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Chromatin Accessibility at a STAT3 Target Site Is Altered Prior to Astrocyte Differentiation

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ABSTRACT. **DNA demethylation of astrocyte-specific gene promoters and STAT3 activation in neural precursor cells (NPCs) are essential for astrogliogenesis in the developing brain. To date, it remains unclear whether DNA methylation is the sole epigenetic determinant responsible for suppressing astrocyte-specific genes. Here, we used mouse embryonic stem cells (TKO ESCs) that lacked all 3 DNA methyltransferase genes, *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, and thereby exhibit complete demethylation of the astrocyte-specific glial fibrillary acidic protein (*Gfap*) gene promoter. We found that although the *Gfap* promoter was demethylated, STAT3 failed to bind to its cognate element to induce *Gfap* transcription, whereas it induced transcription of a different target gene, *Socs3*. Moreover, although the *Gfap* promoter region containing the STAT3-binding site (GSBS) is enriched with transcription-repressive histone modifications, such as methylation of H3 at lysine 9 (H3K9me3) and H3K27me3, the reduction of these modifications in TKO ESCs was not sufficient for binding of STAT3 at GSBS. Furthermore, GSBS was digested by micrococcal nuclease in late-gestational NPCs that express GFAP upon LIF stimulation, but not in cells that show no expression of GFAP even in the presence of LIF, indicating that STAT3 can access GSBS in the former cells. We further showed that expression of NF-1A, which is known to potentiate differentiation of mid-gestational NPCs into astrocytes, increased its accessibility. Taken together, our results suggest that chromatin accessibility of GSBS plays a critical role in the regulation of *Gfap* expression.**

Key words: Astrocyte, cytokine, STAT, chromatin, transcription

Introduction

Neural precursor cells (NPCs) in the central nervous system can self-renew and differentiate into neurons (in midgestation), as well as astrocytes and oligodendrocytes (in late gestation) (Qian *et al.*, 2000; Sauvageot and Stiles, 2002). The mechanisms underlying the systematic changes in competence of NPCs include cell-external cues and cell-internal programs, such as cytokines and epigenetic modifications, respectively (Takizawa *et al.*, 2001; Hsieh and Gage, 2004; Song and Ghosh, 2004; Miller and Gauthier, 2007; Namihira *et al.*, 2008).

The interleukin-6 (IL-6) family of cytokines has been shown to play crucial roles in astrocyte differentiation of NPCs. Upon binding of individual IL-6 family cytokines onto specific receptors on the cell surface, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is activated through formation of a heterodimer between specific receptors and a common receptor component, gp130 (Bonni *et al.*, 1997; Nakashima *et al.*, 1999a; Nakashima and Taga, 2002; Ohno *et al.*, 2006). It has been shown that gp130-knockout mice exhibited a

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marked reduction in the number of cells expressing an astrocyte marker, glial fibrillary acidic protein (GFAP) (Nakashima *et al.*, 1999b). In addition, a dominant-negative form of STAT3 was found to inhibit late-gestational NPCs from differentiating into astrocytes, even when the cells were stimulated with leukemia inhibitory factor (LIF), an IL-6 family cytokine (Nakashima *et al.*, 1999a, 1999b).

Although embryonic NPCs at midgestation can respond to LIF and show STAT3 activation, they are not able to give rise to astrocytes; in other words, activation of the JAK-STAT pathway is not sufficient for differentiation of NPCs into astrocytes (Takizawa *et al.*, 2001). Previous reports by our group suggested that epigenetic modifications in specific genes are essential for astrocyte differentiation (Takizawa *et al.*, 2001; Namihira *et al.*, 2004). We also demonstrated that a CpG dinucleotide within the STAT3-binding site in the *Gfap* gene promoter (GSBS) is highly methylated in NPCs at midgestation when the cells have not yet become potentiated to astrocyte differentiation (Takizawa *et al.*, 2001). Because of DNA methylation, STAT3 is incapable of binding to the *Gfap* promoter to induce *Gfap* expression in mid gestational NPCs. In late gestation, GSBS gets demethylated and can then be bound by STAT3 upon LIF stimulation (Takizawa *et al.*, 2001; Fan *et al.*, 2005). Histone modifications have also been implicated in the regulation of astrocyte differentiation (Song and Ghosh, 2004). At midgestation, GSBS is enriched with dimethylated histone H3 at lysine 9 (H3K9me2), which is a transcriptional repressive modification. As gestation proceeds, H3K9me2 around GSBS is decreased, whereas dimethylation of lysine 4 of the histone H3 (H3K4me2), a transcriptional potentiation maker, increases. These findings indicated the essential roles played by epigenetic modifications in astrocyte differentiation during brain development (Takizawa *et al.*, 2001; Song and Ghosh, 2004; Namihira *et al.*, 2009).

