Cell Cycle 9:24, 4866-4875; December 15, 2010; © 2010 Landes Bioscience

The novel BTB/POZ and zinc finger factor Zbtb45 is essential for proper glial differentiation of neural and oligodendrocyte progenitor cells

Erik Södersten, Tobias Lilja and Ola Hermanson*

Linnaeus Center in Developmental Biology for Regenerative Medicine (DBRM); Department of Neuroscience; Karolinska Institutet; Stockholm, Sweden

Key words: glioma, oligodendroglioma, 19q deletion, transcription, differentiation, gene expression, neural development

Abbreviations: BTB/POZ, broad-complex, tramtrack, bric-a-brac/pox-virus and zinc fingers; BMP, bone morphogenetic protein; BrdU, 5-bromo-2-deoxyuridine; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CNTF, ciliary neurotrophic factor; DIV, days in vitro; FGF2, fibroblast growth factor2; GE, ganglionic eminences; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NCx, neocortex; NSCs, neural stem cells; Th, thalamus

Understanding the regulatory mechanisms controlling the fate decisions of neural stem cells (NSCs) is a crucial issue to shed new light on mammalian central nervous system (CNS) development in health and disease. We have investigated a possible role for the previously uncharacterized BTB/POZ-domain containing zinc finger factor Zbtb45 in the differentiation of NSCs and postnatal oligodendrocyte precursors. In situ hybridization histochemistry and RT-qPCR analysis revealed that Zbtb45 mRNA was ubiquitously expressed in the developing CNS in mouse embryos at embryonic day (E) 12.5 and 14.5. Zbtb45 mRNA knockdown in embryonic forebrain NSCs by siRNA resulted in a rapid decrease in the expression of oligodendrocyte-characteristic genes after mitogen (FGF2) withdrawal, whereas the expression of astrocyte-associated genes such as CD44 and GFAP increased compared to control. Accordingly, the number of astrocytes was significantly increased seven days after Zbtb45 siRNA delivery to NSCs, in contrast to the numbers of neuronal and oligodendrocyte-like cells. Surprisingly, mRNA knockdown of the Zbtb45-associated factor Med31, a subunit of the Mediator complex, did not result in any detectable effect on NSC differentiation. Similar to NSCs, Zbtb45 mRNA knockdown in oligodendrocyte precursors (CG-4) reduced oligodendrocyte maturation upon mitogen withdrawal associated with downregulation of the mRNA expression and protein levels of markers for oligodendrocytic differentiation. Zbtb45 mRNA knockdown did not significantly affect proliferation or cell death in any of the cell types. Based on these observations, we propose that Zbtb45 is a novel regulator of glial differentiation.

Introduction

Deletions on chromosomes 1p and 19q are commonly found in various gliomas, and are primarily associated with a favorable prognosis in oligodendroglioma.[1] Although it has long been known that certain regions of 19q, such as 19q13.4, are rich in genes encoding zinc finger proteins,[2] surprisingly little is known regarding the functional roles of these factors. A subclass of zinc finger factors, the members of the Zbtb family, contain the so called BTB/POZ (Broad-complex, Tramtrack, Bric-a-brac/Pox-virus and zinc fingers) domain and C_2H_2 Zinc fingers.[3] Several Zbtb family members have been implicated in many different biological processes, including DNA damage responses, cell cycle progression and various developmental events such as gastrulation, limb formation, B-cell fate choice and hematopoietic stem cell fate determination.[4-7] While displaying features of classical transcriptional repressors, Zbtb proteins seem to function as molecular docking bays for other transcriptional regulators. For example, the BTB/POZ-domain of the proto-oncogenes PLZF and Bcl-6 interacts with the NCoR/SMRT and mSin3A co-repressors along with HDAC1, while the central region of PLZF and the zinc-fingers of Bcl-6 associate with the ETO co-repressor and class II HDACs, respectively.[6,8,9] The Drosophila Zbtb factor Tramtrack has been shown to repress at least a subset of targets through interaction with dMi-2, the ATPase subunit of the Drosophila nucleosome remodeling and histone deacetylation (dNuRD) complex.[10] Moreover, the central domain of the Zbtb protein HIC-1 has been shown to bind to the co-repressor CtBP via a CtBP-binding motif.[11] Despite these important functional implications and associations with crucial co-regulators,[7,12] only a few Zbtb proteins have yet been studied in the context of nervous system development and neural progenitor differentiation.[13-15]

Telencephalic neural stem cells (NSCs) derived from the embryonic rodent brain at mid-gestation have the capability to

*Correspondence to: Ola Hermanson; Email ola.hermanson@ki.se
Submitted: 07/28/10; Revised: 10/28/10; Accepted: 11/08/10
Previously published online: www.landesbioscience.com/journals/cc/article/14154
DOI: 10.4161/cc.9.24.14154

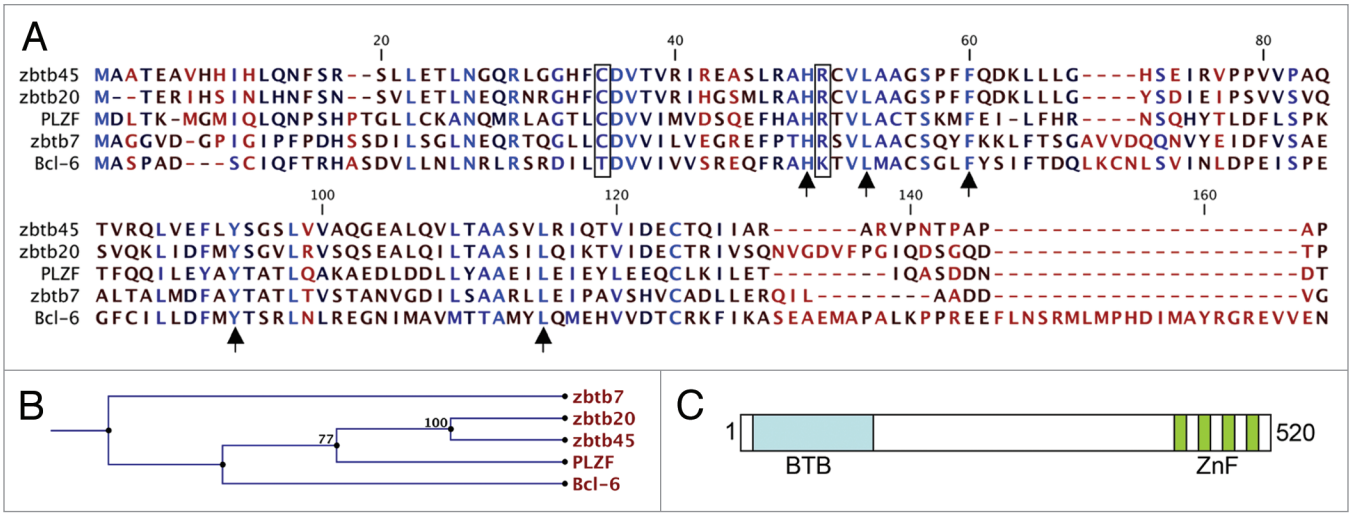

Figure 1. The predicted Zbtb45 amino acid sequence displayed typical features of a Zbtb protein. (A) Alignment of the predicted amino acid sequence of Zbtb45 to Zbtb20, Zbtb7, PLZF and Bcl-6 confirmed that the N-terminal part of the putative protein contained highly conserved residues that contribute to protein stability (black triangles) as well as highly conserved components of a charged pocket that is required for the function of Zbtb proteins (framed). (B) Phylogenetic tree showing that the predicted Zbtb45 amino acid sequence share closest homology to Zbtb20. (C) The Zbtb45 gene was predicted to be encoded into a 520-residue transcript with a BTB/POZ domain proximal to the N terminus and four predicted zinc fingers of C_2H_2-type in the C terminus.

