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Nuclear Factor I/A Coordinates the Timing of Oligodendrocyte Differentiation/Maturation Via *Olig1* Promoter Methylation

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ABSTRACT

Transcription factors (TFs) and epigenetic modifications function cooperatively to regulate various biological processes such as cell proliferation, differentiation, maturation, and metabolism. TF binding to regulatory regions of target genes controls their transcriptional activity through alteration of the epigenetic status around the binding regions, leading to transcription network formation regulating cell fates. Although *nuclear factor I/A* (*Nfia*) is a well-known TF that induces demethylation of astrocytic genes to confer astrocytic differentiation potential on neural stem/precursor cells (NS/PCs), the epigenetic role of NFIA in oligodendrocytic lineage progression remains unclear. Here, we show that oligodendrocyte differentiation/maturation is delayed in the brains of Nfia-knockout (KO) mice, and that NFIA-regulated DNA demethylation in NS/PCs plays an important role in determining the timing of their differentiation. We further demonstrate that the promoter activity of the *oligodendrocyte transcription factor 1* (*Olig1*) gene, involved in oligodendrocyte differentiation/maturation, is suppressed by DNA methylation, which is in turn regulated by *Nfia* expression. Our results suggest that NFIA controls the timing of oligodendrocytic differentiation/maturation via demethylation of cell-type-specific gene promoters.

1. Introduction

Epigenetic modifications, such as histone modifications and DNA methylation, are deeply involved in the regulation of various cellular processes, including transcription, imprinting, and cell differentiation (Feng *et al.* 2007). DNA methylation is maintained by DNA methyltransferases (DNMTs) and is heritable to sister cells. In the process of development from an early embryo, DNA methylation patterns are constructed and adjusted to suit each differentiated cell type.

It is generally known that methylation of gene promoter regions suppresses transcription by preventing the binding of transcription factors (TFs) or recruiting repressor complexes involving methylated-DNA-binding proteins (Tate and Bird 1993; Lister and Ecker 2009). During central nervous system (CNS) development, astrocyte differentiation is strongly influenced by DNA methylation. Astrocyte differentiation is induced through the activation of the Janus kinase/signal transducer activator of transcription (JAK/STAT) signaling pathway. However, in mid-gestational neural stem/precursor cells (NS/PCs), promoters of astrocytic genes such as those encoding *glial fibrillary acidic protein (Gfap)* and *S100 calcium-binding protein B (S100b)* are highly methylated, preventing the binding of STAT3, a JAK-STAT signaling mediator. A decrease in the methylation levels of astrocyte-specific gene promoters during mid-to-late gestation results in the acquisition of astrocyte differentiation potential by NS/PCs (Nakashima 1999; Takizawa *et al.* 2001; Namihira *et al.* 2004). Based on these findings, demethylation of astrocyte-specific gene promoters has been proposed to function as a neuronal-to-glial differentiation switch (Fan 2005).

Recently, we have reported the genome-wide patterns of DNA methylation in developmentally distinct NS/PCs, 3 types of neural lineage cells (neurons, astrocytes, and oligodendrocytes), and embryonic stem cells (Sanosaka *et al.* 2017). During lineage commitment of NS/PCs, DNA demethylation occurs in lineage-specific gene promoters associated

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with putative binding sequences of specific TFs. For example, astrocyte-specific gene promoters containing the binding motif of nuclear factor I (NFI) family TFs are demethylated as development proceeds(Sanosaka *et al.* 2017). Previously we have shown that binding of nuclear factor I/A (NFIA) to the *Gfap* promoter leads to DNA demethylation in mid-gestational NS/PCs, and *Nfia* deficiency results in a failure to differentiate into astrocytes in late-gestational NS/PCs (Namihira *et al.* 2009). Knockout (KO) mice deficient in another NFI family gene, *Nfib*, show severe reduction of GFAP expression similar to that in *Nfia*-KO mice (das Neves *et al.* 1999; Shu *et al.* 2003; Piper *et al.* 2009). These findings suggest that *Nfia* and *Nfib* are required for the onset of astrocyte specification through the induction of DNA demethylation both *in vitro* and *in vivo* (Deneen *et al.* 2006). Interestingly, the *Nfia*-KO mouse exhibits reduced expression of differentiation/maturation oligodendrocyte marker genes in the postnatal brain (Wong *et al.* 2007). Thus, whereas the relationship between glial differentiation and *Nfia* expression has been clarified, the link between NFIA-induced DNA demethylation and oligodendrocyte differentiation/maturation has not yet been elucidated.

In this study, we examined the function of NFIA in mouse oligodendrocyte maturation. We sought to identify NFIA targets that might be regulated by promoter methylation. We also examined oligodendrocyte differentiation/maturation in *Nfia*-KO mice. Our results suggest that NFIA induces DNA demethylation at an oligodendrocyte-specific gene promoter to stimulate oligodendrocyte differentiation/maturation during development.

2. Materials and Methods

2.1. Mice

We purchased *Nfia*$^{+/-}$ mice (B6.129S6-*Nfia*^{tm1Rmg}) (das Neves *et al.* 1999) from MMRRC and maintained them in a C57BL/6N (Japan SLC) background. Postnatal-stage *Nfia*$^{+/-}$ mice were generated by backcrossing to an ICR background for 5 generations. Genotyping was performed as described (das Neves *et al.* 1999). All animal experiments were performed according to the animal experimentation guidelines of the Nara Institute of Science and Technology.