Nucleosome, which is the fundamental repeating unit of eukaryotic chromatin, consists of 147-bp DNA wrapped around a histone octamer (Owen-Hughes and Workman, 1994; Bai and Morozov, 2010). The structure not only helps to pack large eukaryotic genomes into the nuclei but also plays a role in the regulation of a wide range of nuclear functions, such as gene transcription and genome repair. Accessibility of some transcription factors to the gene promoters and regulatory sequences can be under the control of chromatin structures including nucleosome positioning (Owen-Hughes and Workman, 1994; Svaren and Hörz, 1997; Lam *et al.*, 2008; Bai and Morozov, 2010). One example is the regulation of the *PHO5* gene of *Saccharomyces cerevisiae*, which encodes a phosphatase to generate orthophosphoric acid from environmental phosphates (Pi). During high concentrations of Pi, the transcription factor, Pho4, is unable to bind to its recognition sequences in the *PHO5* promoter, due to the presence of nucleosomes. In contrast, when Pi is depleted from media, the nucleosomes are disassembled from the promoter, thereby allowing Pho4 binding to its target site (Svaren and Hörz, 1997; Lam *et al.*, 2008).

In this study, we used several mutants of murine embryonic stem cells (ESCs) that lack either DNA methylation (Tsumura *et al.*, 2006) or histone modifications such as H3K9me and H3K27me (Tachibana *et al.*, 2002; Ura *et al.*, 2008). We observed that the absence of DNA methylation and/or histone modifications is insufficient to induce the expression of an astrocyte marker, *Gfap*. The result of micrococcal nuclease (MNase) digestion of chromatin also showed that GSBS is protected from digestion in both ESCs and NPCs at the midgestation phase, which do not express GFAP in response to LIF, whereas it is efficiently digested in NPCs from late gestation. Furthermore, expression of NF-1A, which is a member of the CCAAT box element-binding transcription factor family (Gronostajski, 2000), and is involved in the acquisition of astrocyte differentiation potential by NPCs (Namihira *et al.*, 2009), increased MNase digestion of GSBS in NPCs from midgestation. Taken together, our findings suggest that alteration of chromatin accessibility around the STAT3-binding site in the *Gfap* promoter together with STAT3 activation plays an essential role in the regulation of *Gfap* expression for the acquisition of astrocyte differentiation potential by NPCs.

Materials and Methods

Cell culture

Common cell culture media and their supplements were procured from Invitrogen (Carlsbad, CA). NPCs prepared from telencephalon of ICR mice at embryonic day (E) 11.5 and E14.5 were cultured as previously described (Nakashima and Taga, 2002). Briefly, the telencephalon was triturated in Hanks' balanced salt solution by gently pipetting with 1-mL pipette tips. Dissociated cells were cultured in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 containing 10 ng/mL of basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN), on culture dishes (Nunc, Waltham, MA) that were precoated with poly-L-ornithine and fibronectin (Sigma-Aldrich, St. Louis, MO). After a day of culture, cells from E11.5 mouse telencephalon, referred from now onwards as E11.5 NPCs, were used for various assays. Cells re-plated after 4 days of culture and allowed to grow for another day were referred to as E14.5 NPCs. Primary astrocytes were prepared from telencephalons of postnatal day 0 ICR mice as previously described (Kohyama *et al.*, 2010). Primary neurons were prepared from telencephalon of E17.5 ICR mice. Specifically, the telencephalon was digested with papain (Sigma-Aldrich, St. Louis, MO), triturated with fire-polished Pasteur pipettes, and plated on poly-L-lysine-coated dishes containing alpha-Minimum Essential Medium (MEM) with 5% fetal bovine serum. After 3 h, the medium was replaced with maintenance Neurobasal Medium supplemented with B27 supplement. Half of the medium was replaced every 3 days with fresh maintenance medium containing