form the major cell types found in the telencephalon, including excitatory and inhibitory neurons, astrocytes and oligodendrocytes, thus providing a versatile model for studying transcriptional control of neural lineage decision.[16-19] Upon mitogen (basic Fibroblast Growth Factor, FGF2) withdrawal, NSCs mainly form neurons and astrocytes, but oligodendrocyte differentiation can be enhanced by administration of thyroid hormone (T3), and efficient astrocytic differentiation can be obtained by treatment with interleukin-6 related compounds, such as CNTF, cardiotrophin-1 (CT-1) or leukemia inhibitory factor (LIF).[16,17,20,21] Several BTB/POZ-interacting co-regulators have been shown to repress differentiation of NSCs. For example NCoR and SMRT are both repressors of astrocyte differentiation whereas SMRT in addition is required for repression of neuronal differentiation and the histone demethylase JMJD3.[17,19,22]

By investigating the expression of a number of genes containing zinc fingers and BTB/POZ domains during nervous system development, we identified the previously uncharacterized Zbtb factor *Zbtb45*, located at 19p13.4, as an ubiquitously expressed gene in the developing forebrain. We therefore aimed at investigating possible functional roles for the Zbtb45 factor in telencephalic NSC differentiation. siRNA mediated loss of Zbtb45 mRNA in NSCs resulted in a rapid upregulation of the mRNA levels of astrocyte lineage-associated markers and a subsequent increase in the number of GFAP-positive astrocytes. In contrast, the mRNA expression of oligodendrocyte markers decreased under the same conditions. Knockdown of a subunit of the Mediator complex, Med31, a Zbtb45-associated co-regulator, in NSCs had no major effect on differentiation or cell death in this context. Similar to NSCs, bi-potent oligodendrocyte progenitors (CG-4)[23] transfected with the Zbtb45 siRNA displayed decreased gene expression and number of immunopositive cells of the oligodendrocyte marker MBP after mitogen withdrawal. Taken together, we propose that Zbtb45 is a novel regulator of glial differentiation of telencephalic NSCs and postnatal oligodendrocyte precursors.

Results

The Zbtb45 gene is conserved in mammals. We seeked to identify factors of the Zbtb family that could putatively play a role in telencephalic and cortical development. The criteria used were (a) sequence similarity with the Notch signaling-associated transcription factor Tramtrack due to its roles in neural development in other species and (b) expression pattern in mid-gestation embryonic telencephalon of such Zbtb factors that showed sequence similarity to Tramtrack as defined by series of in situ histochemistry hybridization experiments. Using these criteria, we identified *Zbtb45* as a previously uncharacterized putative BTB/POZ-domain containing zinc finger factor encoding gene. A phylogenetic analysis showed that the *Zbtb45* gene was conserved in mouse, rat and human. Interestingly, the human *ZBTB45* gene was located in the 19q13.4 arm, which is commonly deleted in gliomas.[24] The mouse protein was predicted to be encoded by a 2,167 bp (ORF bp 120 to 1,682) cDNA sequence (accession number NM_001024699) mapped to the chromosome locus 7a1. Sequence alignment confirmed that the N-terminal part of Zbtb45 shared typical features of the BTB/POZ domain, including components of a hydrophobic core and a charged pocket (**Fig. 1A**).[25] The Zbtb45 amino acid sequence shared closest homology with Zbtb20 (**Fig. 1B**), a transcriptional repressor that have been previously linked to neural development.[13,26] The putative 520-residue transcript contained a BTB/POZ-domain (E = 5e-19) in close proximity to the N terminus and 4 predicted C_2H_2-type predicted zinc finger domains in the C-terminus (E = 0.009, E = 0.014, E = 0.12, E = 0.29)[27] (**Fig. 1C**).