2.2. Cell Culture

NS/PCs were isolated from E14.5 telencephalons as previously described (Takizawa *et al.* 2001). Neural stem cells (NSCs) were derived from E14.5+4DIV NS/PCs and cultured in NS-A medium (Euroclone) supplemented with modified N2 containing bFGF (10 ng/mL, Peprotech) and EGF (10 ng/mL, Peprotech) (Conti *et al.* 2005). To induce astrocyte differentiation, NS/PCs were treated with LIF (50 ng/mL, Millipore) and bFGF for 3 days.

2.3. Retroviruses Production

Retroviral vectors (pMYs-IRES-GFP and pMYs-HA-NFIA-IRES-GFP) were previously described (Namihira *et al.* 2009). To produce retrovirus, retrovirus vectors transfected into Plat-E packaging cells (Morita *et al.* 2000) using Trans-IT 293 (Mirus). On the day after transfection, the culture medium was replaced with new medium for NS/PCs. The culture supernatants were collected 48 h after medium change and removed dead cells and Plat-E by filtration. Collected retrovirus was introduced into NS/PCs by adding these supernatants to the culture media.

2.4. Immunohistochemistry

Animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were isolated and postfixed in 4% PFA overnight at 4°C. Subsequently, the fixed brains were sequentially stored in 10% and 20% sucrose in PBS overnight each. The cryoprotected brains were embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek) and frozen at -80°C. The brains were sectioned at a thickness of 20 µm with a cryostat. The primary antibody was chick anti-myelin basic protein (MBP; 1:1000, Aves Labs). The secondary antibody was FITC-conjugated donkey anti-chick IgY or Cy3-conjugated donkey anti-chick IgY (Jackson ImmunoResearch). The nuclei were stained with bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque). The sections were mounted on glass slides in Immu-Mount (Thermo Scientific) and examined under an LSM 710 microscope (Zeiss).

2.5. Immunocytochemistry

Cells were fixed with 4% PFA and washed twice in PBS. Immunostaining was performed as described (Namihira *et al.* 2004). The primary antibodies were rabbit anti-GFAP (1:2000, Dako) and mouse anti-Nestin (1:500; Millipore, MAB353). The secondary antibodies were FITC-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch) and Cy3-conjugated donkey anti-mouse IgG (1:500, Jackson ImmunoResearch). The nuclei were stained with bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque). The stained cells were mounted in Immu-Mount (Thermo Scientific) and examined under an Axiovert 200M microscope (Zeiss).

2.6. Quantitative Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from NS/PCs and mouse brain, and reverse-transcribed using the Superscript II polymerase kit (Invitrogen). Quantitative RT-PCR was performed using the Mx3000P system (Stratagene).

Gene expression was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The gene-specific mouse *Gapdh*, *Olig,* and *Olig2* primers have been described elsewhere (Kohyama *et al.* 2010; Pasini *et al.* 2010).

2.7. Bisulfite Sequencing

Genomic DNA was extracted from cells, and bisulfite sequencing was performed as previously described (Takizawa *et al.* 2001). PCR amplification of *Gfap* and *Olig1* promoter regions was previously described (Takizawa *et al.* 2001; Sanosaka *et al.* 2017). The amplified products were cloned into pT7Blue vector (Novagen). Over 10 colonies were randomly picked and sequenced. DNA methylation levels were visualized and quantified by QUMA (Kumaki *et al.* 2008).

2.8. Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously (Kimura *et al.* 2008). The antibodies used for immunoprecipitation were anti-HA (Santa Cruz) and anti-DNMT1 (Cosmo Bio). Co-immunoprecipitated DNA was quantified by quantitative PCR. The primers were: *Gfap* (-1.5 kb), 5'-CCTTTTGTGCCCACGAGTGA-3' (forward) and 5'-GCAGTACAAGCTCCCAGCTCAA-3' (reverse); *Gfap* (exon), 5'-AGGAAGTCAGGGGCAGATTT -3' (forward) and 5'-GTGATGCGTCTCCGCTG-3' (reverse); *Olig1* (3.6 kb), 5'-CCAAGCATGTCCTGAATGTGG-3' (forward) and 5'- AGCATGACAGCTGGCAGAGG-3' (reverse); *Olig1* (0.7 kb), 5'- CCTGAGTTCCTGAGCTAACTTTCCA-3' (forward) and 5'- CCCTTAAACCATCAAAACAGCAACT-3' (reverse).