10 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich, St. Louis, MO) and after 10–12 days of culture, the neurons were used for various assays. Mouse embryonic fibroblasts were prepared from E14.5 ICR mouse skin as described previously (Takahashi and Yamanaka, 2006). ES cell lines, including J1, TKO (Tsumura *et al.*, 2006), TT2, G9a knockout (KO) (Tachibana *et al.*, 2002), and embryonic ectoderm development (EED) conditional KO (cKO) (Ura *et al.*, 2008), were maintained on gelatin-coated dishes in the presence of Glasgow's MEM containing 10% fetal bovine serum, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 10 ng/mL LIF (R&D Systems). EED cKO ESCs lack endogenous EEDs but express a major EED isoform, Eed1, whose expression is under the control of a tetracycline (Tet)-off promoter, and therefore, is repressed in the presence of 2 μM tetracycline (Sigma-Aldrich, St. Louis, MO).

Construction of recombinant retrovirus and infection of cells

A NF-1A expression vector was constructed using the retroviral vector pMYs-IRES-GFP (Kitamura *et al.*, 2003) as previously described (Namihira *et al.*, 2009), and was then transiently transfected into the Plat-E packaging cell line (Kitamura *et al.*, 2003) by using Trans-IT 293 transfection reagent (Mirus Bio LLC, Madison, WI). On the following day, culture medium used for transfection was replaced with N2-supplemented DMEM-F12 and cells were cultured for another day. The medium containing viruses was collected, centrifuged at 8000×*g* for 24 h, and the supernatant was added to E11.5 NPCs that had been cultured for 1 day. On the following day, cells were cultured in fresh N2-supplemented DMEM-F12.

Immunostaining

Cells cultured on 6-cm dishes were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, and stained with the following primary antibodies: rabbit anti-Sox2 (1:1000; Millipore, Billerica, MA), mouse anti-Oct3/4 (1:500; Becton Dickinson, Franklin Lakes, NJ), mouse anti-β III-tubulin (1:500; Sigma-Aldrich), guinea pig anti-GFAP (1:2000; Dako, Dusseldorf, Germany), and rabbit anti-s100b (1:500; Sigma-Aldrich). The following secondary antibodies were used: Alexa488-conjugated goat anti-rabbit IgG (1:500; Invitrogen), Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson Immuno Research, West Grove, PA), Cy5-conjugated donkey anti-guinea pig IgG (1:500; Jackson ImmunoResearch). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque, Kyoto, Japan).

Bisulfite analysis

Sodium bisulfite treatment of genomic DNA was performed using the Methylamp DNA Modification Kit (Epigentek, Farmingdale, NY), according to the manufacturer's instructions. A region in the *Gfap* promoter containing the STAT3-binding site was PCR amplified from bisulfite-treated genomic DNA by using the following forward and reverse primers: GFmS (5'-GGGATTTATTA-GGAGAATTTTAGAAGTAG-3') and GFmAS (5'-TCTACCCAT-ACTTAAACTTCTAATATCTAC-3'), respectively. PCR products were cloned into the pT7Blue vector (Novagen, Billerica, MA), and at least 12 randomly selected clones were sequenced.

Western blotting

Cells were lysed in NP40 lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 100 μM protease inhibitor cocktail (Nacalai Tesque), 5 mM EDTA). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Immunoblotting was performed using mouse anti-STAT3 (1:200; Cell Signaling Technology, Danvers, MA) or anti-tyrosine-phosphorylated STAT3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000; GE Healthcare, Little Chalfont, UK) was used as a secondary antibody. Detection was performed using the ECL Detection System (GE Healthcare).

Expression of mRNA

Total RNA was isolated from NPCs and ESCs using Sepasol RNA1 (Nacalai Tesque) and then treated with DNaseI (Promega, Fitchburg, WI). Complementary DNAs were synthesized from 2 μg of total RNA using Superscript II (Invitrogen). Quantitative real-time PCR (qPCR) was performed in the LightCycler 480 (Roche, Penzgerg, Germany) by using the Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA). Expression of the target genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The primers used for qPCR were as follows: *Gfap*, forward (5'-ACCGCATCACCATTCCTGTAC-3') and reverse (5'-TGGCCTTCTGACACGGATTT-3'); and *Socs3*, forward (5'-CCAAGAACCTACGCATCCAGTG-3') and reverse (5'-CGTGGGTGGCAAAGAAAGG-3').

