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ORIGINAL ARTICLE

EPAC Negatively Regulates Myelination *via* Controlling Proliferation of Oligodendrocyte Precursor Cells

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Abstract Increasing evidence suggests that a cyclic adenosine monophosphate (cAMP)-dependent intracellular signal drives the process of myelination. Yet, the signal transduction underlying the action of cAMP on central nervous system myelination remains undefined. In the present work, we sought to determine the role of EPAC (exchange protein activated by cAMP), a downstream effector of cAMP, in the development of the myelin sheath using EPAC1 and EPAC2 double-knockout ($EPAC^{dKO}$) mice. The results showed an age-dependent regulatory effect of EPAC1 and EPAC2 on myelin development, as their deficiency caused more myelin sheaths in postnatal early but not late adult mice. Knockout of EPAC promoted the proliferation of oligodendrocyte precursor cells and had diverse effects on myelin-related transcription factors, which in turn increased the expression of myelin-related proteins. These results indicate that EPAC proteins are negative regulators of myelination and may be promising targets for the treatment of myelin-related diseases.

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Introduction

In the central nervous system (CNS), oligodendrocytes (OLs) provide electrical insulation and trophic support for neurons by myelinating axons [1]. OL precursor cells (OPCs), which are generated from the neural tube during CNS development, differentiate into OLs and migrate throughout the CNS [2]. The transition of OLs from proliferative to differentiated stages is a highly coordinated process and is critical for understanding myelin formation and the repair of demyelinating diseases. To date, more and more molecules have been shown to play important roles in the development of the myelin sheath, including axonally-expressed ligands [3–5], nuclear transcription factors [6–10], and mitogens [11–15].

Cyclic adenosine monophosphate (cAMP) is a key regulator of metabolic activity, survival, proliferation, and differentiation in a variety of cell types. Increasing evidence suggests that a cAMP-dependent intracellular signal drives the process of myelination. For example, an increase or decrease in cAMP level in dorsal root ganglion neurons results in suppressed or enhanced myelination, respectively [16]; and enhanced intracellular cAMP prevents the demyelination in the corpus callosum of mice induced by cuprizone [17]. Yet, the signal transduction underlying the action of cAMP on CNS myelination remains undefined.

cAMP controls complex cellular processes primarily through the activation of two downstream effectors, PKA (protein kinase A) and EPAC (exchange protein activated by cAMP) [18]. The catalytic subunits of PKA

phosphorylate and modulate the activity of cytosolic and nuclear substrates [18]. EPAC directly transduces cAMP signals as a guanine nucleotide exchange factor for the small GTP-binding protein Rap1 [18]. However, their potential roles in the proliferation and differentiation of OPCs are still elusive. In the present work, we sought to examine the role of EPAC in the development of the myelin sheath using EPAC1 and EPAC2 double-knockout ($EPAC^{dKO}$) mice. Our results showed that EPAC deficiency caused a developmentally-dependent increase of myelin sheaths, which may be due to increased proliferation of OPCs. We also showed that EPAC had diverse effects on myelin-related transcription factors, which in turn increased the expression of myelin-relation proteins.

Materials and Methods

Animals

The experiments in the present work were approved by the Animal Experimentation Ethics Committee of Zhejiang University. Original breeding pairs of $EPAC^{dKO}$ mice were obtained from Prof. Youmin Lu [19]. The resulting offspring were genotyped using PCR of genomic DNA. Mice were kept at the Experimental Animal Center of Zhejiang University under temperature-controlled conditions on a 12:12 h light/dark cycle. All experiments were performed blind to genotype in littermates of either sex.

Antibodies and Reagents

Antibodies against EPAC1, EPAC2, cyclin D1, and cyclin-dependent kinase 4 (CDK4) were from Cell Signaling (Danvers, MA). Antibodies against 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), myelin basic protein (MBP), Olig2, TCF4, SOX2, SOX10, CC-1, and GAPDH were from Millipore (Billerica, MA). Antibodies against GPR17, myelin regulatory factor (Mrf), Lamin B, yin-yang 1 (YY1), and CDK5 were from Santa Cruz (Dallas, TX). The antibody against Ki67 was from Abcam (Cambridge, UK). Myelin-associated glycoprotein (MAG) antibody, IgG antibody, and Alexa Fluor-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated secondary antibodies for immunoblotting were from GE Healthcare (Waukesha, WI). Proteinase inhibitor was from Merck Chemicals (Darmstadt, Germany).

Western Blotting

Protein assays were performed using Western blots as in previous work [20]. Primary antibodies and their dilutions used for Western blots were EPAC1 (1:1000), EPAC2 (1:1000), cyclin D1 (1:500), CDK5 (1:500), MBP (1:5,000), MAG (1:2000), CNP (1:5000), TCF4 (1:1000), Mrf (1:1000), YY1 (1:200), SOX10 (1:1000), SOX2 (1:1000), GPR17 (1:2000), LaminB (1:500), and GAPDH (1:10000). The signals on films were scanned and quantitated using ImageJ 1.42q (NIH, Bethesda, MD).

Immunohistochemistry

Immunohistochemistry was performed as in our previous work [20]. Secondary antibodies were diluted at 1:1000. The dilutions of primary antibodies were MBP (1:500), Olig2 (1:500), Ki67 (1:1000), and CC-1 (1:1000).

Electron Microscopy (EM)

EM studies were performed as in previous work [20, 21] using a Philips CM100 microscope (FEI). ImageJ was used to measure the ratio of axonal diameter to fiber diameter (*g*-ratio).