2.9. Construction of *Olig1* Reporter Plasmids

The *Olig1* reporter vector (3.7 kb) was constructed by insertion of 2 fragments (upstream and downstream) of the *Olig1* promoter at an artificial *SpeI* site in pGL3-basic vector (Promega), between the *MluI* and *NcoI* sites. The upstream and downstream *Olig1* promoter fragments were obtained from genomic DNA by PCR with the following primers: Up-Fw, 5'- CGACGCGTAA GGCCTGGTGAAGACAAGAGCGTTGTTAGGGCTG -3', and Up-Rv, 5'- GGACTAGTGTGGAGGGCCC CAGAAGAGAGT - 3', Down-Fw, 5'-ACTAGTTGGA ATACCTAAATGCAGGCTGACTAC -3', and Down-Rv, 5'- CATGCCATGGCTGGGGCGGGGGCTGTGGGG -3'. The PCR amplicons were purified and digested with restriction enzymes (upstream, *MluI* and *SpeI*; downstream, *SpeI* and *NcoI*) and ligated into pGL3-basic vector. The *Olig1* reporter vector (3.5 kb) was made by digesting the *Olig1* reporter vector (3.7 kb) with *SpeI* and *MluI*, blunting, and self-ligation. To methylate the upstream region, the *Olig1* reporter vector (3.7 kb) was digested with *SpeI* and *MluI,* and the upstream fragment was purified and methylated by *SssI* methyltransferase *in vitro*. Then, the methylated fragment was purified and inserted into the *Olig1* reporter vector (3.7 kb) at the *MluI* and *SpeI* sites.

2.10. Reporter Assay

Luciferase assays were performed as described previously (Asano *et al.* 2009). As an internal control, pRL-TK (Promega) was used. Reporter plasmids and pRL-TK were co-transfected using TransIT-LT1 (Mirus Bio) according to the manufacturer's instructions. On the day after transfection, the culture medium was replaced with new medium. On the 2nd day after transfection, cells were solubilized and the luciferase activity was measured according to the manufacturer's recommendations for the Dual Luciferase Reporter Assay System (Promega), using a Wallac 1,420 ARVO/Light (PerkinElmer Life and Analytical Sciences) luminometer.

2.11. ChIP-Seq Data Processing

Sequencing data were obtained from the European Nucleotide Archive under accession number ERR414099 (Mateo *et al.* 2014). Reads were aligned to mouse genome build mm9 with Bowtiw2 2.3.1 (Langmead *et al.* 2009) and SAMtools 1.4.1 (Li *et al.* 2009). Reads mapped to specific regions were normalized by the total number of mapped reads (reads per million). Data were visualized by Integrative Genomics Viewer (IGV) (Thorvaldsdóttir *et al.* 2013).

3. Results

3.1. NS/PCs Derived from *Nfia*-KO Mice Acquire Astrocyte Differentiation Potential in Long-Term Culture

Previously, we reported that NS/PCs isolated from E14.5 *Nfia*-KO mouse forebrains showed impaired astrocyte differentiation when treated with the astrocyte-inducing cytokine leukemia inhibitory factor (LIF) (Namihira *et al.* 2009). However, *Nfia*-KO mice retained partial GFAP expression, showing reduced GFAP levels in the postnatal brain (das Neves *et al.* 1999; Shu *et al.* 2003). These results prompted us to hypothesize that NFIA was not essential for astrocyte differentiation but, rather, regulated the timing of the acquisition of astrocyte differentiation potential by NS/PCs. To test this hypothesis, we first prepared NS/PCs from E14.5 wild-type (WT) and *Nfia*-KO mouse brains, and cultured them for 4 days in the presence of basic fibroblast growth factor (bFGF). After passaging, we treated the cells with LIF and bFGF for an additional 3 days to confirm the potential of astrocyte differentiation (Figure 1a upper). Whereas WT NS/PCs differentiated into GFAP-positive astrocytes, *Nfia*-KO NS/PCs showed no GFAP expression, with most cells still expressing Nestin, a marker for undifferentiated NSCs (Figure 1b). This result shows that 4-day culture with bFGF is not sufficient for *Nfia*-KO NS/PCs to acquire the potential for astrocyte differentiation, likely because

the *Gfap* promoter is still highly methylated (Namihira *et al.* 2009). In our previous study, we concluded that NFIA-induced DNA demethylation occurred passively via cell division (Namihira *et al.* 2009). Therefore, to investigate whether prolonged culture with mitotic factors might promote *Nfia*-KO NS/PCs to acquire the ability to differentiate into astrocytes, we established NSCs from WT and *Nfia*-KO NS/PC by treatment with both bFGF and EGF, which maintain NSCs in symmetrical division and an undifferentiated state (Conti *et al.* 2005). After 3 passages, we observed GFAP-positive cells in *Nfia*-KO NS/PC cultures treated for 3 days with LIF (Figure 1c). Furthermore, the *Gfap* promoter was hypomethylated even in *Nfia*-KO NS/PCs in this extended culture condition (Figure 1d). These results suggest that NFIA is sufficient but not necessary for astrocytic gene promoter demethylation to confer astrocyte differentiation capacity on NS/PCs, and that NS/PC symmetrical division (maintained by prolonged culture with mitotic factors), rather than NFIA, may be the main requirement for demethylation.

