoid granulations, or defects in ependymal cell function, but many cases of congenital hydrocephalus remain unexplained (Del Bigio 2010; Tully and Dobyns 2014; Kahle et al. 2016).

Ependymal cells arise from neural stem cells during fetal development and fully differentiate by the second week of postnatal life (Tramontin et al. 2003; Spassky et al. 2005). Some of the mechanisms that regulate the differentiation of ependymal cells have been identified. Two proteins that are homologous to geminin of ependymal cells have been identified. Two proteins, some of which are necessary for ependymal cell differentiation (Malaterre et al. 2008; Jacquet et al. 2009; Stubbs et al. 2012; Tan et al. 2013; Kyrouri et al. 2015). The transcription factors Trp73 (Yang et al. 2000; Gonzalez-Cano et al. 2016), Yap (Park et al. 2016), and Gli3 (Wang et al. 2014) and the kinase Ulk4 (Liu and Guan 2016) are also required for ependymal cell differentiation. There are also many genes that, when mutated, impair cilia function and can contribute to the development of hydrocephalus (Fliegauf et al. 2007; Tissir et al. 2010; Ohata et al. 2014).

The PR domain-containing (Prdm) family contains 16 members that function as transcriptional regulators and methyltransferases in diverse cell types (Hohenau and Moore 2012). Prdm16 was found originally in leukemias, where truncation mutants are oncogenic (Mochizuki et al. 2000; Nishikata et al. 2003; Shing et al. 2007). Prdm16 promotes stem cell maintenance in the fetal hematopoietic and nervous systems (Chuikov et al. 2010; Aguiló et al. 2011; Luchsinger et al. 2016) as well as brown fat cell differentiation from skeletal muscle precursors (Seale et al. 2008; Cohen et al. 2014). Microdeletion of a chromosome 1 locus that includes Prdm16 in humans causes 1p36 deletion syndrome, affecting one in 5000 newborns. 1p36 deletion syndrome is associated with congenital heart disease, hydrocephalus, seizures, and developmental delay. Prdm16 is necessary for normal heart development in humans (Battaglia et al. 2008) and zebrafish (Arndt et al. 2013). However, it is unknown whether Prdm16 deletion contributes to hydrocephalus.

We and others found that germline deletion of Prdm16 impairs the maintenance of neural stem cells and hematopoietic stem cells during fetal development (Chuikov et al. 2010; Aguiló et al. 2011). However, Prdm16 germline knockout mice die at birth; therefore, it is not known whether Prdm16 is required for the maintenance or differentiation of neural stem cells postnatally. In this study, we conditionally deleted Prdm16 in fetal and adult neural stem cells. We found that Prdm16 was required for adult neural stem cell maintenance and neurogenesis as well as the differentiation of neural stem/progenitor cells into ependymal cells.

Results

Fetal deletion of Prdm16 leads to hydrocephalus and thinning of the SVZ

We assessed the Prdm16 expression pattern in the adult SVZ by localizing β-galactosidase (β-gal) expression in embryos of 3-mo-old Prdm16LacZ/+ [Prdm16Gt(OST67423)Lex] mice (Bjork et al. 2010). β-Gal was broadly expressed in the SVZ of these mice [Fig. 1A–D], including nearly all GFAP+ cells (which include quiescent neural stem cells and astrocytes) [Fig. 1A–D, Doetsch et al. 1999; Codega et al. 2014; Mich et al. 2014], DCX+ neuroblasts [Fig. 1B–D, Gleeson et al. 1999], and S100B+ ependymal cells (Spassky et al. 2005) lining the lateral wall of the lateral ventricle [the SVZ ventricular surface] [Fig. 1C,D]. Thus, Prdm16 is expressed broadly in the adult SVZ, including within neural stem cells, neural progenitors, and ependymal cells. To test whether Prdm16 is required for postnatal neural stem cell function, we conditionally deleted it using Nestin-Cre [Tronche et al. 1999; Cohen et al. 2014]. Most Nestin-Cre, Prdm16fl/fl mice survived into adulthood. We confirmed that Prdm16 was efficiently deleted from the SVZ of 2- to 3-mo-old adult Nestin-Cre, Prdm16fl/fl mice by quantitative RT–PCR (qRT–PCR) on unfractoned SVZ cells [Fig. 1E] as well as neurospheres cultured from the SVZ [Supplemental Fig. S1A]. PCR on genomic DNA from individual neurospheres from Nestin-Cre, Prdm16fl/+ mice showed that 100% exhibited deletion of both Prdm16 alleles [Supplemental Fig. S1A]. Nestin-Cre, Prdm16fl/+ mice had normal body mass [Fig. 1F] and brain mass [Fig. 1G], but brain morphology differed from littermate controls. Littermate controls were a combination of Prdm16fl/+ fl/+ mice, and Prdm16fl/+ mice. We did not observe any phenotypic differences among mice with these genotypes. Adult Nestin-Cre, Prdm16fl/+ mice had hydrocephalus marked by enlarged lateral ventricles [Fig. 1H,I; Fliegauf et al. 2007; Tully and Dobyns 2014]. The SVZ was significantly thinner in adult Nestin-Cre, Prdm16fl/+ mice than in littermate controls [Fig. 1J,K], and the olfactory bulb was significantly smaller [Fig. 1L,M]. However, the corpus callosum and cortex were similar in thickness in Nestin-Cre, Prdm16fl/+ mice and littermate controls [Fig. 1N; Supplemental Fig. S1B]. Thus, Prdm16 deficiency in neural stem/progenitor cells led to hydrocephalus, thinning of the adult SVZ, and reduced olfactory bulb size.