Statistics

Excel 2003 (Microsoft), the SPSS 16.0 statistical program (SPSS), and Igor Pro 6.0 (Wavemetrics), were used for data analysis. Statistical differences were determined using unpaired two-sided Student's *t*-test. The accepted level of significance was $P < 0.05$. "*n*" represents the number of animals or cultures tested. Data in the text and figures are presented as the mean ± SEM.

Results

Expression of EPAC Proteins in Developing Brain

To investigate the roles of EPAC proteins in OL development, we first examined their expression in the postnatal brain. Equal amounts of proteins from mouse brain homogenates at different ages (birth to 4 months) were resolved by SDS-PAGE and immunoblotted with antibodies recognizing EPAC1, EPAC2, and MBP, a vital marker protein of OL maturation. We found that the expression of EPAC1 and EPAC2 increased with age and was roughly proportional to MBP expression (Fig. 1A). However, the changing progress of EPAC1 and EPAC2 appeared different: EPAC1 started to slow down after P30 while EPAC2 increased constantly throughout (Fig. 1A). Since *in vivo* Western blotting could not distinguish EPAC proteins from different types of neural cells, we further used *in vitro* cultures to determine the presence of EPACs in OPCs and mature OLs. Rat OPCs were collected from glial cultures

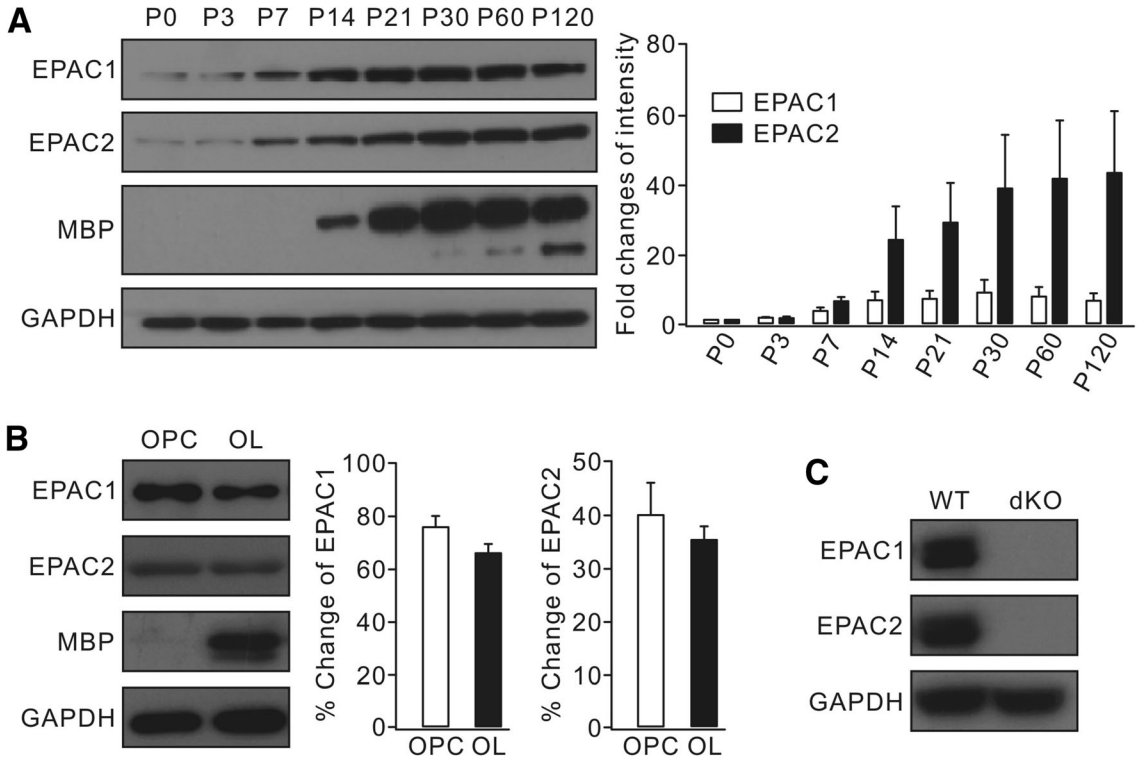


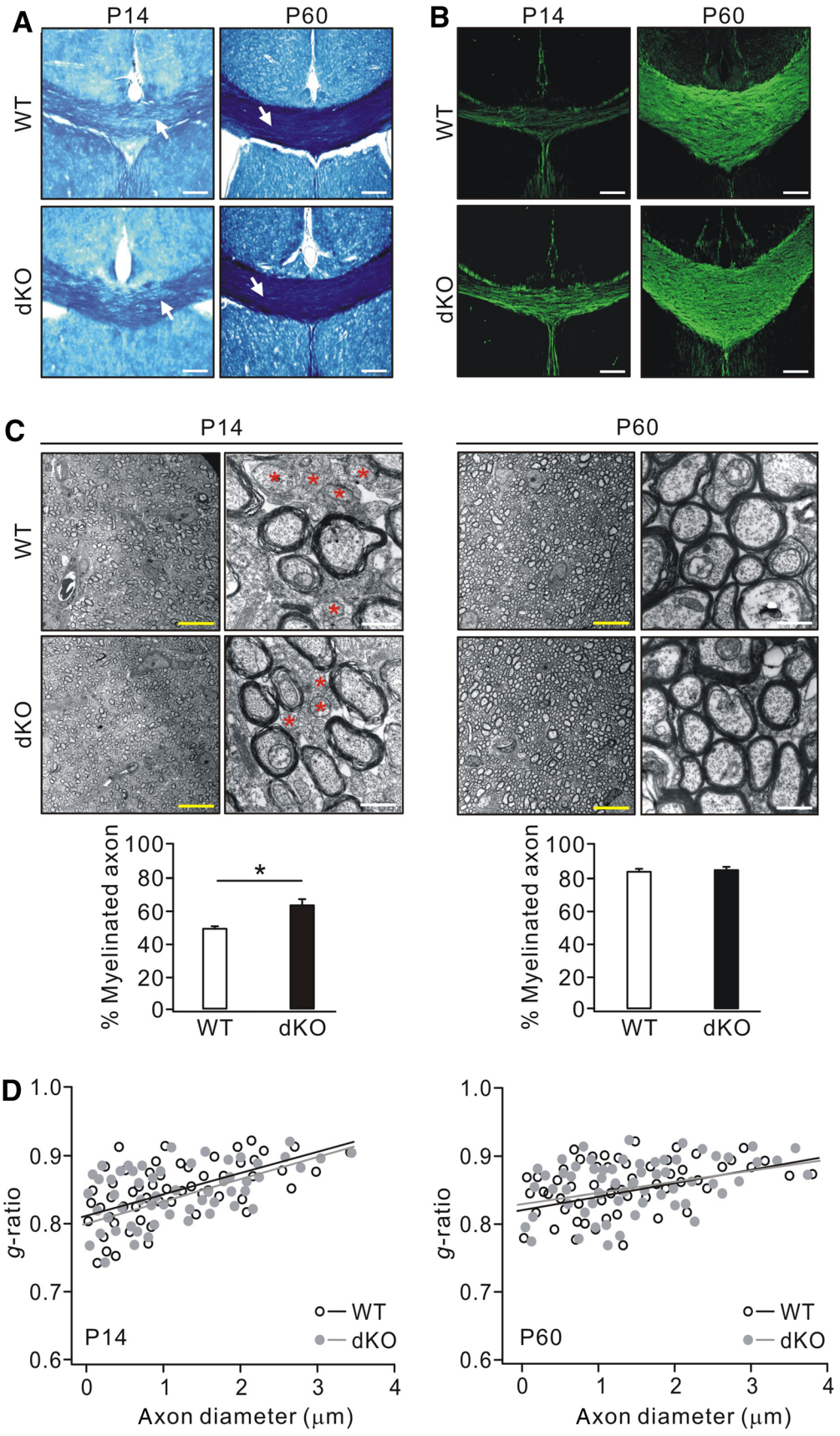
Fig. 1 Expression of EPAC proteins in the brain. **A** Western blots of one out of six independent experiments showing the expressions of EPAC1, EPAC2, and MBP in the brain from postnatal mice. The signal intensities of EPAC1 and EPAC2 were divided by that of GAPDH, the loading control. The ratios were normalized to P0 and fold changes are shown in the histogram. **B** Expression of EPAC1, EPAC2, and MBP in cultured OPCs and OLs. Three-day triiodothyronine treatment was used to stimulate OPC differentiation into mature OLs. GAPDH was the internal control. EPAC1 and EPAC2 were normalized to the corresponding GAPDH and their percentage changes are shown in the bar graphs. EPAC1: 75% ± 4% (OPC) and 66% ± 4% (OL) ($P > 0.05$). EPAC2: 40% ± 6% (OPC) and 35% ± 3% (OL) ($P > 0.05$). The experiment was repeated 4 times. **C** Western blots of EPAC1 and EPAC2 in the brain from WT and EPAC^{dKO} mice (P14). The experiment was repeated 4 times.