3.2. Oligodendrocyte Differentiation Maturation is Delayed in the Postnatal Brains of Nfia-KO Mice

In addition to the effect on astrocyte differentiation, the postnatal brains of *Nfia*-KO mice also exhibited downregulation of genes related to oligodendrocyte maturation (das Neves *et al.* 1999; Wong *et al.* 2007). To examine whether impaired oligodendrocytic maturation can be rescued in *Nfia*-KO mice during development similarly to astrocyte differentiation, we performed immunohistochemistry of WT and *Nfia*-KO mouse brains at postnatal day 7 (P7) and P16. At P7, expression of MBP, a maker of mature

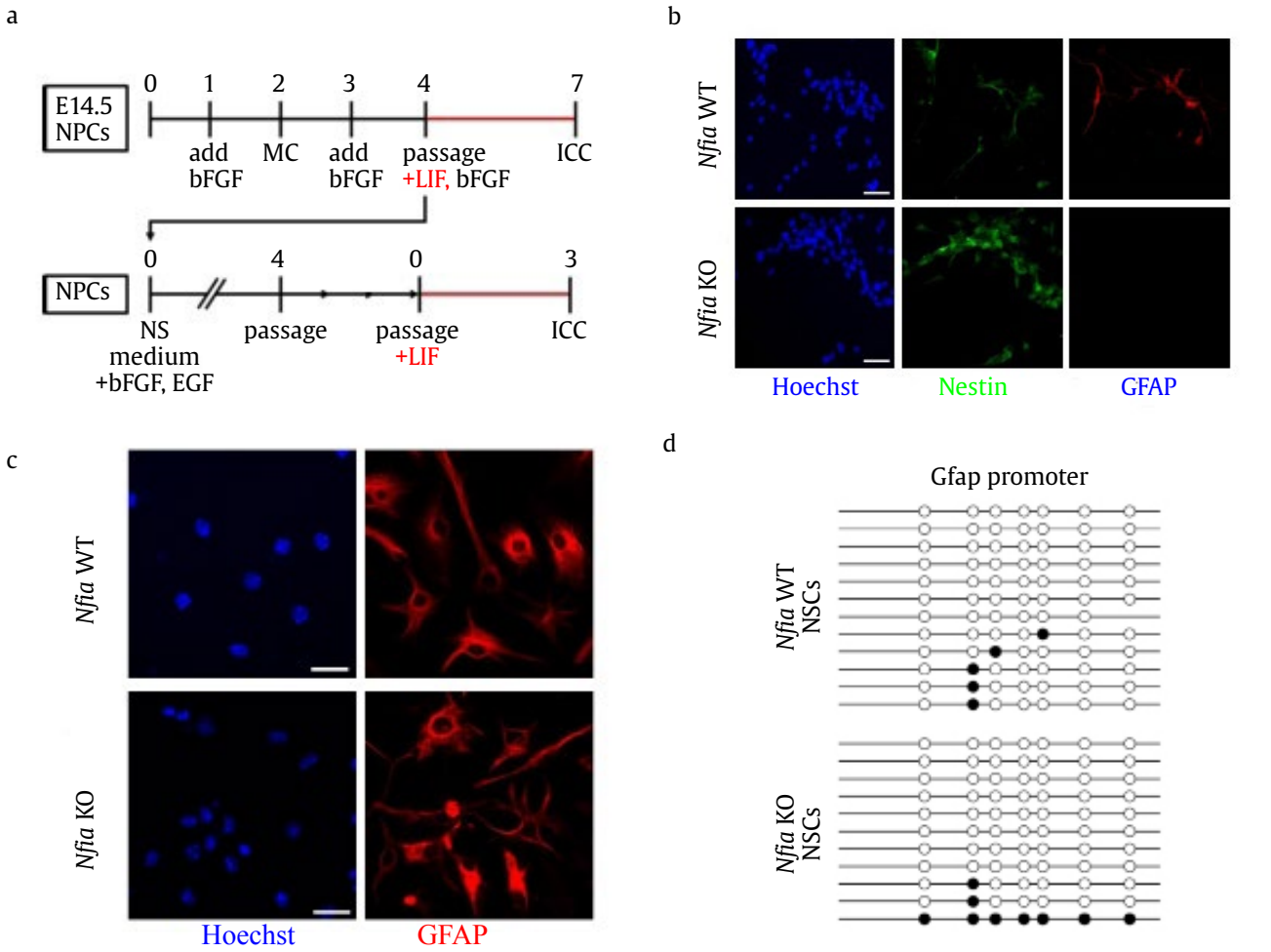

Figure 1. NS/PCs derived from *Nfia*-KO mice show GFAP expression after long-term culture. (a) Schematic representation of the NS/PC culture procedure;(b) Immunocytochemical staining for Nestin (green) and GFAP (red). Nuclei were stained with Hoechst33258 (blue). NS/PCs were derived from E14.5 WT and *Nfia*-KO mouse forebrains and cultured with bFGF for 7 days. GFAP expression was induced by treatment with LIF and bFGF for 3 days. Scale bar, 25 µm;(c) Immunocytochemical staining for GFAP (red). Nuclei were stained with Hoechst33258 (blue). NS/PCs were treated with LIF for 3 days to induce the expression of GFAP. Scale bar, 25 µm;(d) Methylation status of the STAT3-binding site in the *Gfap* promoter. Closed and open circles indicate methylated and unmethylated CpG sites, respectively; bFGF, basic fibroblast growth factor; *Gfap*, glial fibrillary acidic protein; KO, knockout; LIF, leukemia inhibitory factor; Nfia, nuclear factor I/A; NS/PCs, neural stem/precursor cells; STAT3, signal transducer activator of transcription 3; WT, wild-type

oligodendrocytes, had not started in *Nfia*-KO mice, in contrast to WT mice, in which MBP expression was already observed (Figure 2a). However, the MBP levels in *Nfia*-KO mice had "caught up", and were comparable with those in WT mice at P16 (Figure 2b), indicating that the delay in oligodendrocyte maturation in *Nfia*-KO mice was rescued as development proceeded. Thus, these results demonstrate that the timing of oligodendrocyte maturation is also regulated by NFIA.