Prdm16 is required for adult neural stem cell function and neurogenesis

To determine whether Prdm16 regulates adult neural stem cells, we analyzed neural stem cell function and neurogenesis in the adult SVZ. Quiescent neural stem cells (type B cells), which can contribute to the development of hydrocephalus (Fliegauf et al. 2007; Tissir et al. 2010; Ohata et al. 2014). The Prdm16 expression pattern in the adult SVZ was significantly thinner in adult Nestin-Cre, Prdm16fl/+ mice than in littermate controls [Fig. 1J,K], and the olfactory bulb was significantly smaller [Fig. 1L,M]. The corpus callosum and cortex were similar in thickness in Nestin-Cre, Prdm16fl/+ mice and littermate controls [Fig. 1N; Supplemental Fig. S1B]. Thus, Prdm16 deficiency in neural stem/progenitor cells led to hydrocephalus, thinning of the adult SVZ, and reduced olfactory bulb size.

Prdm16 is required for adult neural stem cell function and neurogenesis

To determine whether Prdm16 regulates adult neural stem cells, we analyzed neural stem cell function and neurogenesis in the adult SVZ. Quiescent neural stem cells (type B cells), which can be identified as GFAP+ Sox2+S100B+ cells [Doetsch et al. 1999], were profoundly depleted in the SVZ of 2- to 3-mo-old Nestin-Cre, Prdm16fl/+ mice as compared with littermate controls [Fig. 2A]. GLAST<sup>−/−</sup>EGFR<sup>high</sup> PlexinB2<sup>high</sup>CD24<sup>low</sup>O4/PSA-NCAM<sup>low</sup>Ter119/CD45<sup>−</sup> (GEP/COT) cells, which are highly enriched for neurosphere-initiating cells [Mich et al. 2014], were also significantly depleted in the SVZ of Nestin-Cre, Prdm16fl/+ mice as compared with littermate control mice [Fig. 2B; Supplemental Fig. S1C]. Dissociated SVZ cells from Nestin-Cre, Prdm16fl/+ mice formed significantly fewer neurospheres than control SVZ cells [Fig. 2C,D], and the Prdm16-deficient neurospheres were...
significantly smaller than control neurospheres (Fig. 2C, E). In contrast to control neurospheres, almost no Prdm16-deficient neurospheres underwent multilineage differentiation into TuJ1+ neurons, GFAP+ astrocytes, and O4+ oligodendrocytes (Fig. 2F). Prdm16-deficient neurospheres also failed to form multipotent daughter neurospheres upon dissociation and subcloning into secondary cultures, in contrast to control neurospheres (Fig. 2G). These data suggest that Prdm16 is required for the formation or maintenance of neural stem cells and neurosphere-initiating cells in the adult SVZ.

Prdm16 was also required for normal neurogenesis in the SVZ. Although we did not detect any difference in the frequency of cells undergoing cell death in the SVZ of Nestin-Cre; Prdm16(Δ/Δ) mice compared with control mice (Fig. 2H), we did observe significantly fewer dividing cells in the Nestin-Cre; Prdm16(Δ/Δ) SVZ based on Ki67 staining (Fig. 2I,J) and incorporation of a 2-h pulse of BrdU (Fig. 2K). Consistent with this, neurogenesis was profoundly reduced in the SVZ of Nestin-Cre; Prdm16(Δ/Δ) mice as compared with control mice, with significantly fewer DCX+ cells (Fig. 2L,M) and PSA-NCAM+CD24+ neuroblasts (Fig. 2N).