by shaking procedures and triiodothyronine was added to cultures and incubated for 3 days to allow OPC differentiation [21, 22]. Both EPAC1 and EPAC2 were abundantly expressed in OPC cultures (Fig. 1B). Triiodothyronine promoted OPC differentiation, as indicated by the expression of MBP (Fig. 1B). The expression of EPAC1 and EPAC2 was slightly lower in mature OLs than in OPCs, but there was no significance between two types of cell (Fig. 1B).

EPAC Deficiency Accelerates CNS Myelination

We next generated EPAC^{dKO} mice to evaluate their roles in CNS myelination. The deletion of EPACs was confirmed by Western blotting using mouse brain tissues (Fig. 1C). EPAC^{dKO} mice were viable and did not exhibit gait abnormality during adulthood.

Several experiments were conducted to investigate whether the ablation of EPACs affects CNS myelination. First, eriochrome cyanine staining showed that EPAC^{dKO} mice (P14) had a significantly higher density of white matter tracts of the corpus callosum than wild-type (WT) mice (Fig. 2A). Interestingly, the knock-out effect was age-dependent because the tracts displayed no difference between WT and EPAC^{dKO} mice at P60. Second, immunohistochemical staining indicated that EPAC^{dKO} mice had more MBP-positive fibers than WT mice at P14 (Fig. 2B). Similarly, the intensity of MBP staining in EPAC^{dKO} mice was close to that in WT mice at P60 (Fig. 2B). Third, we examined myelin sheaths in the cerebral cortex from WT and EPAC^{dKO} littermates using EM. The number of myelinated axons was significantly higher in EPAC^{dKO} than in WT mice at P14 (Fig. 2C). This phenotype again faded away in EPAC^{dKO} mice by P60 (Fig. 2C). Together, these results suggest that EPAC proteins act as negative regulators and their deficiency accelerates the progress of CNS myelination. To investigate whether other phenotypes occur in parallel with the reduced percentage of myelinated axons, we measured the thickness of myelin sheaths by morphometric quantification. The results showed that the *g*-ratios of optic nerves in EPAC^{dKO} mice did not differ from those in WT mice at both P14 and P60 (Fig. 2D).