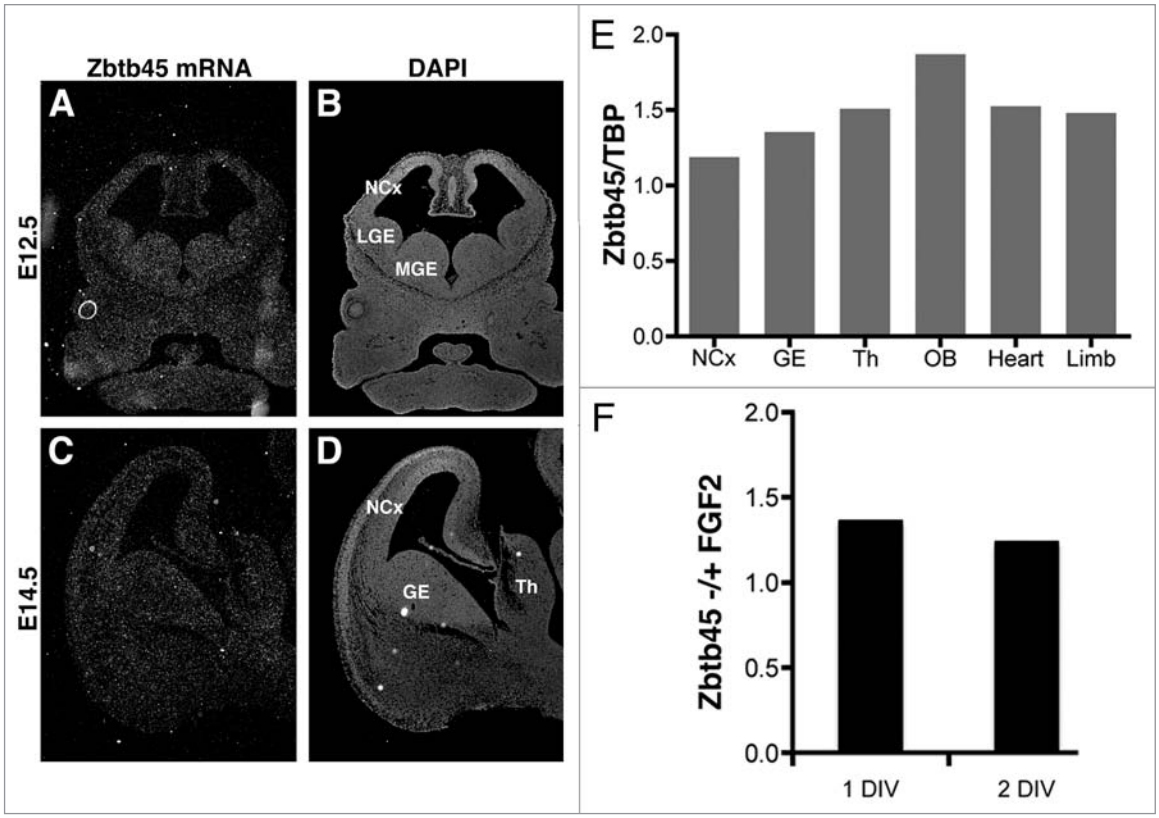

Figure 2. Ubiquitous expression of Zbtb45 mRNA in the developing mouse forebrain. (A and C) In situ hybridization histochemistry of coronal brain sections from embryonic mice revealed that Zbtb45 mRNA was present in the developing forebrain at (A) E12.5 and (C) E14.5. (B and D) DAPI staining on adjacent and same sections. (E) The ubiquitous expression pattern was further confirmed by qPCR analysis of Zbtb45 mRNA levels relative to TATA-binding protein in rat E15 cortex (neocortex; NCx), ganglionic eminences (GE; lateral GE—LGE, medial GE—MGE), thalamus (Th), olfactory bulb (OB), heart and forelimb (Limb), showing only minor regional variations. (F) RT-qPCR analysis of neural stem cells (NSCs) revealed that FGF2 withdrawal had little effect on the expression of Zbtb45 at 1 and 2 DIV. Bars represent the levels of Zbtb45 in FGF2-conditions relative to the levels in $FGF2^{+}$ conditions. Results from representative experiments are shown (A–F).

Zbtb45 mRNA is ubiquitously expressed in the developing mouse forebrain. To investigate the expression pattern of Zbtb45 mRNA during mouse development, we performed in situ hybridizations on forebrain sections from mouse embryos at embryonic day (E) 12.5 and E14.5, using ^{35}S-labeled RNA probes. In contrast to most previously characterized members of the Zbtb family, Zbtb45 mRNA was expressed at relatively even levels in the developing forebrain (**Fig. 2A–D**). Further analysis of Zbtb45 mRNA expression in various tissues dissected from E15 rat embryos (olfactory bulb, pre-thalamus, cortex, ganglionic eminences, heart and forelimb) by quantitative (q) RT-PCR, confirmed that there were only minor regional variations in the levels of Zbtb45 mRNA expression (**Fig. 2E**).

Zbtb45 mRNA knockdown in NSCs results in increased astrocyte and decreased oligodendrocyte gene expression. To investigate a putative role for Zbtb45 in differentiation events during forebrain development, we utilized siRNA mediated knockdown of Zbtb45 mRNA in embryonic telencephalic multipotent neural stem cells (NSCs) isolated from rat cortices and expanded in the presence of fibroblast growth factor-2 (FGF2). One passage after isolation, these NSCs constitute a homogenous progenitor population positive for stem cell markers such as nestin and negative for markers of terminal differentiation.[16,17] These telencephalic NSCs has repeatedly been shown to have the ability to differentiate into neurons, astrocytes and oligodendrocytes as assessed by morphology and cell fate markers,[16,28] and experiments under clonal conditions have previously shown that a single NSC can give rise to at least two of these fates.[16,19,28,29]

We first investigated the expression of Zbtb45 mRNA in the NSCs by RT-qPCR. Similar to the results from embryonic brain regions (**Fig. 2E**), Zbtb45 mRNA levels were in the same range as TATA-box binding protein (TBP), and no significant differences in expression levels of Zbtb45 mRNA were detected after 1, 2 or 7 days (DIV) of mitogen (FGF) withdrawal alone or in combination with treatment with ciliary neurotrophic factor (CNTF), bone morphogenetic protein 4 (BMP4) or thyroid hormone (T3; **Fig. 2F** and data not shown). We transfected small interference RNA (siRNA) directed against Zbtb45 mRNA to the NSCs by nucleofection. The transfected NSCs were further expanded for 24 h in the presence of FGF2 and then allowed to differentiate for 1 or 2 DIV upon mitogen withdrawal. The expression levels of a selection of markers for lineage choice and differentiation were consequently monitored by RT-qPCR, using TBP as endogenous control. Upon delivery of Zbtb45-directed