3.3. Oligodendrocyte Transcription Factor 1 (*Olig1*) Expression is Impaired in P7 *Nfia*-KO Mouse Brains

Oligodendrocyte precursor differentiation and maturation are regulated by the interplay of epigenetic and TF-mediated events, similar to astrocytic differentiation. *Olig1* and *Olig2* are classified as basic helix-loop-helix TFs and play critical roles in oligodendrocyte differentiation and maturation (Lu *et al.* 2002; Zhou and Anderson 2002). To investigate whether these TFs are involved in abnormal differentiation and maturation of oligodendrocytes in the *Nfia*-KO mouse brain, we examined *Olig1* and *Olig2* expression levels in P0, P7, and P16 WT and *Nfia*-KO mouse brains by qPCR (Figure 2c). While no differences in *Olig2* expression were found between WT and *Nfia*-KO brains, a significant reduction of *Olig1* expression was observed in *Nfia*-KO brains compared to WT brains at each postnatal stage (Figure 2c). This result suggests that *Olig1* functions downstream of *Nfia* and is important for oligodendrocyte differentiation/maturation.

3.4. NFIA Binds the *Olig1* Promoter and Regulates its Methylation

The *Olig1* promoter undergoes demethylation as NS/PCs transition from mid to late gestational stages

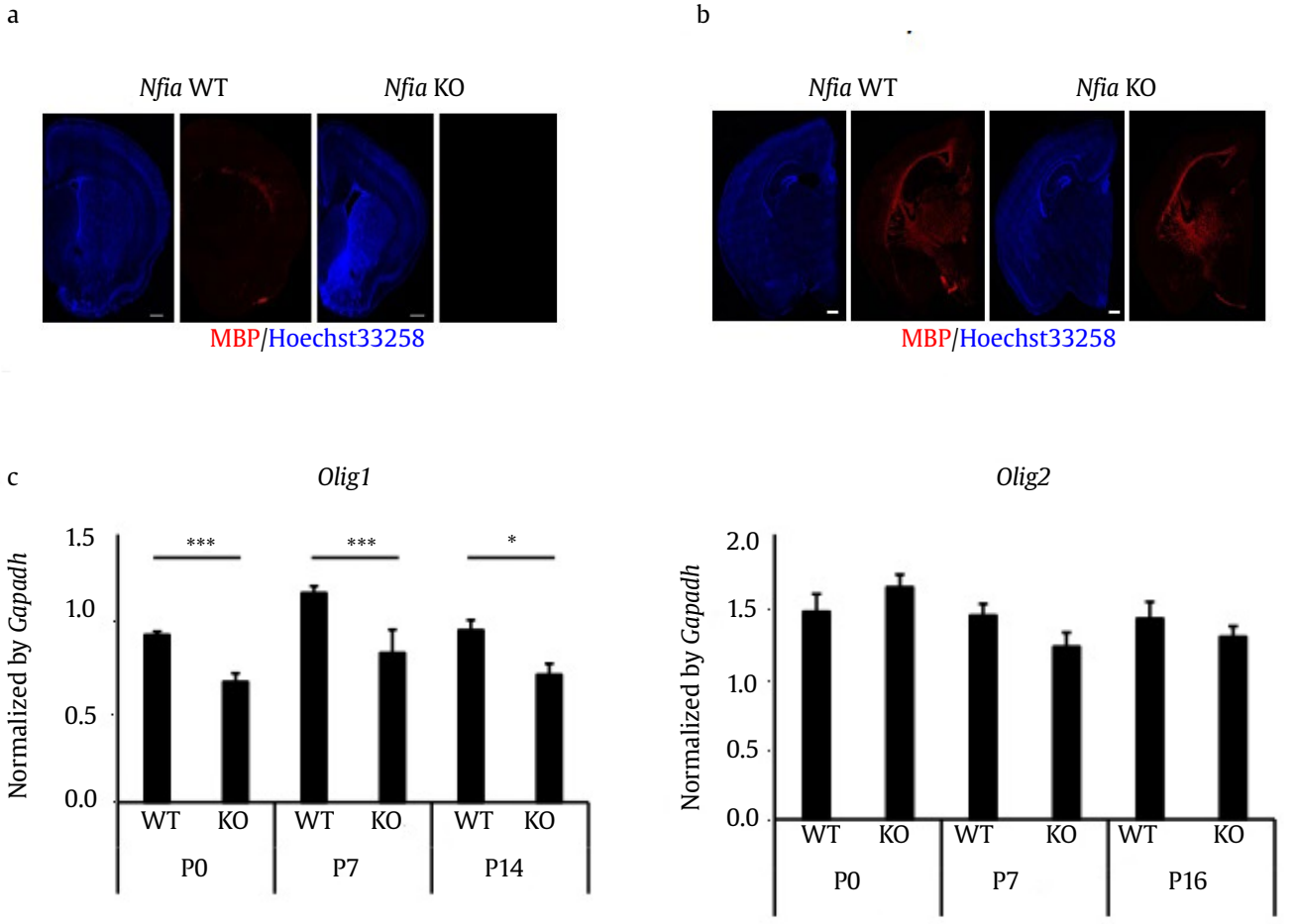

Figure 2. Delayed oligodendrocyte myelination and decreased *Olig1* expression in *Nfia*-KO mice. (a) Immunocytochemistry of P7 WT and *Nfia*-KO mouse brains. Sections were stained using anti-MBP antibody (red). Nuclei were stained with Hoechst33258 (blue). Scale bar, 500 µm;(b) Immunocytochemistry of P16 WT and *Nfia*-KO mouse brains. Sections were stained using anti-MBP antibody (red). Nuclei were stained with Hoechst33258 (blue). Scale bar, 500 µm;(c) Expression of *Olig1* and *Olig2* was analyzed by quantitative RT-PCR in P0 and P7 mouse forebrains. Signals were sequentially normalized by *Gapdh* and P0 WT values; KO, knockout; LIF, leukemia inhibitory factor; MBP, myelin basic protein; Nfia, nuclear factor I/A; NS/PCs, stem/precursor cells; *Olig1/2*, oligodendrocyte transcription factor 1/2; RT-PCR, reverse transcription PCR; WT, wild-type