A
P14
P60
WT
dKO
B
P14
P60
WT
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C
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% Myelinated axon
100
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60
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20
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*
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dKO
% Myelinated axon
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1.0
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Axon diameter (μm)
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1.0
0.9
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P60
WT
dKO
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4
Axon diameter (μm)

◀**Fig. 2** EPAC ablation increases myelination at P14 but not P60. **A** Cyanine staining showing myelin tracts (arrows) in the corpus callosum of WT and $EPAC^{dKO}$ mice at P14 and P60 (scale bars, 200 μm). **B** Immunohistochemistry reveals an increase of MBP-positive fibers in the corpus callosum of $EPAC^{dKO}$ mice compared with WT mice at P14, but not at P60 (scale bars, 200 μm). **C** EM images from optic nerves of WT and $EPAC^{dKO}$ mice at P14 and P60 (red asterisks, unmyelinated axons; yellow scale bars, 10 μm; white scale bars, 1 μm). Bar graphs in the lower panel show percentages of myelinated axons. P14: 48% ± 1% (WT) and 62% ± 2% ($EPAC^{dKO}$); n = 4/group, $^*P < 0.05$. P60: 83% ± 1% (WT) and 84% ± 1% ($EPAC^{dKO}$); n = 4/group. **D** Relationships between axonal diameters and g-ratios of WT and $EPAC^{dKO}$ mice at P14 and P60. In mice at P14, the regression equations were y = 0.032x + 0.81 (WT; n = 56) and y = 0.033x + 0.80 ($EPAC^{dKO}$; n = 61) ($P > 0.05$). For mice at P60, the regression equations were y = 0.02x + 0.82 (WT; n = 60) and y = 0.016x + 0.83 ($EPAC^{dKO}$; n = 70) ($P > 0.05$).

EPAC Deficiency Promotes the Proliferation of OPCs

To investigate how EPAC deficiency affects the progress of CNS myelination, we counted the number of OLs in WT and $EPAC^{dKO}$ mice using antibodies selective for OLs. Our results showed that the total number of OL lineage cells, as indicated by Olig2 staining, significantly increased in the corpus callosum of $EPAC^{dKO}$ mice at P14 (Fig. 3A). Moreover, the number of differentiated OLs, which were stained by both CC-1 and Olig2, was 23% higher in $EPAC^{dKO}$ mice than in WT mice at P14 (Fig. 3A). These results suggested that EPAC deficiency affects myelination through promoting OPC proliferation. The proliferative capacity of OPCs was further assessed by analyzing Ki67, which is expressed in active phases of the cell cycle. Immunohistochemical staining showed that there were more proliferating OLs (Ki67+/Olig2+) in the corpus callosum of $EPAC^{dKO}$ mice than in WT mice at P14 (Fig. 3B), confirming that EPACs contribute to OPC proliferation. The expression of Olig2 and CC-1 was also evaluated in $EPAC^{dKO}$ mice at P60. Statistical results showed that, at this age, the total numbers of Olig2+ and differentiated CC-1+/Olig2+ OLs were normal in the corpus callosum of $EPAC^{dKO}$ mice (Fig. 3C). The percentage of CC-1+/Olig2+ cells among Olig2+ cells was unchanged as well in $EPAC^{dKO}$ mice compared with WT mice at P60 (Fig. 3C). Thus, these results provided further evidence showing the age-dependent regulation of myelination by EPACs.

EPACs Regulate Early Expression of Myelin-specific Proteins

Myelin-related proteins appear in OLs prior to the onset of myelination and are continuously produced by OLs during anabolism and catabolism of the myelin sheath [23]. Myelin-related proteins are not only major components of myelin but also characteristic indicators of the myelination capacity. Hence, we examined myelin-specific proteins to define the effects of EPAC-knockout on myelination. Our results showed that the expression of MAG, CNP, and MBP was significantly higher in the cerebral cortex from $EPAC^{dKO}$ mice than WT mice at P14 (Fig. 4A). Consistent with the results from immunohistochemistry (Fig. 2), myelin-specific proteins were generally not affected by EPAC deletion at P21 and P60, with one exception that CNP expression increased at P21 (Fig. 4A).

Myelin maturation is earlier in the spinal cord than the cerebral cortex. Do EPAC proteins also regulate myelination in the spinal cord? To address this question, we examined MAG, CNP, and MBP in the spinal cord at three postnatal stages (P4, P14, and P21), and found that their expression was increased at P4 but unchanged at P14 and P21 in $EPAC^{dKO}$ mice compared with WT mice (Fig. 4B). The changes of myelin-specific proteins induced by EPAC ablation in the spinal cord were consistent with those in the cerebral cortex, strengthening the idea that the effect of EPACs on myelination is age-dependent.

How are myelin proteins regulated by EPACs? We assessed the mRNA levels of myelin-related genes in $EPAC^{dKO}$ mice to answer this question. Our results showed that the mRNA levels of MAG, CNP, MBP, and PLP were significantly higher in $EPAC^{dKO}$ mice than in WT mice at P14 (Fig. 4C), implying that EPAC ablation up-regulates the transcription of myelin-related genes.