Prdm16 was also required for neurogenesis in the dentate gyrus. The morphology of the dentate gyrus was distorted in Nestin-Cre; Prdm16(Δ/Δ) mice as compared with littermate controls (Fig. 2O). Nestin-Cre; Prdm16(Δ/Δ) mice had significantly reduced frequencies of Ki67+ cells (Fig. 2P), cells that incorporated a 2-h pulse of BrdU (Fig. 2Q), DCX+ cells (Fig. 2R), and NeuroD1+ cells (Fig. 2S) in the subgranular layer as compared with littermate controls.
Pdm16 is required for adult neural stem cell function

In the experiments above, it was not clear to what extent the defects in brain morphology, stem cell function, and neurogenesis in Pdm16-deficient mice reflected a developmental requirement for Pdm16 versus ongoing functions in the adult forebrain. To address this, we conditionally deleted Pdm16 from young adult neural stem/progenitor cells using Nestin-CreERT2 (Balordi and Fishell 2007). Although Nestin is expressed at a lower level in quiescent neural stem cells as compared with neurosphere-initiating cells (Codega et al. 2014), Nestin-CreERT2 recombines in both cell populations (Mich et al. 2014). Nestin-CreERT2; Pdm16fl/fl mice and littermate controls were administered tamoxifen for 1 mo starting at 2 mo of age and then analyzed 2 wk, 3 mo, or 6 mo after finishing tamoxifen treatment [at 3.5, 6, or 9 mo of age]. Nestin-CreERT2; Pdm16fl/fl mice and littermate controls rarely died during these experiments and had similar body masses (Supplemental Fig. S2A).

One month after tamoxifen treatment, we confirmed that Pdm16 was efficiently deleted from the SVZ of Nestin-CreERT2; Pdm16fl/fl mice by qRT–PCR using unfractionated SVZ cells [Fig. 3A]. PCR analysis of genomic DNA from individual neurospheres cultured from the SVZ of Nestin-CreERT2; Pdm16fl/fl mice showed that 96% exhibited deletion of both Pdm16 alleles [24 of 25 neurospheres from three mice] (Supplemental Fig. S2B). We did not detect any signs of hydrocephalus in Nestin-CreERT2; Pdm16fl/fl mice at 3 mo after tamoxifen treatment [Fig. 3B,C]. This demonstrates that the hydrocephalus observed in Nestin-CreERT2; Pdm16fl/fl mice reflected a loss of Pdm16 function during fetal and/or early postnatal development.

At 2 wks after tamoxifen treatment, the frequencies of GFAP^+Sox2^+S100B^+ quiescent neural stem cells [Doetsch et al. 1999] and GEPOT neurosphere-initiating cells [Mich et al. 2014] in the SVZ did not significantly differ between Nestin-CreERT2; Pdm16fl/fl mice and littermate controls [Fig. 3D,E]. However, the frequencies of...
Figure 3. Prdm16 acts intrinsically within adult neural stem/progenitor cells to regulate self-renewal potential and multipotency. Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ and littermate controls (Con) were treated with tamoxifen for 1 mo beginning at 2 mo of age and then analyzed 0.5, 3, or 6 mo after the completion of tamoxifen treatment. All data represent mean ± SD. (A) Prdm16 transcript levels in the SVZ by qRT–PCR 1 mo after tamoxifen treatment. (B) Representative images of hematoxylin and eosin-stained coronal sections. (C) Lateral ventricle cross-sectional area (four to five sections per mouse, cut 300 μm apart). (D) The number of GFAP+Sox2+ S100B+ type B neural stem cells per cubic millimeter in the SVZ. (E) The frequency of GEPCOT neurosphere-initiating cells as a percentage of all live SVZ cells. (F) The percentage of SVZ cells that formed primary neurospheres (>50 μm in diameter) at clonal density in culture. (G) The percentage of SVZ cells that formed neurospheres that underwent multilineage differentiation. (H,J) Representative images (H) and diameters (J) of neurospheres. (I) The number of secondary multipotent neurospheres produced per single primary neurosphere upon subcloning (three to six primary neurospheres subcloned per mouse from five independent experiments). The statistical significance of differences between genotypes was assessed using the Mann-Whitney test [A], Welch’s test [A], Student’s t-tests [C], or two-way ANOVAs with Sidak’s multiple comparisons tests [D–G,J]. [∗] P < 0.05; [∗∗] P < 0.01; [∗∗∗] P < 0.001. The numbers of replicates in each treatment are shown at the top of each graph.

GFAP+Sox2+S100B+ quiescent neural stem cells at 6 mo after tamoxifen treatment and GEPCOT neurosphere-initiating cells at 3 and 6 mo after tamoxifen treatment were significantly lower in the SVZs of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice [Fig. 3D,E]. Dissociated SVZ cells from Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice formed significantly fewer neurospheres—and fewer neurospheres that underwent multilineage differentiation—as compared with control SVZ cells at 2 wk, 3 mo, and 6 mo after tamoxifen treatment (Fig. 3F,G; Supplementary Fig. S2C–E). Prdm16-deficient neurospheres were significantly smaller than control neurospheres at each time point analyzed [Fig. 3H,I] and failed to self-renew upon subcloning to secondary cultures (Fig. 3J). Therefore, there is an ongoing requirement for Prdm16 in adult neural stem cells and neurosphere-initiating cells in the SVZ in vivo.