(Hatada *et al.* 2008; Sanosaka *et al.* 2017). Combined with our above-mentioned findings, these reports led us to hypothesize that *Olig1* promoter demethylation during development might also be regulated by NFIA binding. To test this possibility, we analyzed publicly available NFI ChIP-seq data (Mateo *et al.* 2014) and performed ChIP-qPCR of E11.5 NS/PCs overexpressing a hemagglutinin-tagged NFIA protein (HA-NFIA). Analysis of the ChIP-seq data showed that NFI was enriched at around 3.6 kb upstream of the transcription start site of the *Olig1* gene (region R1; Figure 3a).

We confirmed association of NFIA with the R1 region, as well as with the STAT3-binding site of the *Gfap* promoter, by ChIP-qPCR (Figure 3b). We have previously implicated NFIA in the mechanism of developmental-stage-dependent demethylation in astrocyte-specific genes by showing that NFIA overexpression leads to DNMT1 dissociation from astrocyte-specific gene promoters in E11.5 NS/PCs, resulting in the induction of demethylation (Namihira *et al.* 2009). Similarly, DNMT1 dissociation was observed in the NFIA-binding region at the *Olig1* promoter when HA-NFIA was expressed in E11.5 NS/PCs (Figure 3c). Accordingly, *Olig1* expression was dramatically increased in HA-NFIA-expressing E11.5 NS/PCs (Figure 3d). Taken together, these results suggest that NFIA expression induces *Olig1* promoter demethylation, in turn leading to an increase in *Olig1* expression, similar to the NFIA regulation of astrocytic genes.

3.5. Methylation of the *Olig1* Promoter Reduces its Activity

We next asked if methylation of the *Nfia*-binding region of the *Olig1* promoter regulated its