Effects of EPAC Ablation on Transcription Factors and Cell Cycle-associated Proteins

A number of transcriptional regulators are critical for OPC differentiation and maturation. For example, SOX10, Olig2, Mrf, and YY1 promote OPC differentiation [24] whereas SOX6 arrests it [25]. Thus, the next question was whether EPACs modulate the expression of myelination-related transcription factors. In $EPAC^{dKO}$ mice at P14, we found that the expression of TCF4 and YY1 increased and that of GPR17 was reduced. However, the expression of the other transcription factors, Mrf, SOX10, and SOX2 was not changed by EPAC ablation (Fig. 5A, B). Since EPAC deficiency promoted the proliferation of OPCs (Fig. 3), we examined the expression of cell cycle-associated proteins and found that the total expression of several critical proteins essential to the cell cycle, including cyclin D1, CDK4, and CDK5, was not changed in $EPAC^{dKO}$ mice compared with WT mice at P14 (Fig. 5C). Taken together, these results suggest that EPAC ablation has complex effects on transcription factors and cell cycle-associated proteins.

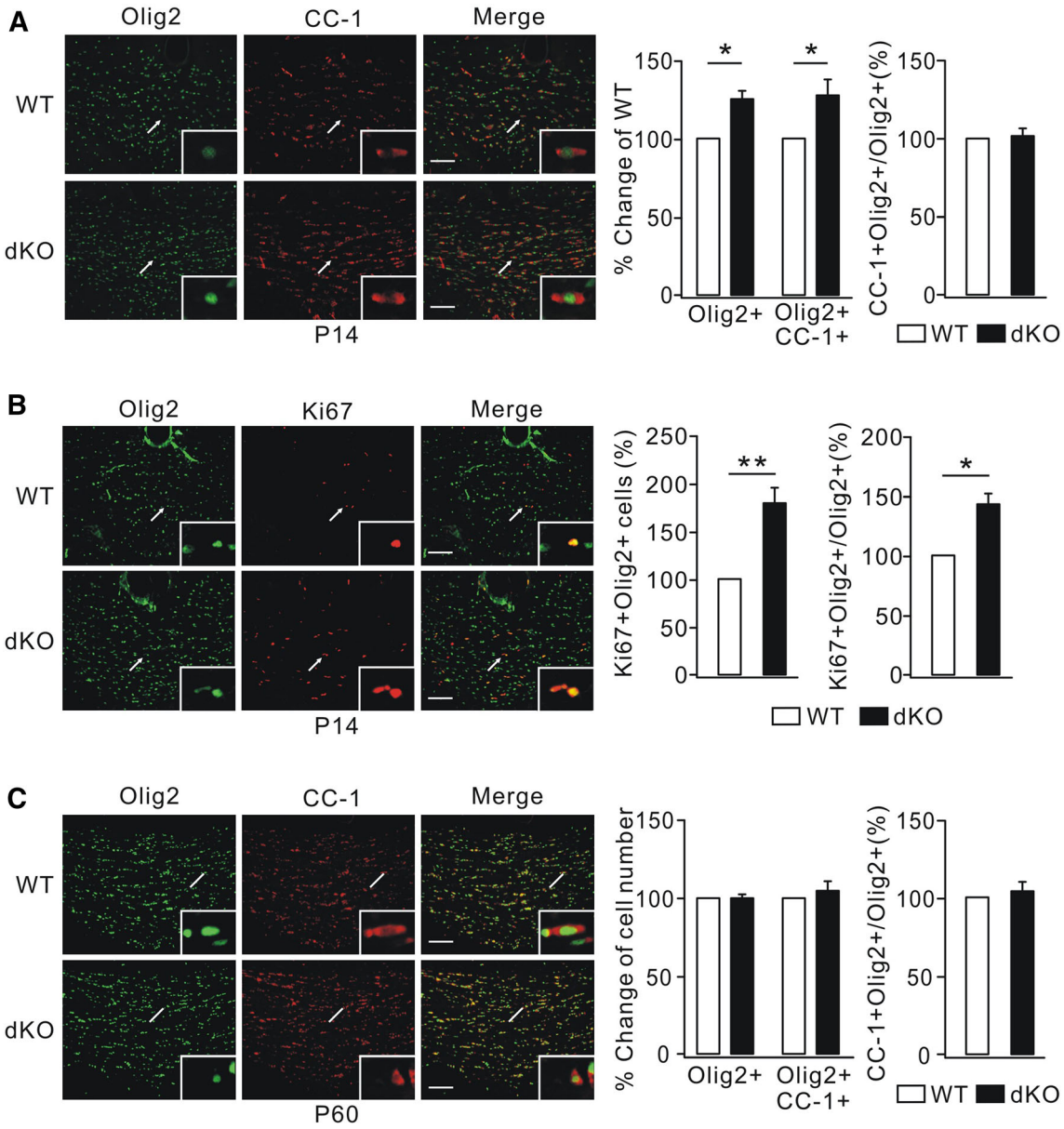

Fig. 3 EPAC ablation promotes OPC proliferation. **A** Double-staining of Olig2 and CC1 showing total (Olig2+) and differentiated (CC-1 + Olig2+) OLs in the corpus callosum from WT and EPAC^{dKO} mice at P14. Higher magnifications in boxes show the cells stained by Olig2 and CC-1 (arrows). Scale bars, 100 μm. The numbers of Olig2+ and CC-1+Olig2+ cells of EPAC^{dKO} mice were normalized to those of WT mice and the percentage changes (Olig2: 126% ± 5%, n = 4/group, $P < 0.05$; CC-1 + Olig2+: 128% ± 10%, n = 4/group, $P < 0.05$) are shown in the bar graphs. The ratio of CC-1 + Olig2+/Olig2+ cells of EPAC^{dKO} mice was normalized to that of WT mice and the percentage change (102% ± 5%, n = 4/group, $P > 0.05$) is also shown in bar graphs. **B** Double-staining of Olig2 and Ki67 showing total (Olig2+) and proliferating (Ki67+Olig2+) OLs in the corpus callosum from WT and EPAC^{dKO} mice at P14. Higher magnifications in boxes show the cells stained by both Olig2 and Ki67 (arrows). Scale bars, 100 μm. The number of Ki67 + Olig2+ cells of EPAC^{dKO} mice was normalized to that of WT mice and the percentage change (180% ± 16%, n = 4/group, $P < 0.01$) is shown in the bar graph. The ratio of Ki67 + Olig2+/Olig2+ cells of EPAC^{dKO} mice was normalized to that of WT mice and the percentage change (144% ± 8%, n = 4/group, $P < 0.05$) is also shown in the bar graphs. **C** Double-staining of Olig2 and CC1 showing total (Olig2+) and differentiated (CC-1 + Olig2+) OLs in the corpus callosum from WT and EPAC^{dKO} mice at P60. Higher magnifications in boxes show the cells stained by both Olig2 and CC-1 (arrows). Scale bars, 100 μm. The numbers of Olig2+ and CC-1 + Olig2+ cells of EPAC^{dKO} mice were normalized to those of WT mice and the percentage changes (Olig2: 100% ± 2%, n = 4/group, $P > 0.05$; CC-1 + Olig2+: 105% ± 6%, n = 4/group, $P > 0.05$) are shown in the bar graphs. The ratio of CC-1 + Olig2+/Olig2+ cells of EPAC^{dKO} mice was normalized to that of WT mice and the percentage change (105% ± 6%, n = 4/group, $P > 0.05$) is also shown in the bar graphs. *$P < 0.05$. **$P < 0.01$.