There was also an ongoing requirement for Prdm16 for progenitor proliferation in the adult SVZ. We did not detect any difference in the frequency of cells undergoing cell death in the SVZ of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ as compared with control mice [Supplemental Fig. S2H]. The frequency of dividing cells in the SVZ of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice did not significantly differ from littermate controls at 2 wk after tamoxifen treatment based on the frequency of Ki67+ cells or the incorporation of BrdU [Fig. 4A–C]; however, the frequencies of Ki67+ cells and BrdU+ cells were significantly reduced in the SVZs of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice as compared with littermate controls at 3 and 6 mo after tamoxifen treatment [Fig. 4B,C].

Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice also exhibited reduced neurogenesis. The frequency of DCX+ neuroblasts did not significantly differ between the SVZs of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ and littermate control mice at 2 wk after tamoxifen treatment but was significantly reduced in Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice at 3 and 6 mo after tamoxifen treatment [Fig. 4D,E]. The frequency of PSA-NCAM+CD24+ neuroblasts was also significantly reduced in the SVZs of Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice as compared with control mice at 3 and 6 mo after tamoxifen treatment [Fig. 4F]. The frequency of newborn BrdU+ NeuN+ neurons in the olfactory bulb was also significantly reduced in Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice as compared with control mice after tamoxifen treatment [Fig. 4G,H].

There was also an ongoing requirement for Prdm16 for proliferation and neurogenesis in the dentate gyrus of the adult hippocampus. The morphology of the dentate gyrus was not significantly altered in Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice as compared with littermate controls [Supplemental Fig. S2G]. However, Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ mice had significantly fewer Ki67+ cells, BrdU+ cells, DCX+ cells, and NeuroD1+ cells in the subgranular layer as compared with littermate controls after tamoxifen treatment [Supplemental Fig. S2H–K].

To assess whether Prdm16 is required for neural stem cell function in the adult SVZ, we treated Nestin-CreER	extsuperscript{T2}, Prdm16	extsuperscript{fl/fl};Δ/Δ and littermate control mice with three daily doses of temozolomide starting 2 wk after the end of tamoxifen treatment [Fig. 4I]. Temozolomide depletes dividing progenitors, including GEPCOT.
Prdm16 is required for the formation of ependymal cells

To better understand the consequences of Prdm16 deletion in the SVZ, we examined ependymal cells in Nestin-Cre; Prdm16<sup>fl/fl</sup> mice [Fig. 1H–K]. The number of GFAP<sup>−</sup>Sox2<sup>−</sup>S100B<sup>−</sup> ependymal cells [Brazel et al. 2005; Kuo et al. 2006] was significantly reduced in Nestin-Cre; Prdm16<sup>fl/fl</sup> mice as compared with littermate control mice [Fig. 5A,B]. We rarely observed such cells in control mice [Fig. 5B]. The GFAP<sup>−</sup>Sox2<sup>−</sup>S100B<sup>−</sup> cells had a radial glial cell-like morphology with long processes that extended into the cortex [Fig. 5A]. Ependymal cells in littermate controls did not have such processes [Fig. 5A]. Given that Nestin-Cre; Prdm16<sup>Δ-Δ</sup> SVZ cells were unable to form multipotent neurospheres in culture [Fig. 2F], the GFAP<sup>−</sup>Sox2<sup>−</sup>S100B<sup>−</sup> cells on the ventricular surface could not have been functional radial glia. Nonetheless, these observations suggested that Prdm16 is required for the formation of normal ependymal cells and that Prdm16 deficiency caused the ventricular surface to be lined by glia rather than ependymal cells.