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to a previous study (Kimura *et al.*, 2008), with the following modifications. Cells (2×10^6 cells in a 60-mm dish) were exposed to 1% formaldehyde for 10 min and then incubated with glycine at a final concentration of 200 mM for another 5 min. Antibodies used for immunoprecipitations were rabbit anti-STAT3 (Santa Cruz Biotechnology), anti-histone mAb (H3K9me2, H3K9me3, and H3K27me3; a gift from Kimura [Kimura *et al.*, 2008]), normal rabbit IgG and mouse IgG (Santa Cruz Biotechnology). Co-immunoprecipitated DNA was detected via qPCR by using the following primers: *Gfap*, forward (5'-CAG-GCCTTGTCTGTAAGCTGAAGAC-3') and reverse (5'-TTATC-CCAGGATGCCAGGATGTCAG-3'); and *Socs3*, forward (5'-GCACAGCCTTTCAGTGCAGAG-3') and reverse (5'-GTATT-TACCCGGCCAGTACGC-3').

MNase assay

Mononucleosomes were isolated from native chromatin in each cell type as previously described (Carey and Smale, 2007), with some modifications. Briefly, cells were lysed in NP40 lysis buffer (0.5% NP40, 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.15 mM spermine, 0.5 mM spermidine) to extract intact nuclei. Isolated nuclei were divided into aliquots (5×10^5 nuclei) and digested in MNase buffer (10 mM Tris-HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) with 2000 gel units of MNase (New England BioLabs, Ipswich, MA) for 60 min at 25°C. Reactions were stopped by adding EDTA to a final concentration of 10 mM. MNase digests were subsequently treated with RNase A for 90 min at 37°C, followed by Protease K treatment for over 4 h at 55°C. The resulting DNA was then purified by phenol extraction, dissolved in 250 μl of H_2O, and used as a template for qPCR. Primers used for qPCR around GSBS were as follows (base pairs are measured from the STAT3 binding site; GSBS location is 0–9 bp): –50 bp forward (5'-CTTTTGTGCCCAACGAGTGAC-3') and reverse (5'-AAGCATCGAAGGAGGCCTGG-3'); –40 bp forward (5'-CAACGAGTGACTCACCTTGG-3') and reverse (5'-ACTTCTCGGAAAGCATCGAAG-3'); +10 bp (GSBS) forward (5'-GCCTGCTTCCCGCTGTGCTC-3') and reverse (5'-GGATGCCAGGATGTCAGCCC-3'); +25 forward (5'-GCTGTGCTCCAGGCCTCCTTC-3') and reverse (5'-GCTTTTATCCCAGGATGCCAG-3'); +80.5 forward (5'-ACTGCACCCGGGGCTGACATC-3') and reverse (5'-AATAGAGCCTTGTTCTCTGC-3'); +140 forward (5'-TGCCATATGCCTCACTGGCG-3') and reverse (5'-ACCCCTGCCTTCCTCTGCCC-3'); +164.5 forward (5'-CGGCAGAGAACAAGGCTC-3') and reverse (5'-TTCTGGAACACAGACAGCTC-3'). Primers used for *H1foo* qPCR were as follows, forward (5'-CATCCCCTTCTGGATCATGTG-3') and reverse (5'-TCTCCCGGTTTCCAGGGACC-3').