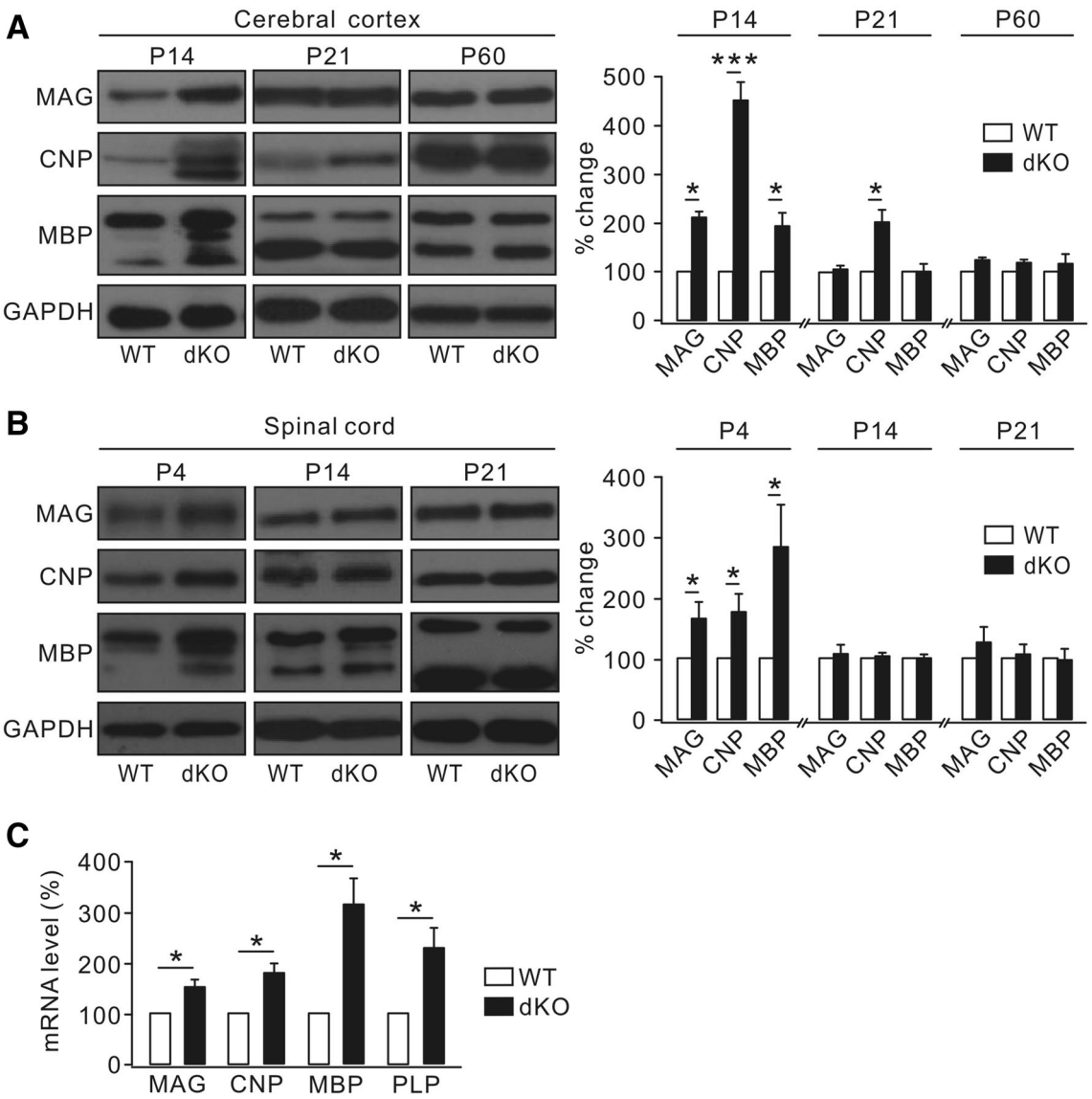

Fig. 4 Effects of EPAC deletion on myelin-specific proteins. **A** Western blots of protein expression in the cerebral cortex of WT and EPAC^{dKO} mice at P14. The protein levels of EPAC^{dKO} mice were normalized to those of WT mice and the percentage changes are shown in the bar graphs on the right. **B** Western blots of protein expression in the spinal cord of WT and EPAC^{dKO} mice at P14. The protein levels in EPAC^{dKO} mice were normalized to those in WT mice and the percentage changes are shown in the bar graphs on the right. **C** mRNA levels of myelin genes were quantified by the comparative Ct method using WT and EPAC^{dKO} mice at P14. The ratios of myelin genes *versus* control GAPDH of EPAC^{dKO} mice were calculated, normalized to those of WT mice, and percentage changes are shown in the bar graphs. The percentage changes in EPAC^{dKO} mice were 152% ± 15% (MAG), 179% ± 19% (CNP), 314% ± 52% (MBP), and 228% ± 40% (PLP). n = 4/group. $^{*}P < 0.05$. $^{***}P < 0.001$.

Discussion

The present work reveals new roles of EPACs in the CNS by showing an age-dependent regulatory effect of EPAC1 and EPAC2 on myelin development, as their deficiency caused more myelin sheaths in early postnatal (P14) but not late adult (P60) mice. Furthermore, we found that EPAC deletion promoted the proliferation of OPCs through immunohistochemical staining. Finally, we showed that EPACs had diverse effects on myelin-related transcription factors, which in turn increased the expression of myelin-related proteins. Together, our results indicate that EPAC proteins are negative regulators of myelination and may be promising for the treatment of myelin-related diseases.

The cAMP-mediated signaling pathway, which includes the downstream effectors EPAC and PKA, regulates a multitude of physiological and pathological processes [18]. EPAC and PKA not only share homologous cAMP-binding domains but also possess unique domains, such as a Ras exchange motif as well as Ras association and CDC25-