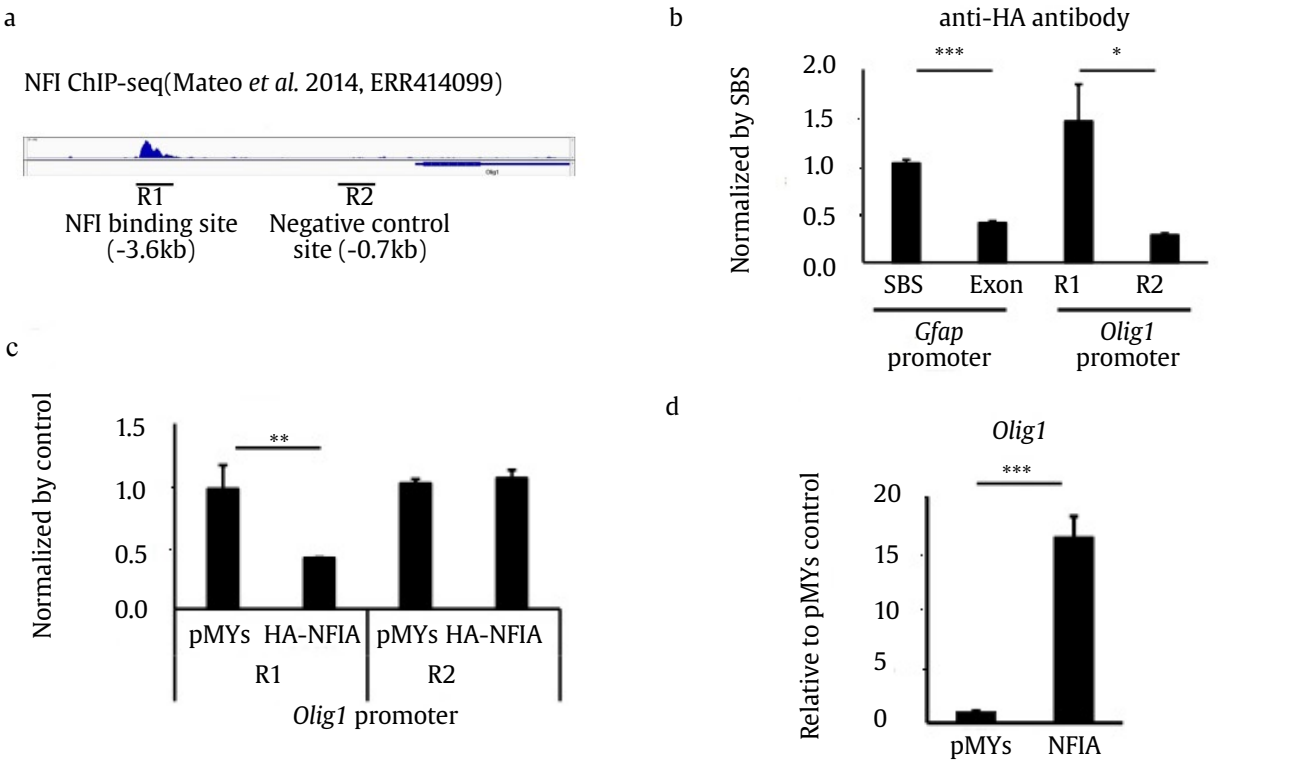

Figure 3. NFIA binds specific region in *Olig1* promoter where DNA demethylation is induced. (a) NFIA binding profiles in the *Olig1* promoter. Vertical axis shows reads per million per nucleotide (RPM). ChIP- qPCR primers were designed to R1 and R2 regions; R2 was used as a negative control;(b) E11.5 NS/PCs were infected with HA-NFIA-expressing retrovirus, and cultured for 2 days in the presence of bFGF. A ChIP assay of HA-NFIA-expressing NS/PCs was performed with an HA-specific antibody. Results of qPCR with primers specific for the R1 and R2 regions of the *Olig1* promoter;(c) E11.5 NS/PCs were infected with HA-NFIA-expressing retrovirus and cultured for 2 days in the presence of bFGF. A ChIP assay was performed with a DNMT1-specific antibody. Results of qPCR with primers specific for the R1 and R2 regions of the *Olig1* promoter;(d) Expression of *Olig1* mRNA was analyzed by quantitative RT-PCR. RNA was isolated from E11.5 NS/PCs infected with pMYs-IRES-GFP or pMYs-HA-NFIA-IRES-GFP retrovirus. Expression levels of *Olig1* mRNA were normalized by those of *Gapdh*. bFGF, basic fibroblast growth factor; ChIP, chromatin immunoprecipitation; DNMT1, DNA methyltransferase 1; HA, hemagglutinin tag; Nfia, nuclear factor I/A; NS/PCs, neural stem/precursor cells; *Olig1*, oligodendrocyte transcription factor 1; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR

transcriptional activity. To answer this question, we performed a luciferase assay using a reporter plasmid containing an *Olig1* promoter with or without the *Nfia*-binding region (R1; Figure 3a). We observed substantial activity in NS/PCs transfected with the 3.7 kb *Olig1* promoter plasmid with the R1 region and a decreased activity with the plasmid without the region, suggesting that R1 enhanced transcriptional activity (Figure 4a). Next, we constructed a region-specific methylated reporter plasmid and found that methylation of R1 region reduced promoter activity to a level similar to that observed with the reporter plasmid without R1 (Figure 4b). Furthermore, E14.5 *Nfia*-KO NS/PCs, which had a higher methylation status in the R1 region compared to that in WT NS/PC, showed decreased *Olig1* expression (Figure 4c), indicating an inverse correlation between *Olig1* expression and the promoter methylation level. Finally, we examined whether *Nfia* could enhance promoter activity directly. *Nfia* overexpression in E11.5 NS/PCs did not affect the promoter activity of an unmethylated reporter plasmid (Figure 4d). Collectively, these findings suggest that *Nfia* binds the *Olig1* promoter to render the gene transcription-competent through demethylation, but does not induce gene expression on its own.