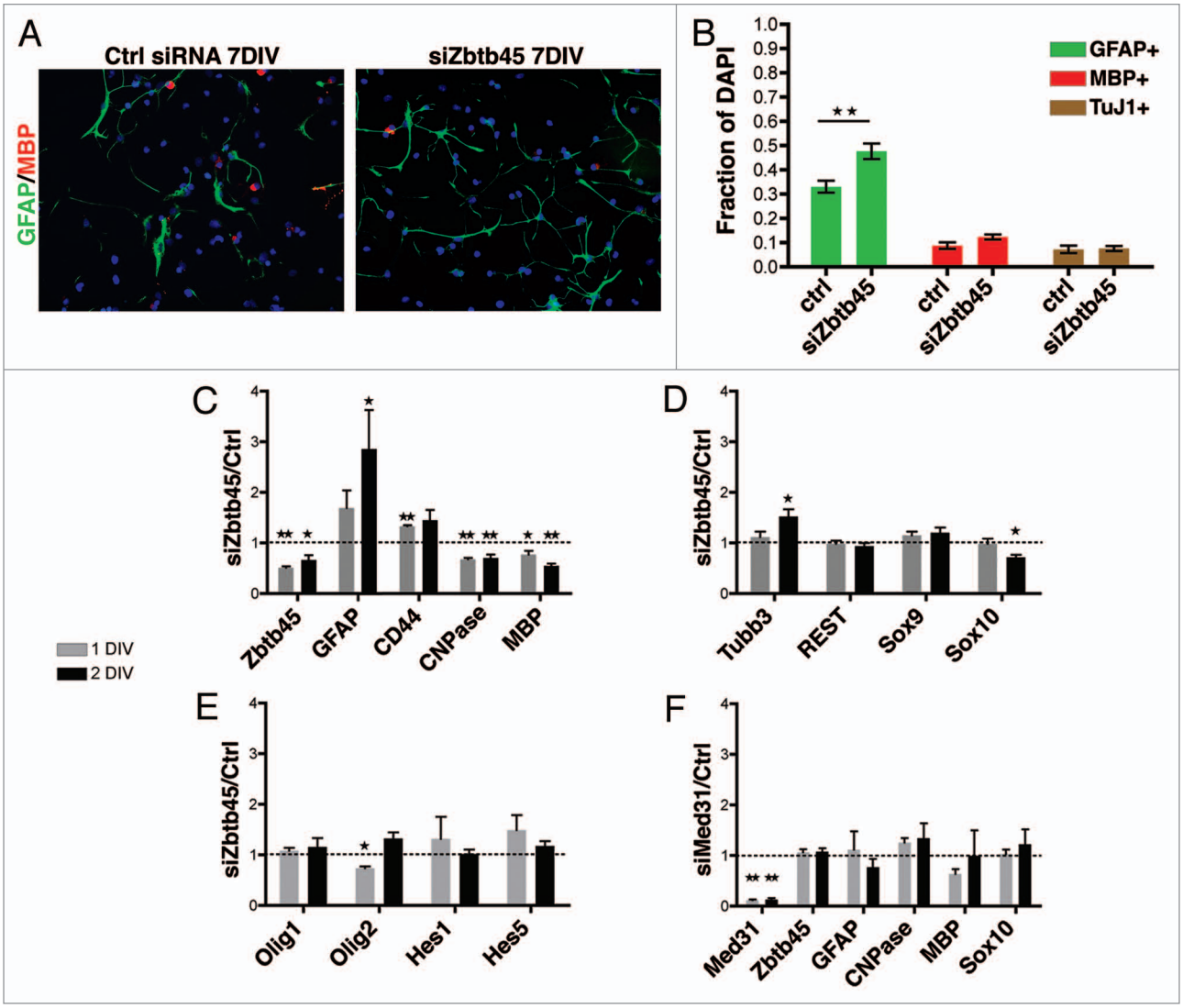

Figure 3. Zbtb45 siRNA delivery to NSCs yielded increased astrocyte- and decreased oligodendrocyte gene expression. (A) siZbtb45 (right part) lead to an increased number of astrocytes after 7 DIV compared to control (left part). Immunocytochemistry with GFAP antibody (green) and MBP (red) with DAPI (blue) counterstain. 20x magnification. (B) NSC cultures lacking Zbtb45 contained significantly increased numbers of GFAP$^+$ cells, whereas numbers of MBP$^+$ and TuJ1 cells were unaffected compared to control. (C–E) RT-qPCR analysis of mRNA levels in NSCs after 1 (gray bars) and 2 DIV (black bars) revealed that siRNA against Zbtb45 led to significant changes in expression levels of markers for astrocytes and oligodendrocytes but only moderate changes in gene expression of various transcription and signaling factors. Bars indicate mRNA levels of siZbtb45 cells relative to control. (F) RT-qPCR analysis of mRNA levels in NSCs after 1 (gray bar) and 2 DIV (black bar) suggested that siRNA mediated knockdown of the Zbtb45-associated factor Med31 had no effect on differentiation. n = 3 (B) or 5 (C–F) independent experiments (*$p < 0.05$, **$p < 0.01$), error bars = SEM.

siRNA, Zbtb45 mRNA levels decreased to levels of in average 50.7% ± 3.5 at 1 DIV post-transfection and 66.1% ± 10 at 2 DIV compared to control siRNA transfected cells (**Fig. 3C**). The expression of the closely related factor Zbtb20/HOF was rather slightly increased at 1 DIV (131.5% ± 6.3) and not significantly changed at 2 DIV [108.6% ± 7.4; data not shown; all numbers expressed as percentage of control (100%)]. Furthermore, Zbtb45 RNA knockdown did not result in cell death as assessed by quantification of the number of pyknotic nuclei (10.8% ± 3.2 in control cultures versus 3.19% ± 1.0 after Zbtb45 siRNA delivery at 1 DIV after Zbtb45 RNA knockdown). All results described were reproduced with an alternative siRNA sequence (see Materials and Methods).

Interestingly, knockdown of Zbtb45 RNA in NSCs resulted in a significant increase in mRNA expression of the astrocyte marker glial fibrillary acidic protein (GFAP), and the astrocyte-associated protein CD44 at 1 DIV (169.0% ± 38 and 133.2% ± 2.0, respectively) and at 2 DIV (284.7% ± 84 and 145.3% ± 24, respectively) upon FGF2 withdrawal, as compared to NSCs transfected with control siRNA (**Fig. 3C**). Accordingly, an assessment of the number of GFAP-positive cells by immunocytochemistry revealed a significant increase in the relative abundance of astrocytes after 7 DIV (145.8% ± 4.5, $p < 0.01$) (**Fig. 3A and B**). These findings were unexpected since we did not detect any significant downregulation of Zbtb45 mRNA levels 6–72 h after CNTF administration and thus initiation of astrocytic

differentiation (data not shown). The increase in astrocyte differentiation was accompanied with a downregulation of the mRNA levels of oligodendrocyte related genes such as the early oligodendrocyte specification marker, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; 67.9% ± 2.8 at 1 DIV, 70.3% ± 7.3 at 2 DIV), as well as the maturation marker, myelin basic protein (MBP; 76.7% ± 8.2 at 1 DIV, 54.9% ± 4.8 at 2 DIV) compared to control (**Fig. 3C**). In contrast to the elevated relative abundance of astrocytes in the cultures after 7 DIV, the decrease in expression of different oligodendrocyte related genes at 1 and 2 DIV was not associated with less MBP staining by immunocytochemistry 3 or 7 DIV after FGF2 withdrawal (**Fig. 3A and B**), in line with previously observed auto-regulatory mechanisms of oligodendrocyte numbers.[30]

Thyroid hormone (T3) can enhance oligodendrocyte differentiation of the telencephalic NSCs.[16,29] To investigate whether this treatment could overcome the rapid decrease in the expression of oligodendrocyte-related genes caused by Zbtb45 RNA knockdown, we stimulated NSCs that received either siRNA against Zbtb45 or control siRNA with T3 for 2 DIV. No significant change in the observed effects of Zbtb45 RNA knockdown in NSCs was observed in the presence or absence of T3 as assessed by GFAP, (310.8% ± 179), CNPase (61.5% ± 5.1) or MBP (79.6% ± 24) expression levels.

To elucidate a putative effect on neuronal differentiation of Zbtb45 RNA knockdown, we analyzed the mRNA levels of the neuronal marker, β-tubulin III by RT-qPCR at 1 and 2 DIV after siRNA transfection, and the number of TuJ1-positive cells by immunocytochemistry. Zbtb45 RNA knockdown did not cause any difference in β-tubulin III mRNA levels at 1 DIV (112.0% ± 11) (**Fig. 3D**). At 2 DIV, a moderate increase in β-tubulin III mRNA levels was observed (152.6% ± 15, $p < 0.01$) although no significant difference in TuJ1-positive cells was detected in the cultures that had received Zbtb45 siRNA versus control siRNA (**Fig. 3B and D**). Thus, in contrast to most previous observations regarding transcription factors influencing lineage decision, the increased astrocytic differentiation after Zbtb45 knockdown did not seem to be associated by a decrease in neuronal differentiation.

Zbtb45 mRNA knockdown results in moderate changes in gene expression of several transcription and signaling factors. To find putative effector and/or target genes that could be associated with the effects of Zbtb45 knockdown, we analyzed the mRNA expression of a number of transcription factors and signaling molecules involved in stem cell characteristics, glial differentiation and telencephalic development. In all experiments, we observed an upregulation to a varying extent (148.8 ± 34%) at 1 DIV of Hes5 mRNA (**Fig. 3E**), a bHLH-factor and downstream effector of Notch signaling that has been previously linked to inhibition of myelin gene expression.[31] The expression of Mash1, another bHLH factor implicated in oligodendrocytic differentiation, was unaffected (data not shown). In accordance with the decrease of oligodendrocyte-associated genes, Olig2 expression was slightly downregulated (73.6% ± 4.0) at 1 DIV followed by decreased Sox10 expression at 2 DIV (71.9% ± 4.6) (**Fig. 3D and E**). Furthermore, there was no significant changes in the expression levels of Sox9, Olig1, Hes1, BMP4 or BMP7 mRNA, signaling factors known to regulate astrocytic and oligodendrocytic gene expression and differentiation of telencephalic NSCs[21] (**Fig. 3D and E** and data not shown). In line with the relatively mild effect on neuronal differentiation, Zbtb45 knockdown did not affect the expression of the transcription factor REST, a well-established repressor of neuronal gene expression (**Fig. 3D**).