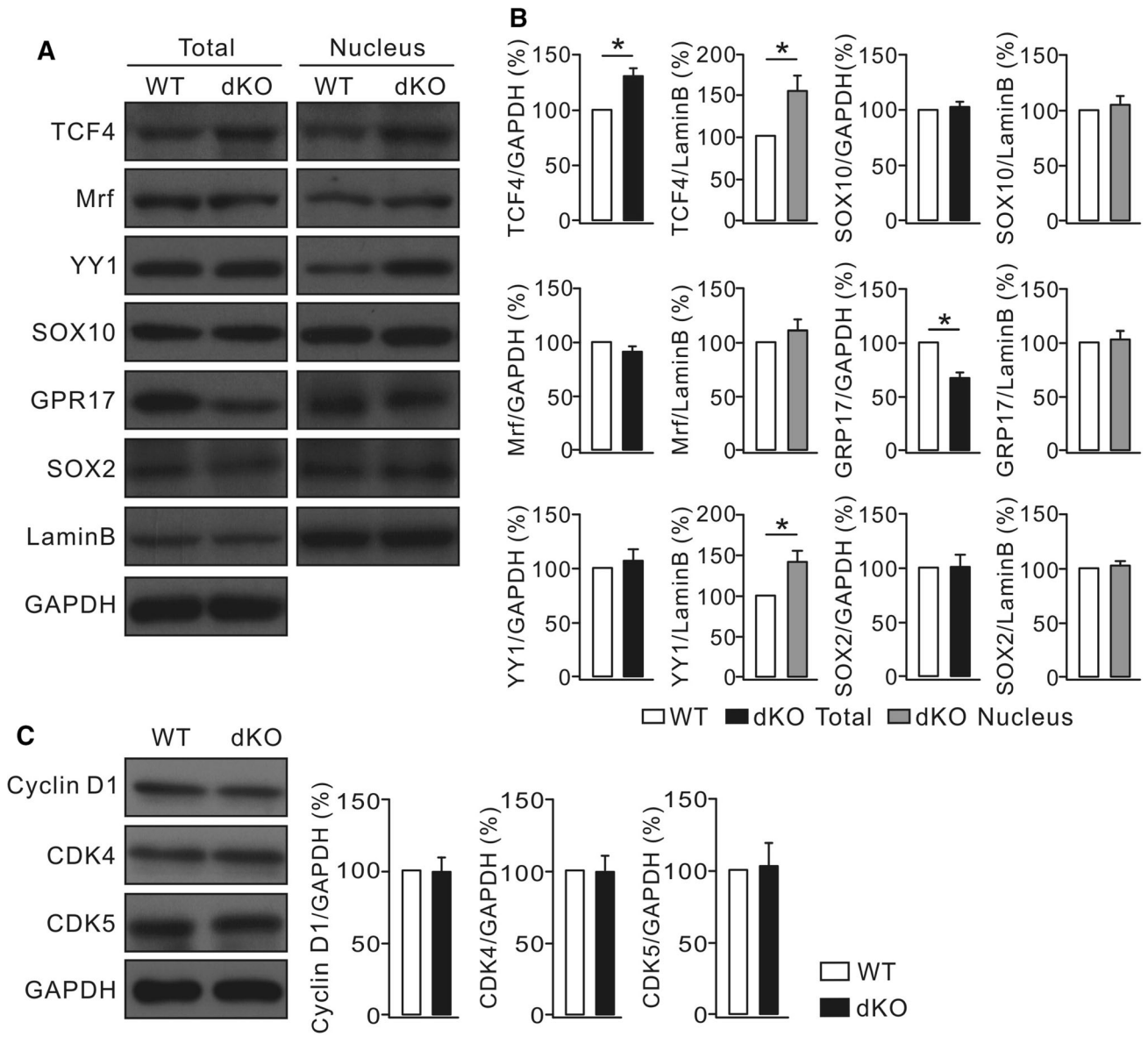

Fig. 5 Effects of EPAC deletion on transcription factors and cell cycle-related proteins. **A** Total and nuclear expression of transcription factors in the cerebral cortex from WT and EPAC^{dKO} mice (P14). Lysates were immunoblotted with antibodies against TCF4, Mrf, YY1, SOX10, GPR17, SOX2, LaminB, and GAPDH. **B** Percentage changes in ratios of transcription factors *versus* control GAPDH (total) or LaminB (nucleus) of EPAC^{dKO} mice normalized to those of WT mice. **C** Brain lysates from WT and EPAC^{dKO} mice (P14) were immunoblotted with antibodies against Cyclin D1, CDK4, CDK5, and GAPDH. Histograms show percentage changes in the ratios of proteins *vs* control GAPDH of EPAC^{dKO} mice normalized to those of WT mice. $^{*}P < 0.05$.

homology domains [18]. Previously, a study reported that elevated intracellular cAMP controls the proliferation and differentiation of cultured Schwann cells (SCs) [26]. Their results suggest that proliferation requires PKA activation and differentiation into myelin-forming cells requires EPAC activation in SCs [26]. Thus, the balance between EPAC and PKA may switch the action of cAMP between proliferation and myelinating phenotypes in SCs and possibly in OLs. Although the conclusion that EPAC is required for SC differentiation has been drawn [26, 27], it should be noted that the actions of PKA and EPAC in SCs are still paradoxical. For example, the same work shows that EPAC activation alone is not sufficient to drive a full differentiation response and there is no clear correlation between the application of PKA analogs and the induction of differentiation [26]. Considering that EPACs and PKA share homologous domains, it cannot be excluded that the analogs may fail to distinguish them and may operate in an integrated manner to achieve a net physiological effect. In other words, results derived from pharmacological treatments in cultured SCs are not sufficient to oppose our finding showing that EPAC proteins promote the

proliferation of OLs. Now, it is of interest to investigate how EPACs regulate the myelination process in the peripheral nervous system using EPAC double-knockout mice. In addition, a higher density of OPCs might cause more differentiation *in vitro* [28]. Thus, more evidence is needed to exclude potential side-effects of OPC proliferation.