Ependymal cells are multiciliated and promote directional CSF flow. We visualized ependymal cilia by
Figure 5. Prdm16 is required for the generation of ependymal cells. Two-month-old to 3-mo-old Nestin-Cre; Prdm16fl/fl (Δ/Δ) and littermate controls (Con). All data represent mean ± SD from three independent experiments. (A,B) GFAP+SOX2+S100B+ cells with long processes were observed in the SVZ of Nestin-Cre; Prdm16fl/fl mice but not in littermate controls (arrows indicate radial glial-like processes). (C) GFAP-Sox2+S100B+ ciliated ependymal cells were abundant on the ventricular surface of the control mouse SVZ but not the Nestin-Cre; Prdm16fl/fl mouse SVZ. (D) Scanning electron microscopy revealed abundant ciliated ependymal cells on the lateral wall of the lateral ventricle in control mice but not in Nestin-Cre; Prdm16fl/fl mice. (E,F) γ-Tubulin-β-catenin+ ciliated ependymal cells were abundant on the ventricular surface of the control SVZ but not the Nestin-Cre; Prdm16fl/fl SVZ. (G–I) Representative images of bead flow tracks on the ventricular surface of SVZ specimens in culture. Beads tended to flow anteriorly over control SVZs and posteriorly/ventrally over Nestin-Cre; Prdm16fl/fl SVZs. (D) indicates dorsal, and “A” indicates anterior. (I) Anterior and posterior regions of the SVZ were selected rostral or caudal to the adhesion point (three SVZs per genotype in three independent experiments). Each circle indicates 5% of the total number of microbeads. The statistical significance of differences between genotypes was assessed using the Mann-Whitney test (I, posterior), Welch’s test (G), and Student’s t-tests (C,F,I anterior). [*] P < 0.05, [**] P < 0.01, [***] P < 0.001. The numbers of replicates or beads analyzed in each treatment are shown at the top of each graph.
Prdm16 is required postnatally for the normal differentiation of ependymal cells. Nestin-CreERT2; Prdm16+/− mice (Δ/Δ) and littermate controls (Con) were treated with tamoxifen at P0 and P2 and then analyzed 1 mo later. All data represent mean ± SD. (A) A schematic of the experiment. (B) Prdm16 transcript levels in the SVZ by qRT–PCR 1 mo after tamoxifen treatment. (C) For comparison purposes, representative images of hematoxylin and eosin-stained coronal sections from Nestin-Cre; Prdm16+/− and littermate control mice at 1 mo of age. (D) Representative images of hematoxylin and eosin-stained coronal sections 1 mo after tamoxifen treatment of Nestin-CreERT2; Prdm16+/− and littermate control mice. (E) Lateral ventricle cross-sectional area (four to five sections per mouse, cut 300 µm apart, beginning at the rostral end of the lateral ventricle) of Nestin-CreERT2; Prdm16+/− and littermate control mice. (F) The number of GFAP+Sox2+S100B+ ependymal cells was decreased in the SVZ of Nestin-CreERT2; Prdm16+/− mice compared with littermate controls. (G) GFAP+S100B+Sox2+ cells with long processes were common in the SVZ of Nestin-CreERT2; Prdm16+/− mice but not littermate controls. (H) Representative images of SVZ sections showing the increased numbers of GFAP+S100B+Sox2+ cells with long processes on the ventricular surface of Nestin-CreERT2; Prdm16+/− mice. (I) En face images of the lateral wall of the lateral ventricle surface showing that β-catenin+ ependymal cells showed unusual GFAP expression in Nestin-CreERT2; Prdm16+/− mice. (J,K) Nearly all β-catenin+ cells on the lateral ventricle surface were also γ-tubulin+ in mice of both genotypes. The statistical significance of differences between genotypes was assessed by Mann-Whitney [E–G]. (*) P < 0.05; (**) P < 0.001. The numbers of replicates in each treatment are shown at the top of each graph.

Figure 6. Prdm16 is required postnatally for the normal differentiation of ependymal cells. Nestin-CreERT2; Prdm16+/− mice (Δ/Δ) and littermate controls (Con) were treated with tamoxifen at P0 and P2 and then analyzed 1 mo later. All data represent mean ± SD. (A) A schematic of the experiment. (B) Prdm16 transcript levels in the SVZ by qRT–PCR 1 mo after tamoxifen treatment. (C) For comparison purposes, representative images of hematoxylin and eosin-stained coronal sections from Nestin-Cre; Prdm16+/− and littermate control mice at 1 mo of age. (D) Representative images of hematoxylin and eosin-stained coronal sections 1 mo after tamoxifen treatment of Nestin-CreERT2; Prdm16+/− and littermate control mice. (E) Lateral ventricle cross-sectional area (four to five sections per mouse, cut 300 µm apart, beginning at the rostral end of the lateral ventricle) of Nestin-CreERT2; Prdm16+/− and littermate control mice. (F) The number of GFAP+Sox2+S100B+ ependymal cells was decreased in the SVZ of Nestin-CreERT2; Prdm16+/− mice compared with littermate controls. (G) GFAP+S100B+Sox2+ cells with long processes were common in the SVZ of Nestin-CreERT2; Prdm16+/− mice but not littermate controls. (H) Representative images of SVZ sections showing the increased numbers of GFAP+S100B+Sox2+ cells with long processes on the ventricular surface of Nestin-CreERT2; Prdm16+/− mice. (I) En face images of the lateral wall of the lateral ventricle surface showing that β-catenin+ ependymal cells showed unusual GFAP expression in Nestin-CreERT2; Prdm16+/− mice. (J,K) Nearly all β-catenin+ cells on the lateral ventricle surface were also γ-tubulin+ in mice of both genotypes. The statistical significance of differences between genotypes was assessed by Mann-Whitney [E–G]. (*) P < 0.05; (**) P < 0.001. The numbers of replicates in each treatment are shown at the top of each graph.

CreERT2; Prdm16+/− mice had a significantly reduced number of GFAP+ Sox2+ S100B+ ependymal cells (Fig. 6F, H) and a significantly increased number of GFAP+ Sox2+ S100B+ cells lining the lateral ventricle (Fig. 6C). Although ependymal cells do not usually express GFAP, the GFAP+ Sox2+ S100B+ cells observed in Nestin-CreERT2; Prdm16+/− mice [Fig. 6I] were ciliated [Fig. 6J, K]. These data suggest that postnatal deletion of Prdm16 from Nestin-expressing stem/progenitor cells led to abnormally differentiated ependymal cells that retained GFAP expression. Nonetheless, these cells retained adequate ependymal cell function to avoid the development of hydrocephalus. Taken together, our data indicate that Prdm16 acts primarily during fetal development to promote the formation of ependymal cells, but there is an ongoing requirement for Prdm16 postnatally for normal differentiation.