RNA knockdown of the Zbtb45-associated transcriptional co-regulator Med31 does not directly influence glial gene expression in NSCs. Although the BTB-domain has been shown to interact with many different co-regulators, the only confirmed protein interaction of ZBTB45/Zbtb45 reported so far is Med31, a subunit of the Mediator complex, as identified in a global yeast two-hybrid assay.[32] The Mediator complex can function both in repressing and activating transcription when recruited to target promoters, by coordinating RNA polymerase II binding.[33] Despite its ubiquitous requirement at RNA polymerase II promoters, some Mediator sub-complexes have been reported to confer distinct regulatory functions during development.[34-37] Med31 mRNA levels in NSCs were detected in a similar range as TBP, and siRNA against Med31 resulted in a dramatic decrease of the mRNA levels compared to control cultures (to 11.5–13.2% compared to control) (**Fig. 3F**). Employing the same assays as described above for Zbtb45 mRNA knockdown, we did not find any significant difference in NSC survival or differentiation after Med31 mRNA knockdown compared to siRNA control (**Fig. 3F**). These experiments thus failed to support any role for Med31 in Zbtb45 siRNA mediated effects on glial differentiation in NSCs.

Loss of Zbtb45 in CG-4 oligodendrocyte precursors inhibits oligodendrocyte maturation. To further investigate a potential shift in glial fate proportions as induced by Zbtb45 knockdown in NSCs, we turned to the bi-potent oligodendrocyte precursor cell line CG-4. CG-4 cells spontaneously differentiate into mature oligodendrocytes upon mitogen withdrawal (FGF2 and platelet-derived growth factor (PDGF-BB)),[23] but can also form astrocytes when grown in 10% FBS. It should be noted that astrocyte differentiation of CG-4 cells has not been reported without stimulating the cells with FBS. Zbtb45 siRNA was introduced by lipofection and changes in gene expression were monitored 1 and 3 DIV post-transfection. Zbtb45 mRNA expression was decreased to levels around 40% of control culture levels at both time points (**Fig. 4A**). While the relative levels of gene expression is shown in **Figure 4A**, it should be noted regarding the absolute levels that upon mitogen withdrawal, control cells at 1 DIV expressed high levels of CNPase but only basal levels of MBP. At 3 DIV, CNPase levels were increased and MBP expression was clearly present in the control cultures. Although no significant effect on oligodendrocyte related gene expression was seen after 1 DIV, Zbtb45 knockdown caused a reduction of CNPase (61.1% ± 3.7 compared to control) and MBP (25.3% ± 4.6 compared to control) mRNA expression after 3 DIV, in accordance with the results from the NSCs (**Fig. 4A**). The inhibitory effect on oligodendrocyte maturation was confirmed by immunocytochemistry using an antibody against MBP, 7 DIV after transfection of

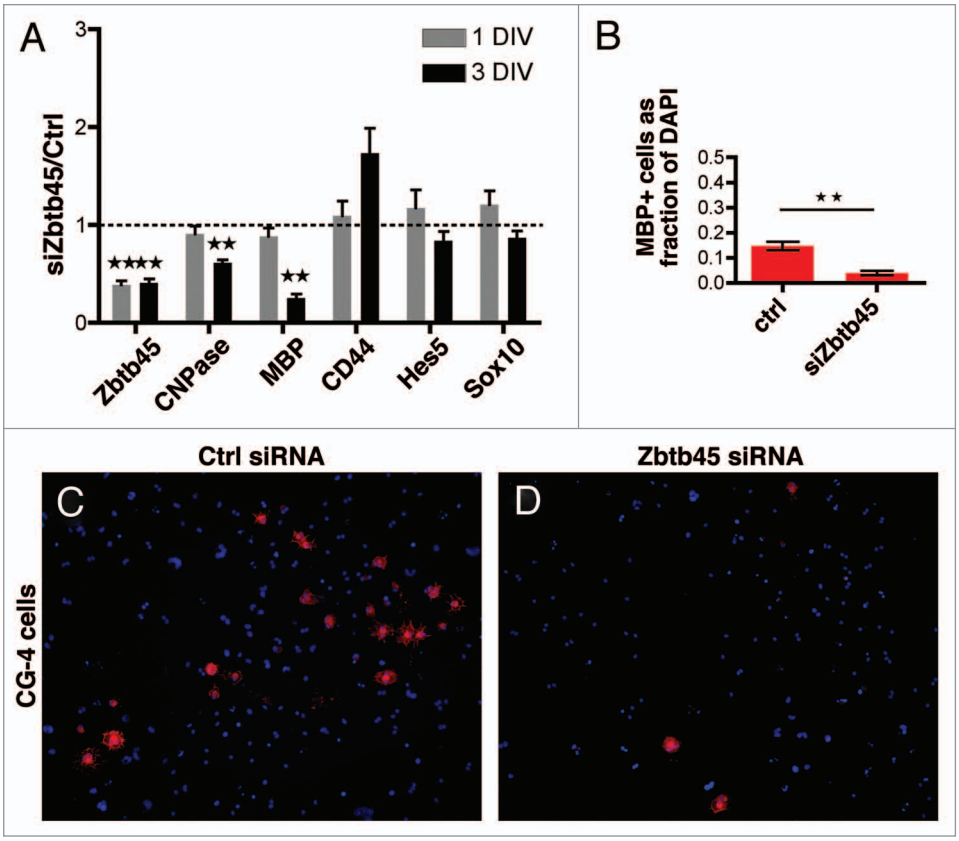

Figure 4. Delivery of Zbtb45 siRNA to CG-4 oligodendrocyte precursors inhibited oligodendrocyte maturation. (A) RT-qPCR analysis of mRNA levels in NSCs after 1 (gray bars) and 3 DIV (black bars) revealed that siRNA against Zbtb45 led to significant changes in expression levels of markers oligodendrocytes along with an upregulation of the astrocyte related gene CD44. Bars indicate mRNA levels of siZbtb45 cells relative to control. (B) CG-4 cultures lacking Zbtb45 contained significantly less MBP$^+$ cells. (C and D) Immunocytochemistry with MBP (red) with DAPI (blue) counterstain. 20x magnification. siZbtb45 lead to a marked decrease in mature oligodendrocytes after 7 DIV compared to control. n = 3 independent experiments (**p < 0.01), error bars = SEM.

Zbtb45 siRNA and mitogen withdrawal (**Fig. 4B–D**). siZbtb45 transfected cultures displayed a striking reduction in mature oligodendrocytes in contrast to the fully differentiated control cells (24.8% ± 7.3 MBP-positive cells compared to control, $p < 0.01$) (**Fig. 4B–D**). The differentiating CG-4 cells did not express GFAP mRNA at any time point after mitogen withdrawal but interestingly the expression of the astrocyte-associated marker CD44 was increased after 3 DIV in all experiments but to a varying extent (173.4% ± 31) (**Fig. 4A**). Hes5 and Sox10 mRNA levels remained largely unaffected (**Fig. 4A**).