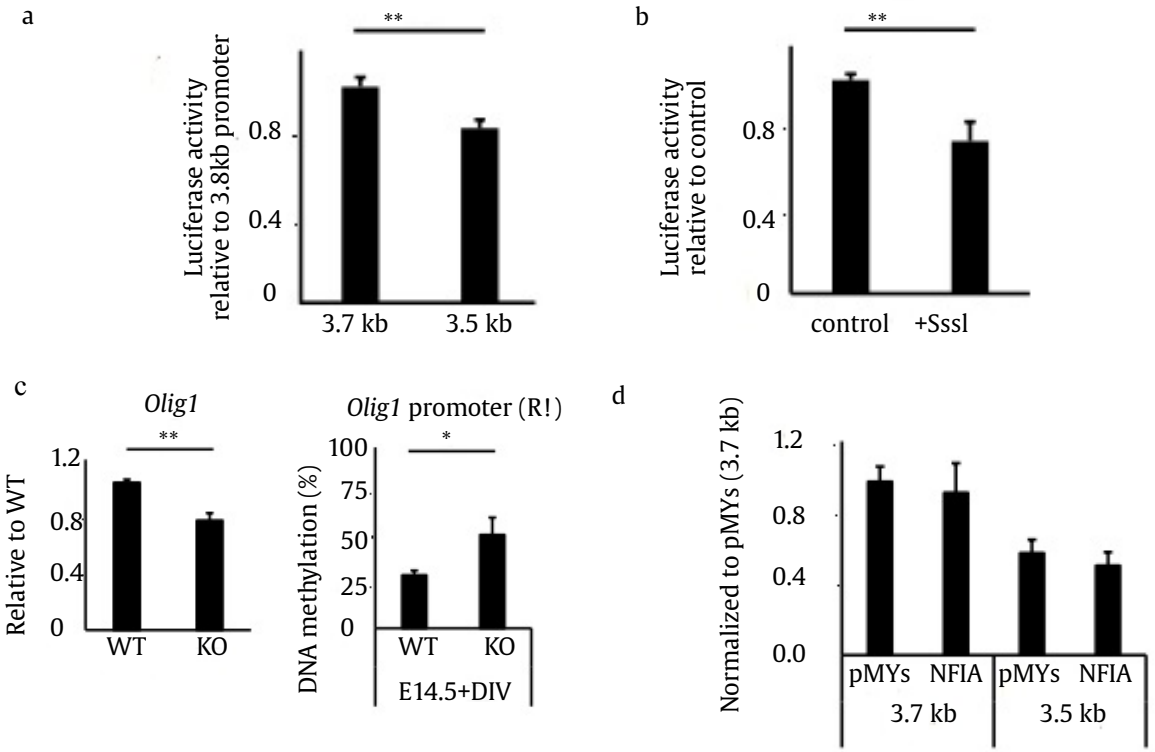

Figure 4. Methylation regulates *Olig1* promoter activity. (a) Activity of the *Olig1* promoter in NS/PCs. NSCs were transfected with pGL-*Olig1*(-3.7 kb) or pGL-*Olig1*(-3.5 kb) plasmid. Renila luciferase (Rluc) was used as an internal control;(b) Activity of the *Olig1* promoter in NS/PCs. Unmethylated and methylated pGL-*Olig1*(-3.7 kb) plasmids were transfected into NS/PCs. Rluc was used as an internal-control;(c) Expression of *Olig1* mRNA was analyzed by quantitative RT-PCR. RNA was isolated from E14.5 NS/PCs derived from WT or *Nfia*-KO mouse forebrain. NS/PCs were cultured with bFGF for 5 days. The *Olig1* signals were normalized by *Gapdh*;(d) Methylation status of the *Olig1* promoter was determined using genomic DNA prepared from E14.5 NS/PCs derived from WT or *Nfia*-KO mouse forebrain. NS/PCs were cultured with bFGF for 5 days. bFGF, basic fibroblast growth factor; KO, knockout; *Nfia*, nuclear factor I/A; NS/PCs, neural stem/precursor cells; *Olig1*, oligodendrocyte transcription factor 1; RT-PCR, reverse transcription PCR; WT, wild-type

4. Discussion

In this study, we observed delays in astrocytic differentiation in *Nfia*-deficient NS/PCs (*in vitro)* and in oligodendrocyte differentiation/maturation in *Nfia*-KO brains (in vivo). Together with other methylation-related results of the present study (Figure 1d and 4c), these findings allow us to propose that *Nfia* is important for initiation of glial differentiation via induction of demethylation in astrocyte-specific and *Olig1* gene promoters. *Olig1* is involved in oligodendrocyte differentiation/maturation in the CNS, whereas *Olig2* can compensate for *Olig1* functions associated with oligodendrocyte maturation (Dai *et al.* 2015). In the present study, we found *Olig1* expression to be decreased in *Nfia*-KO mouse brains from P0 to P14 (Figure 2c). However, the *Nfia* deficiency did not affect *Olig2* expression (Figure 2c), suggesting that *Olig1* expression is also involved in regulating the timing of oligodendrocyte differentiation/maturation. We further revealed that *Olig1* promoter methylation was involved in the regulation of *Olig1* expression (Figure 4a-c). Given that *Nfia* overexpression did not activate the *Olig1* promoter (Figure 4d), the main function of *Nfia* is likely to be induction of DNA demethylation. Therefore, *Nfia*-induced DNA demethylation may play a critical role not only in astrocyte, but also in oligodendrocyte differentiation/maturation.

Nfia-induced demethylation of astrocytic gene (Namihira *et al.* 2009) and *Olig1* promoters is attributable to passive demethylation following DNMT1 dissociation from the promoter (Figure 3c). NS/PCs derived from *Nfia*-KO mice can acquire astrocyte differentiation potential during prolonged in vitro culture (Figure 1c). The *Olig1* promoter methylation level slightly and gradually decreased from E11.5 to E14.5 even in the absence of *Nfia* expression (Figure 4c), indicating that demethylation of astrocytic gene and *Olig1* promoters is spontaneously induced in a time-dependent manner. Alternatively, other TFs inducing DNA demethylation may be involved. In summary, our results show that *Nfia* contributes to the strict control of the acquisition by NS/PCs of the potential to differentiate into glial cells via demethylation of glial-lineage-specific promoters.

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