Results

ESCs do not express Gfap even in the absence of DNA methylation

We have previously shown that DNA demethylation in the STAT3-binding site located ~1.5 kbp upstream to the transcription start site (TSS) of an astrocyte-specific gene, *Gfap*, is necessary for NPCs to respond to astrocyte-inducing cytokines and to express the gene during brain development (Takizawa *et al.*, 2001). These findings prompted us to ask whether DNA methylation is the sole epigenetic modification responsible for suppressing the *Gfap* gene and whether demethylation is sufficient for its expression. To address this question, we used triple knockout (TKO) ESCs that lack a maintenance DNA methyltransferase, Dnmt1, and *de novo* DNA methyltransferases, Dnmt 3a and 3b (Tsumura *et al.*, 2006). TKO ESCs barely carry DNA methylation on their genomes but still show characteristic features of ESCs, such as proliferation in an undifferentiated state (Tsumura *et al.*, 2006). We first performed bisulfite sequencing analysis of GSBS. As expected, the results showed that the region containing GSBS was hypomethylated in TKO ESCs, as well as in the late gestational NPCs prepared from the E14.5 telencephalon, which are potentiated to express *Gfap* in response to LIF (Fig. 1A). In contrast, the region was almost completely methylated in mid-gestational (E11.5) NPCs and wild type (WT) ESCs. We also found that LIF failed to induce the *Gfap* expression in TKO ESCs, as judged by immunostaining, whereas pluripotent markers such as Oct3/4 and Sox2 were readily detected (Fig. 1B). Neither another astrocyte marker S100β nor a neuronal marker βIII-tubulin was detected in TKO ESCs, yet these two markers and GFAP were readily detected in E14.5 NPCs-derived differentiated cultures (Fig. 1B). Moreover, *Gfap* mRNA was not detected regardless of LIF stimulation in the ESCs (WT and TKO) or E11.5 NPCs, suggesting that the expression is inhibited at transcription levels (Fig. 1C). Although TKO and WT ESCs did not express *Gfap* upon LIF stimulation, it does not mean that the LIF signaling pathway was not activated in these cells. In fact, these cells responded to LIF stimulation and expressed *suppressor of cytokine signaling 3* (*Socs3*), which is another target for the JAK-STAT pathway (Fig. 1D). Furthermore, STAT3 was found to be phosphorylated to the same extent in TKO and WT ESCs, as well as in E14.5 NPCs (Fig. 1E). These results clearly demonstrate that hypomethylation in GSBS alone is not sufficient to induce *Gfap* expression.

STAT3 cannot bind to the Gfap promoter in TKO ESCs

We next probed the binding of STAT3 to its cognate sequences in TKO ESCs by chromatin immunoprecipitation (ChIP). Consistent with a previous report (Takizawa *et al.*, 2001), GSBS in E14.5 NPCs, but not in WT or TKO ESCs, was bound by STAT3 in response to LIF stimulation (Fig. 2A). In contrast to *Gfap*, the STAT3 target site in the *Socs3* promoter was bound by STAT3 after LIF stimulation in all cell types tested (Fig. 2B), which was in agreement with the induction of *Socs3* mRNA expression *via* the JAK-STAT pathway in these cells, as shown in Fig. 1E. In NPCs, a small but significant amount of STAT3-binding to its cognate sequences in the both *Gfap* and *Socs3* promoter was observed even without LIF stimulation (Fig. 2A and B). This is most likely due to activation of STAT3 by IL-6 family of cytokines secreted from E14.5 NPCs themselves (Nakashima *et al*., 1999c). Taken together, these findings show that the absence of DNA methylation in the *Gfap* promoter is not sufficient for STAT3 binding to its target site in ESCs.

In addition to DNA methylation, transcriptionally repressive histone modifications have also been shown to reduce binding of transcription factors to their target sites. For instance, the reduction of H3K9me2 modification around