Loss of Zbtb45 does not affect cell proliferation. To assess the possibility that the change in glial gene expression and differentiation seen in the NSCs and CG-4 cells upon Zbtb45 knockdown would be due to changes in proliferation thereby causing a shift in lineage proportions, we first investigated the expression of cyclin-dependent kinase inhibitors (CKis) in these cultures. CKis have been implicated in controlling cell cycle exit and also been shown to influence the differentiation of neural stem cells, oligodendrocytes and myelinating Schwann cells.[38-41] In NSCs, levels of $p21^{Cip1}$ and $p27^{Kip1}$ were slightly positively affected by Zbtb45 mRNA knockdown at 2 DIV, whereas a significant temporal decrease in $p57^{Kip2}$ mRNA expression was detected after 1 DIV (**Fig. 5A**). In contrast, no significant changes in the expression levels of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ mRNA expression could be detected in the CG-4 cells after 1 or 3 DIV (**Fig. 5B**).

To investigate whether Zbtb45 siRNA had a more general effect on progenitor proliferation, we determined the distribution of cells in different phases of the cell cycle by 5-bromo-2-deoxyuridine (BrdU) analysis. Zbtb45 siRNA was delivered to NSCs and CG-4 cells by nucleofection and lipofection respectively, and the cells were subsequently grown in proliferating or differentiating conditions. We then treated NSCs and CG-4 cells for 2 h pulse with 20 mM BrdU to estimate the ratio of cells in S-phase 24 h post siRNA delivery. No significant differences in BrdU incorporation in NSCs or CG-4 cells were detected after Zbtb45 knockdown versus control cultures in any of the conditions tested (**Fig. 5C** and data not shown). It should be noted that we have previously shown that NSC-derived astrocytes can remain proliferative several days after initiation of differentiation,[41] and that the differentiation thus may not per se result in a major effect on proliferation in these cultures. Our results suggest that the effects on glial differentiation and associated gene expression by Zbtb45 knockdown were independent of the expression of these cell cycle inhibitors and major direct effects on proliferation.

Discussion

Increased and/or precocious astrocytic gene expression and differentiation have been observed after gene deletion or knockdown of a limited number of transcription factors. Double mutant mice harboring deletions in Olig1/Olig2 or Mash1/Ngn2 show increased astrocytic differentiation in the spinal cord and forebrain, respectively,[42,43] and the bHLH factor Hes6 has been shown to repress astrocytic differentiation and promote neuronal differentiation.[44] In addition, NSCs isolated from mice harboring gene deletion for the BTB/POZ domain-interacting co-repressors NCoR or SMRT show spontaneous astrocytic differentiation even in the presence of FGF2.[17,19] The results from the present study suggest that Zbtb45 is a novel factor essential for proper glial differentiation as Zbtb45 mRNA knockdown results in increased astrocytic gene expression and

differentiation, and aberrant oligodendrocyte gene expression and differentiation in multipotent telencephalic NSCs and oligodendrocyte precursors.

The underlying mechanism/s for the shifts in gene expression levels caused by Zbtb45 knockdown are yet to be elucidated. The ubiquitous expression pattern of Zbtb45 in the developing forebrain opens for the possibility that Zbtb45, like other Zbtb proteins,[45] can be part of several specific transcriptional complexes, a subset of which seems to be essential for the proper regulation of glial differentiation in neural progenitors. Because of the reported interaction with Med31, a member of the Mediator complex,[32] we used a similar approach as with Zbtb45 to investigate whether loss of Med31 mRNA should show any similarities with Zbtb45 knockdown. These experiments failed however to support any role for Med31 in the Zbtb45-dependent events in NSCs (**Fig. 3F**).

The highly conserved BTB/POZ domain of Zbtb proteins have been shown to interact with various co-repressors such as CtBP, NCoR and SMRT that in turn are part of HDAC-containing and chromatin remodeling complexes.[46] Loss of either SMRT or NCoR results in increased astrocytic gene expression and differentiation, and Akt kinase-mediated translocation of NCoR from the nucleus to the cytoplasm is sufficient to induce astrocytic differentiation, a repressor mechanism protected by protein phosphatase-1 (PP1).[17] We have not detected any rapid or significant downregulation of NCoR or SMRT levels after Zbtb45 knockdown (Lilja T and Hermanson O, unpublished observations), but this observation does not exclude certain post-translational effects, such as a change in the subcellular localization of NCoR or SMRT. Future studies will aim at elucidating whether the effect of Zbtb45 knockdown in NSCs could be a result of impaired recruitment of NCoR and/or SMRT to key regulatory elements.

Despite the developmental impact of Zbtb proteins and their implications in stem cell differentiation, little is understood regarding the mechanisms by which Zbtb proteins regulate gene expression in the developing nervous system. However, the ubiquitous expression of Zbtb45 in the developing CNS suggests a more general function than specifically regulating glial differentiation of NSCs or glia progenitors. It gives further support to view Zbtb proteins as a part of the transcriptional machinery acting as molecular docking bays for co-factors and other transcription factors, rather than being the end-point in a given signaling pathway or acting as a bona fide transcriptional repressor. In addition to Med31, CtBP and NCoR/SMRT, an additional possibility is that Zbtb45 is involved in the recruitment of Polycomb-group complexes through the BTB/POZ domain as this interaction has been proposed for the Drosophila transcriptional repressor Tramtrack and the Tramtrack interacting GAGA factor.[47] Current studies are therefore focusing on the relation between Zbtb45 and various complexes in NSCs in detail. We conclude that Zbtb45 is a novel factor required for proper glial differentiation of multipotent NSCs and glial precursors, and we suggest that this may be due to loss or aberrant recruitment of specific co-regulatory complexes to crucial genes as a consequence of reduced levels of Zbtb45.