Prdm16 is required for the expression of Foxj1

Radial glial cells proliferate during embryogenesis and differentiate into ciliated ependymal cells by the second postnatal week (Spaskey et al. 2005). Using Prdm16fl/fl mice, we confirmed that Prdm16 was expressed by most Nestin+ neural stem cells in the SVZ at P4 (Fig. 7A). Prdm16 was efficiently deleted from the SVZ of Nestin-Cre; Prdm16+/− mice by P4 (Fig. 7B). At this stage, the number of Sox2-positive neural stem/progenitor cells did not significantly differ between Nestin-Cre; Prdm16+/− mice and littermate controls (Fig. 7C). To assess the mechanism by which Prdm16 regulates the formation of ependymal cells, we performed RNA sequencing [RNA-seq] analysis on P4 SVZ cells from Nestin-CreERT2; Prdm16+/− mice but not littermate controls [n = 3 independent samples per genotype, each from different mice]. We identified 130 gene products that were significantly differentially expressed between Prdm16-deficient and control SVZ cells [fold change > 2.5; false discovery rate [FDR] q < 0.005; fragments per kilobase per million mapped fragments [FPKM] > 1 in all of the samples from at least one of the cell populations]. Of these, 103 were expressed at significantly lower levels in Prdm16-deficient cells. These included Foxj1 and Myb [transcription factors that promote ependymal cell
differentiation] [Malaterre et al. 2008; Yu et al. 2008; Jacquet et al. 2009; Tan et al. 2013] as well as 18 ciliary structural proteins (Ak7, Spata18, Ccdc113, Dynlrb2, Ciap126, Rshp1, Tek4, Spag16, Ethc1, Ccn0, Ccde135, Ccde170, Rshp4a, Ak8, Dnaib13, Spef2, Spa17, and Fam161a). Based on an analysis of cilium-related gene sets, we estimated that there are ~400 genes that encode products required for the formation or function of cilia in the genome (~1.7% of the genome). This indicates that cilium-related genes were highly enriched among genes that were down-regulated in Prdm16-deficient SVZ cells (P < 0.0001 by binomial test).

Gene set enrichment analysis revealed six gene sets that were significantly enriched [FDR q < 0.001] among genes that were down-regulated in Prdm16-deficient cells [Supplemental Table S3]. Five of the six gene sets were cilium-related, including “ciliary cytoplasm,” “axoneme,” “axoneme assembly,” “cilium movement,” and “motile cilium” [Fig. 7D,E]. We found nine gene sets that were significantly enriched [FDR q < 0.001] among genes that were up-regulated [NES ≥2.3] in Prdm16-deficient cells [Supplemental Table S4]. None were related to cilia. Most were gene sets related to neuronal differentiation.

Foj1 is required for the differentiation of radial glia into ependymal cells [Yu et al. 2008; Jacquet et al. 2009]. Therefore, the reduced expression of Foxj1 in the absence of Prdm16 offers a potential mechanism to explain the reduced generation of ependymal cells in Nestin-Cre; Prdm16fl/fl mice. (NES) Normalized enrichment score; (FDR) false discovery rate. (Δ) Gene set enrichment analysis showing “axoneme” and “cilium movement” gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. (Δ/Δ) Mice lacking Prdm16 in the absence of Nestin in the SVZ at P4. [B] qRT–PCR analysis of Foxj1 transcript levels in the SVZ. [C] The number of Sox2+ neural stem/progenitor cells per cubic millimeter in the SVZ. [D,E] Gene set enrichment analysis showing “axoneme” and “cilium movement” gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. [F] qRT–PCR analysis of Sox2 transcript levels in the SVZ. [G] The number of Foxj1+ cells per cubic millimeter in the SVZ. [H] Representative images of Foxj1, Sox2, and DAPI staining in the SVZ. [I–M] The number of K67+ proliferating cells per cubic millimeter in the SVZ; [J] representative images of K67, phospho-Histone 3 (pH3), and DAPI staining in the SVZ; [K] the number of pH3+ proliferating cells per cubic millimeter in the SVZ; [L] and the number TUNEL+ dying cells per SVZ section. [M]. The statistical significance of differences between genotypes was assessed using Welch’s test (F) and Student’s t-tests (B,C,G,I,J,L,M). [∗] P < 0.05; [∗∗] P < 0.01; [∗∗∗] P < 0.001. The numbers of replicates in each treatment are shown at the top of each graph.