Many proteins are involved in the cell cycle of eukaryotic cells, which comprises the G1, S, G2, and mitosis phases. As key factors, cyclin and cyclin-dependent protein kinases affect cell proliferation, differentiation, and apoptosis by regulating the length of the cell cycle. Cyclin D1 and CDK4 are positive regulators of the G1/S phase and accelerate cell division [29, 30]. CDK5 interacts with regulatory subunits such as p35 and P39 to regulate cell proliferation and apoptosis. In order to determine how EPAC deletion causes the increased proliferation of OPCs, we assessed the expression of cyclin D1, CDK4, and CDK5. Our results showed that their expression did not differ between $EPAC^{dKO}$ and WT mice. However, these results are not enough to rule out the potential regulation of the cell cycle by EPACs, because the phosphorylation of cyclin protein kinases as well as other factors included in the cell cycle may be subject to regulation by EPAC. Alternatively, EPAC may synergistically enhance the activation of neuregulin co-receptor ErbB2-ErbB3 [31] and the downstream MEK-ERK and Akt pathways [32]. It has been shown that cAMP is able to induce the phosphorylation of ErbB2 on a threonine residue, Thr-686, and in turn enhances tyrosine phosphorylation of ErbB2 and ErbB3, activation of ERK and Akt, and cell proliferation [31].

We showed that EPAC proteins are negative regulators of CNS myelination, which makes them unique. We previously reported that the GSK3β–β-catenin signaling is a positive regulator of OPC differentiation by increasing the expression of the positive transcription factors SOX10, Olig2, Mrf, and YY1 [22]. In contrast, the present work showed that EPAC deletion did not influence the expression of most transcription factors, suggesting the distinct roles of negative and positive regulators of transcription factors. In fact, very little is known about how transcription factors are regulated *via* cAMP sensing [33].

To conclude, the results presented here indicated that EPAC proteins play important roles in OPC proliferation and myelination. A major remaining challenge is to explore how EPAC proteins act at the early stage of myelin development. The answer to this question will unveil the functional connection of EPAC proteins to CNS myelination and provide a potential target for the treatment of demyelinating diseases, for which a negative regulator of myelination may be more effective, as previously shown for LINGO-1 [5]. In addition, we could not exclude the possibility that abnormalities in other types of cells may indirectly impair the development of myelin. The mice with specific deletion of EPAC 1 and EPAC2 in OL lineage cells are a better model for a clear conclusion.

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Conflict of interest The authors claim that there are no conflicts of interest.

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