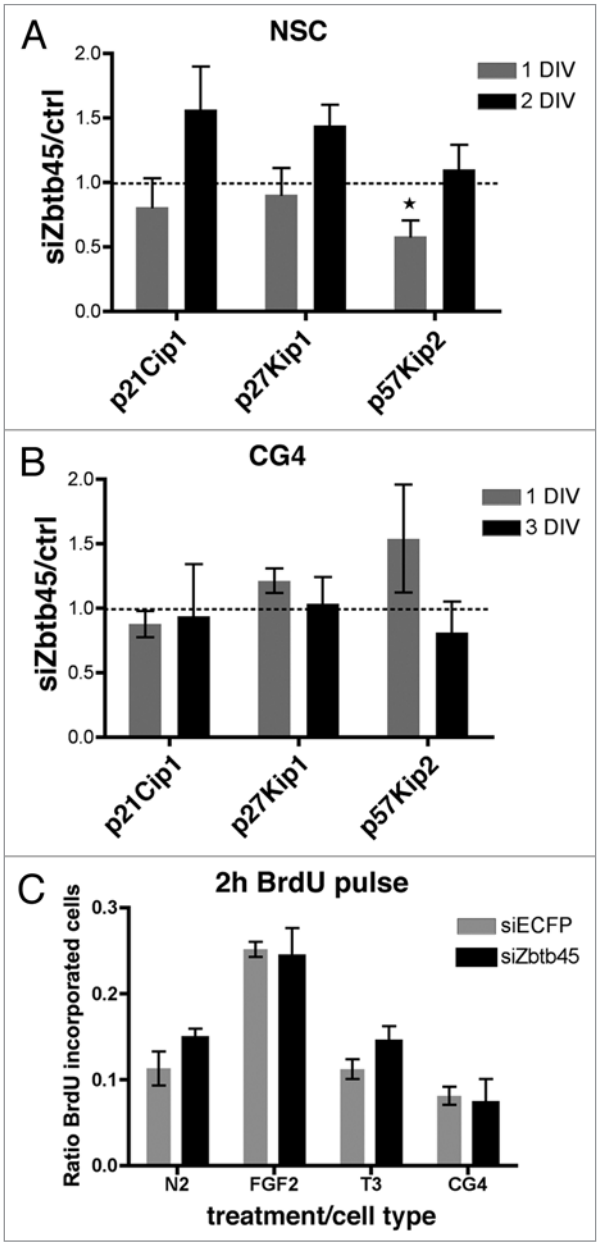

Figure 5. Zbtb45 siRNA did not significantly affect proliferation or cell cycle inhibitor expression. (A) RT-qPCR analysis of mRNA levels of cyclin-dependent kinase inhibitors in NSCs 1 and 2 DIV after Zbtb45 knockdown. A temporal, significant downregulation of $p57^{kip2}$ mRNA levels was observed at 1 DIV (B) RT-qPCR analysis of mRNA levels of cyclin-dependent kinase inhibitors in CG-4 cells 1 and 3 DIV after Zbtb45 knockdown. (C) Bars indicate numbers of cells incorporating BrdU after a 2 h pulse relative to total number of nuclei. No significant difference in BrdU incorporation was detected in NSCs lacking Zbtb45 relative to control in N2 media, presence of FGF2 or thyroid hormone (T3) or CG-4 cells in proliferating conditions. n = 5 (A) or 3 (B and C) independent experiments (*$p < 0.05$), error bars = SEM.

Materials and Methods

Sequence analysis and alignments. Amino acid sequences for mouse Zbtb45, Zbtb20, Zbtb7, PLZF and Bcl-6 were obtained from NCBI databases, and aligned using default parameters

(gap open cost = 100; Gap extension cost = 1.0) in the CLC Free Workbench software, along with a neighbor-joining phylogenetic tree construction (default parameters; bootstrap analysis, 100 replicates). Conserved domains of the primary Zbtb45 amino acid sequence were predicted using the CDD tool[27] provided by NCBI Basic Local Alignment Search Tool (BLAST).

In situ hybridization. *Tissue preparation.* Heads from embryonic day (E) 12.5 and E14.5 of mouse embryos were fixed in 4% paraformaldehyde (Sigma) for 2–5 h and then transferred to 30% sucrose at +4°C over night before frozen in Tissue-Tek (Sakura Finetek) on dry ice and kept in -70°C until cryostat-sectioned (Microm) onto SuperFrost Plus glasses (Menzel-GmbH & Co., KG) as 12 μm sections.

Radio-labeled in situ hybridizations. Two different ^{35}S-labeled mRNA probes were made by 3'-labeling of the synthetic oligonucleotides 5'-GCT TCG CCC TGT GCC ACC ACC AAC GAG CCG CTG TAA AGG AAC T-3' and 5'-TGC GGC AGT GGC TAC ACT CAT AGG CGG GTT GCT CAG CAC CTT G-3' (CyberGene AB, Huddinge, Sweden) using terminal deoxynucleotidyl transferase (GE Healthcare, Amersham, UK) in a cobalt-containing buffer with ^{35}S-dATP (Perkin-Elmer, Waltham, MA) to a specific activity of 1–4 x 10^9 d.p.m./μg and purified in QIAquick spin columns (Qiagen, Hilden, Germany). Tissue-sections were air-dried and incubated for 16 h at 42°C with 0.5 ng of each labeled Zbtb45 probe(s). The probes were diluted in a hybridization solution containing 50% deionized formamide (Sigma), 4x standard saline citrate, 1x Denhart's solution, 1% N-lauroylsarcosine (Sigma), 10% dextran sulfate (Sigma), 500 μg/mL heat-denatured salmon sperm DNA (Sigma) and 200 mM dithiotreitol (DTT; Sigma). After hybridization, the sections were rinsed in 1x SCC four times for 15 min at 55°C, followed by 45 min at room temperature. The sections were then dehydrated in ethanol, and dipped in NTB2 nuclear track emulsion (Kodak, Rochester, NY, USA). After two to three weeks, the slides were developed in D-19 developer (Kodak), counterstained and coverslipped. The sections were examined in a Nikon (Tokyo, Japan) Mocrophot-FX microscope equipped with a dark field condenser. To control for specificity, additional sections were incubated with hybridization cocktail containing an excess (100x) of non-labeled probe. In addition, a scrambled, non-sense probe were used for additional control. Animals were treated in accordance with institutional and national guidelines, ethical permit. [Ethical permit no. N110-07, approved by the ethics committee for animal research in Stockholm (norra), Sweden].

Embryonic neural stem cell cultures and rat tissue dissections. For analysis of Zbtb45 expression by RT-qPCR, various tissues and regions of the developing brain were dissected in animals at E15. Heart and forelimb structures were easily separated while the dissection of the nervous system regions was validated by expression of the transcription factors Ascl1 (Mash1), Neurog2 (Ngn2), together with OMB as a marker of the olfactory bulb. The regions were defined as follows: olfactory bulb: Ascl1$^+$/Neurog2$^+$/OMB$^+$; pre-thalamus: Ascl1$^+$/Neurog2$^+$/OMB$^-$; cortex: Ascl$^-$/Neurog2$^+$/OMB$^-$; ganglionic eminences: Ascl1$^+$/Neurog2$^-$/OMB$^-$, using Zbtb45 expression as standard.

The cortical neural stem cell culture was obtained as previously described in reference 16, 21, 41 and 48. Briefly, rat cortical tissue from E15.5 was dissociated and 800 x 10^3 cells were plated per 10 cm dish, previously coated with poly-L-ornithine and fibronectin (Sigma-Aldrich). Neural stem cells were then expanded in N2 media with 10 ng/mL of basic fibroblastic growth factor (FGF2; R&D Systems) and passaged by dissociation in the presence of HBSS, $NaHCO_3$ and HEPES (from Invitrogen and Sigma-Aldrich). NSCs were differentiated for up to 7 days in vitro (DIV), in absence of FGF2 or with CNTF (R&D Systems, 10 ng/mL), BMP4 (R&D Systems, 10 ng/mL) or thyroid hormone (T3; Sigma-Aldrich, 50 ng/mL). Factors were added daily and media was changed every second day. After 3 or 7 DIV, cells were fixed with 10% formalin (Sigma-Aldrich) and analyzed by immunocytochemistry or RT-qPCR after 1 or 2 DIV. Animals were treated in accordance with institutional and national guidelines [Ethical permit no. N310-05 and 109-07, approved by the ethics committee for animal research in Stockholm (norra), Sweden].