Figure 7. Prdm16 is required for the expression of Foxj1 in neural stem/progenitor cells at P4, when ependymal cells are normally differentiating. All of the analyses were performed on Nestin-Cre, Prdm16fl/fl (Δ/Δ), Nestin-Cre; Prdm16fl/fl, or littermate control (Con) mice at P4. All data represent mean ± SD from two or three independent experiments. [A] In Prdm16fl/fl mice, β-gal colocalized with Nestin in the SVZ at P4. [B] qRT–PCR analysis of Prdm16 transcript levels in the SVZ. [C] The number of Sox2+ neural stem/progenitor cells per cubic millimeter in the SVZ. [D,E] Gene set enrichment analysis showing "axoneme" and "cilium movement" gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. [NES] Normalized enrichment score; (FDR) false discovery rate. (Δ) Gene set enrichment analysis showing “axoneme” and “cilium movement” gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. [Δ/Δ] Mice lacking Prdm16 in the absence of Nestin in the SVZ at P4. [B] qRT–PCR analysis of Foxj1 transcript levels in the SVZ. [C] The number of Sox2+ neural stem/progenitor cells per cubic millimeter in the SVZ. [D,E] Gene set enrichment analysis showing “axoneme” and “cilium movement” gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. [NES] Normalized enrichment score; (FDR) false discovery rate. (Δ) Gene set enrichment analysis showing “axoneme” and “cilium movement” gene sets that were significantly depleted in Nestin-Cre; Prdm16fl/fl mice. [Δ/Δ] Mice lacking Prdm16 in the absence of Nestin in the SVZ at P4.
for Foxj1 expression by neural stem/progenitor cells in the SVZ.

To investigate whether Prdm16 directly regulates Foxj1, we performed ChIP-seq [chromatin immunoprecipitation (ChIP) combined with high-throughput sequencing] analysis using a Prdm16 polyclonal antibody in P4 SVZ samples. However, we did not find convincing Prdm16-binding sites associated with Foxj1 or other cilia-related genes whose expression declined in the absence of Prdm16 (data not shown). Indeed, we observed very few convincing Prdm16-binding sites genome-wide. There is increasing evidence that Prdm16 regulates the expression of some genes by binding to protein complexes that are difficult to detect by ChIP-seq rather than by direct binding to DNA (Harms et al. 2015; Iida et al. 2015; Ishibashi and Seale 2015). Therefore, it remains unclear whether Prdm16 directly or indirectly regulates the expression of Foxj1 and other genes required for ciliogenesis.

We did not observe any difference in the numbers of Ki67+ or pH3+ proliferating cells in Nestin-Cre; Prdm16fl/fl mice as compared with littermate control mice at P4 (Fig. 7J-L) but did observe significantly increased apoptosis in the SVZ of Nestin-Cre; Prdm16fl/fl mice as compared with control mice at P4 (Fig. 7M). These data suggest that in the absence of Prdm16, at least some neural stem/progenitor cells in the postnatal SVZ undergo cell death. Given the accumulation of an unusual population of GFAP+Sox2+S100B+ cells with radial processes at the ventricular surface of Nestin-Cre; Prdm16fl/fl, but not control, mice (Fig. 5A,B), some of the Sox2+ cells that would normally differentiate to ependymal cells may acquire an abnormal glial identity.

Discussion

1p36 microdeletion syndrome, which affects a genomic locus that includes Prdm16 as well as other genes, is associated with severe mental retardation, seizures, distinctive facial features, hydrocephalus, and heart abnormalities. Although there is evidence that loss of Prdm16 contributes to the craniofacial and cardiac defects in this syndrome [Bjork et al. 2010; Arndt et al. 2013], the cause of hydrocephalus in 1p36 microdeletion syndrome is not understood. Our study indicates that, in addition to being required for fetal and adult neural stem cell function, Prdm16 is required for the differentiation of neural stem cells into ciliated ependymal cells (Fig. 7). Prdm16 was required in neural stem/progenitors for the expression of Foxj1, a transcription factor necessary for ependymal cell differentiation [Yu et al. 2008; Jacquet et al. 2009]. The hydrocephalus that we observed after deletion of Prdm16 using Nestin-Cre is consistent with the hydrocephalus observed after deletion of Foxj1 (Jacquet et al. 2009).

Using ChIP-seq analysis, we were unable to detect convincing localization of Prdm16 to the Foxj1 promoter or to other loci that encode genes involved in ciliogenesis. However, Prdm16 appears to often regulate the expression of genes by binding to, and regulating the function of, other transcription factors [Nishikata et al. 2003; Harms et al. 2015; Iida et al. 2015]. Therefore, it remains unclear whether Prdm16 directly or indirectly regulates the expression of genes involved in ciliogenesis.