CG-4 OLP cultures. The post-natal OLP cell line was cultured as previously described in reference 23. Briefly, cells were plated in flasks coated with poly-L-ornithine and fibronectin (Sigma-Aldrich) and expanded in N1 media supplemented with FGF2 (10 ng/mL) and PDGF-BB (10 ng/mL, both from R&D Systems). Cells were passaged with trypsin (Invitrogen) and DNase I (Roche), and differentiated by removal of the mitogens. For lipofection or differentiation assays, CG-4 cells were plated at 1 x 10^5 cells in 35 mm plates (Corning).

Transfection by nucleofection and lipofection. siRNA delivery to NSCs were made by nucleofection in a Nucleofector (Amaxa), using program A-33, under conditions recommended by the manufacturer. 3 μg of DNA were used per nucleofection. siRNA delivery to CG4 cells were made by lipofection using Lipofectamine 2000 (Invitrogen). 3 μg of siRNA were used per 35 mm plate. Opti-MEM was used to prepare the complexes. Cells were changed to D-MEM medium (Invitrogen) and incubated with the complexes for 4 hours, before returning to their original medium. Cells were collected for immunocytochemistry or RT-qPCR analysis after 1 or 3 DIV. Lipofection of siRNA in differentiated conditions was performed after 3 DIV in differentiating conditions. The following target sequences for siRNAs (Dharmacon or Invitrogen) were used:

ECFP control: 5'-GAA GAA CGG CAU CAA GGC C-3',

Zbtb45: 5'-UGA AGA AGG UGA AGG CGU U-3',

Zbtb45 alternative: 5'-CAG UGC ACC ACA UCC ACC UAC AGA A-3',

Med31: 5'-UGG AAA UCG ACU UCG GUU U-3'.

Primer design. Gene specific primers were achieved from Universal ProbeLibrary Assay Design Center (Roche Applied Science) or designed with the Primer3 software.[49] The specificity of PCR primers was determined by BLAST run of the primer sequences. All primers were purchased from MWG Biotech. All primer sequences can be obtained on request.

Reverse transcription and qPCR. Total RNA was isolated from embryonic tissue, NSCs and CG-4 cells using RNeasy extraction kit (Qiagen), with DNAse treatment in-column.

200 ng of total RNA were used per synthesis reaction with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Alternatively, Superscript II reverse transcriptase (Invitrogen) was used. RT-qPCR reactions were performed in duplicates for each sample. Each PCR reaction had a final volume of 25 μl and 1 μl of 25 x diluted cDNA. RT- was run for a few samples in each run to discard genomic DNA amplification. Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used, according to the manufacturer's instructions (but with a 4 x dilution from the original mastermix, instead of 2 x). The following thermo cycling program was used: 50°C for 2 min, 94°C for 2 min and then 40 cycles of 94°C for 30 s, 59/60°C for 30 s and 72°C for 30 s on the ABI PRISM 7000 Detection System (PE Applied Biosystems, Foster City, CA, USA). A melting curve was obtained for each PCR product after each run, in order to confirm that the SYBR Green signal corresponded to a unique and specific amplicon. Then, the ΔΔCt method was used for quantifying expression levels relative to TATA-binding protein (TBP), or in a few instances, hypoxanthine guanine phosphoribosyl transferase (HPRT) as endogenous controls. For each individual experiment, sample values were normalized by the average sample value of the experiment.

Immunocytochemistry and cell quantification. Cells were fixed in 10% formalin for 15 min and washed in PBS with 0.1% Triton x100, 3 times for 5 min. After an overnight incubation at 4°C with the primary antibody rat MBP (MAB386, Chemicon, 1:250), mouse TuJ1 (Biosite #MMS-435P, 1:500), rabbit GFAP (DakoCytomation #Z0334, 1:500) in PBS with 1% bovine serum albumin (BSA) and 0.1% Triton x100, cells were washed six times 5 min with PBS with 0.1% Triton x100 followed by a 2 h incubation with the appropriate secondary antibody (conjugated to Alexa 488 and Alexa 546/594, 1:500, Molecular Probes/Invitrogen) in PBS with 1% BSA and 0.1% Triton X100. After three washes for 5 min with PBS, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Images were acquired with a Zeiss Axioskop 2 microscope (objectives: 5x/0.25, Zeiss Fluar ∞/0.17, 10x/0.3, Zeiss NeoFluar ∞/0.17 and 20x/0.50, Zeiss Neo-Fluar ∞/0.17) and collected with a Zeiss AxioCam camera MRm (with Axiovison Rel 4.6 software). Images were processed with Adobe Photoshop CS2 version 9.0.2. For BrdU assay, NSCs and CG-4 cells where transfected with Zbtb45 siRNA along with ECFP control siRNA by nucleofection or lipofection. Then after 1 DIV, the cells where exposed to a 20 μM BrdU concentration in differentiating medium conditions for 2 h followed by immunocytochemical analysis of BrdU incorporation. Quantitative immunocytochemical data of NSCs and CG4 cultures represents means ± SEM, obtained from five non-overlapping 10 x fields in each condition, from three separate independent experiments, after normalization with the number of DAPI positive cells present in the same fields. For DAPI quantification, a macro was programmed in Image J. Cells present in clusters were counted manually with the Cell Counter Plug-in. Pyknotic and viable cell nuclei were counted by defining nuclei as being > 20 pixels and viable nuclei being > 70 pixels on 10x fields.

Statistical analysis. Statistical analysis and graphs were performed using the software Prism 4 (Graph Pad). When comparing to a hypothetical control value (100%), one sample t-test was used. When two groups were compared, statistical analysis was performed using ANOVA followed by two-tailed unpaired t-test. Comparisons were performed between all treatments, unless stated otherwise. The threshold value for statistical significance (α value) was set at 0.05 (*$p < 0.05$, **$p < 0.01$). In all graphs, results are expressed as mean ± standard error mean (SEM). The statistics in the RT-qPCR experiments were based on three (CG4) or five (NSCs) independent experiments, and in the immunocytochemistry on five non-overlapping 10x fields from three separate independent experiments in each condition as described in detail above.

Acknowledgements

We would like to thank Dr. J.C. Louis (Amgen) for the CG4 cell line, Stephanie Robertson, Gonçalo Castelo-Branco, Anna Cascante, and other members of the Hermanson lab for input, and Christian Broberger and Kylie Foo for valuable technical advice. This study was supported by grants from the Swedish Research Council (VR), the Swedish Cancer Society (CF), the Jeansson Foundation, the Åke Wiberg Foundation, the Åhlén Foundation, the Swedish Medical Society, Karolinska Institutet, the Swedish Foundation for Strategic Research (SSF), K&A Wallenberg Foundation (*CLICK*), and the Swedish Childhood Cancer Foundation (BCF) to O.H.

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