Although some ciliated cells remained in Nestin-Cre; Prdm16fl/fl mice, the cilia movement was abnormal and caused abnormal CSF flow (Fig. 5). Although a loss of motile cilia is known to cause hydrocephalus in mice [Jacquet et al. 2009; Del Bigio 2010; Tissir et al. 2010; Ohata et al. 2014], magnetic resonance imaging (MRI) scanning studies show that CSF overproduction, CSF absorption defects, and aqueduct stenosis can also lead to hydrocephalus in humans [Kahle et al. 2016]. Due to the small size of the mouse brain, it is technically challenging to test these possibilities in mice by MRI. Therefore, the simplest interpretation of our data is that the hydrocephalus observed in Nestin-Cre; Prdm16fl/fl mice is caused by a failure of ependymal cell function and abnormal CSF flow, although it is possible that other congenital defects in the brain contribute to this phenotype.

In contrast to the defects in ependymal cell differentiation and hydrocephalus in Nestin-Cre; Prdm16fl/fl mice, conditional deletion of Prdm16 during adulthood in Nestin-CreERT2; Prdm16fl/fl mice did not lead to defects in ependymal cell differentiation, cilia function, or hydrocephalus [Supplemental Fig. S3]. Nonetheless, these mice continued to exhibit SVZ neural stem cell depletion, little ability to form multipotent or self-renewing neurospheres in culture (Fig. 3), and reduced SVZ proliferation and neurogenesis (Fig. 4). Thus, these defects in neural stem cell function are not secondary consequences of hydrocephalus, as they were observed even in Nestin-CreERT2; Prdm16fl/fl mice that never developed hydrocephalus. Thus, Prdm16 independently appears to regulate multiple aspects of neural stem cell function, including neural stem cell maintenance, neurogenesis, and ependymal cell differentiation.

Materials and methods

Mice

All mice were housed at the Animal Resource Center of the University of Texas Southwestern Medical Center. All protocols were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee. Mice were housed in standard cages that contained three to five mice per cage, with water and standard diet ad libitum and a 12-h light/dark cycle. Both male and female mice were analyzed in all experiments. Nestin-Cre mice were obtained from Jackson Laboratory (Tromche et al. 1999). Nestin-CreERT2 mice were provided by G. Fishell (Balordi and Fishell 2007). Prdm16fl/fl mice were provided by B. Spiegelman [Cohen et al. 2014]. Prdm16Gt(OST67423)Lex mice were obtained from the National Institutes of Health Mutant Mouse Regional Resource Center [http://www.mmrrc.org]. All mice were backcrossed onto a C57BL/Ka background for at least three generations prior to analysis. Control mice were a combination of Prdm16fl/fl, Prdm16fl/+, and Prdm16+/+ mice. We did not observe any phenotypic differences among mice with these genotypes. For tamoxifen treatment, tamoxifen [Sigma, T5648] was dissolved in 90% corn oil/10% ethanol at 20 mg/mL and injected at 100 mg/kg per day intraperitoneally for three consecutive days.
and tamoxifen citrate [Sigma or Spectrum Chemical] was given in the chow for 1 mo at 400 mg/kg with 5% sucrose [Harlan] starting at 2 mo of age. Mice were fed a standard diet for at least 2 wk before analysis. For tamoxifen treatment of postnatal mice, tamoxifen was dissolved in 90% corn oil/10% ethanol at 2.5 mg/mL, and 20 µL was injected into the stomachs of P0 and P2 mice. Mice were analyzed 1 mo after tamoxifen treatment.

SVZ cell preparation

SVZs from adult [2- to 10-mo-old] mice were dissected and dissociated as described [Mich et al. 2014]. Briefly, SVZs were minced and digested with 700 µL of trypsin solution [Ca and Mg-free Hank’s buffered salt solution [HBSS], 10 mM HEPES, 0.5 mM EDTA, 0.25 mg/mL trypsin [EMD Millipore], 10 µg/mL DNase I [Roche] at pH 7.6] for 15 min at 37°C. Digestion was quenched with 2 vol of staining medium [440 mL of Leibovitz L-15 medium, 50 mL of water, 5 mL of 1 M HEPES at pH 7.3–7.4, 5 mL of 100X penicillin–streptomycin [Pen-Strep], 1 g of bovine serum albumin [Sigma, A7030] and 100 µg/mL trypsin inhibitor [Sigma, T6522]. Digested cells were centrifuged at 491 L by gently drawing them into a P1000 pipetman and expelling them 20 times without forming bubbles. The cell suspension was then filtered through a 45-µm mesh, counted on a hemocytometer, and added to culture or processed for flow cytometry. Neuropeptide formation, self-renewal, and differentiation assays were performed as described previously [Mich et al. 2014].

For additional methodological details concerning PCR genotyping of mice, immunohistochemistry, flow cytometry, microbead flow analysis, scanning electron microscopy, hematoxylin and eosin staining, analysis of RNA-seq data, cell quantification, and statistics, see the Supplemental Material.

Accession numbers

RNA-seq data have been deposited in the Sequence Read Archive public database with accession number PRJNA339165 and submission number SUB1780202.

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