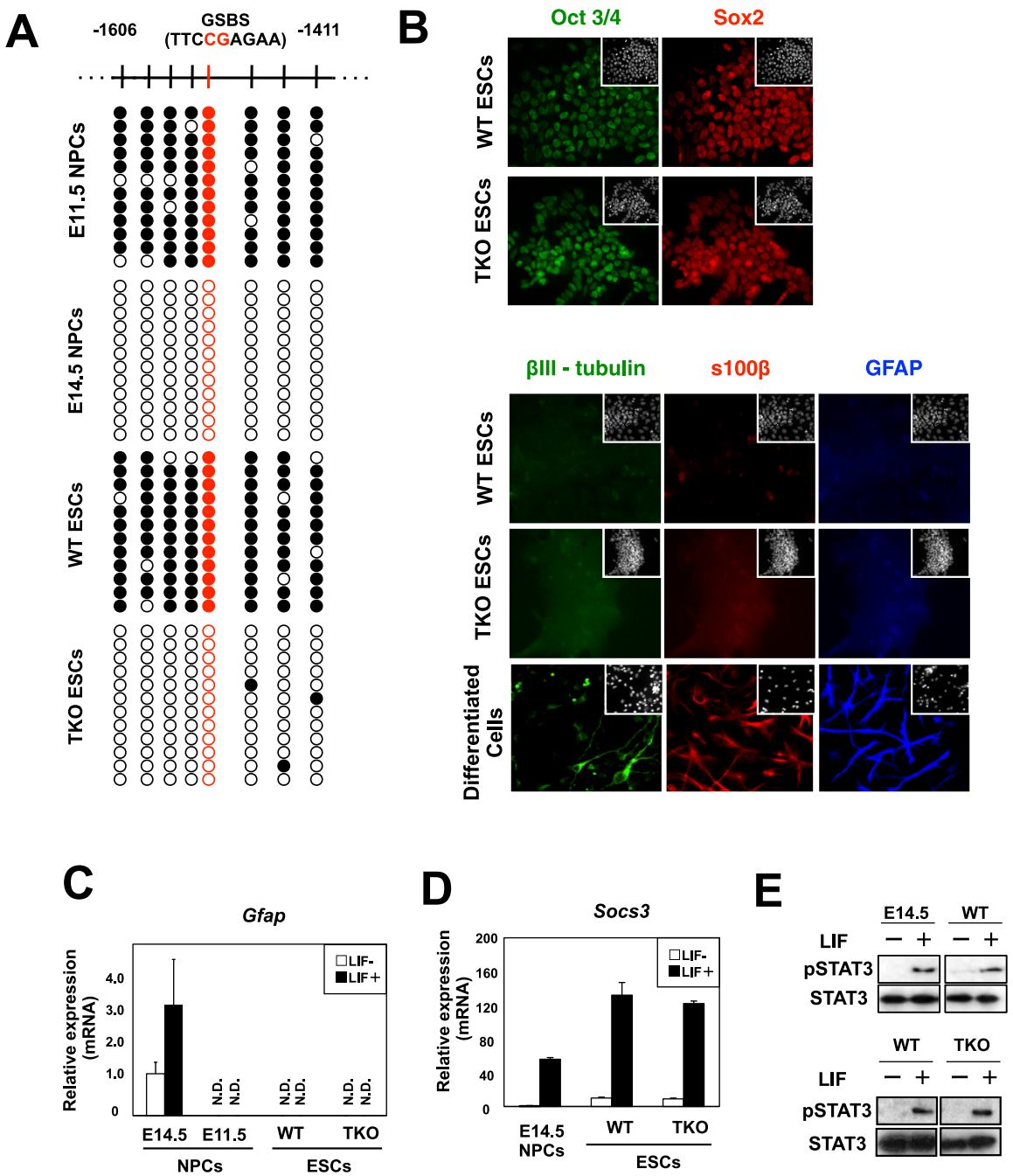

Fig. 1. Analysis of the glial fibrillary acidic protein (*Gfap*) expression in neural precursor cells (NPCs) and embryonic stem cells (ESCs). (A) The DNA methylation status around GSBS in E11.5 and E14.5NPCs, as well as wild type (WT) and triple knockout (TKO) ESCs. Open circle, unmethylated CpG; filled circle, methylated CpG. (B) The expression of specific makers for ESCs or neural cells. WT and TKO ESCs were stained with antibodies against Oct3/4 (green) and Sox2 (red) (upper two rows), as well as βIII-tubulin (green), S100β (red), and GFAP (blue) (lower two rows). E14.5 NPCs-derived differentiated cells (differentiated cells) were used as positive controls for stanining of βIII-tubulin, S100β, and GFAP. Nuclei were stained with Hoechst 33258 (white). (C and D) Expression of *Gfap* (C) and *Socs3* (D) mRNA; *Gfap* and *Socs3* mRNA expression were detected by quantitative PCR (qPCR) after LIF stimulation for 2 h. (E) Results of western blotting analysis of tyrosine-phosphorylated STAT3 and total STAT3. WT, wild-type ESCs (J1); N.D., not detected. Error bars represent standard deviation from mean of three independent experiments.

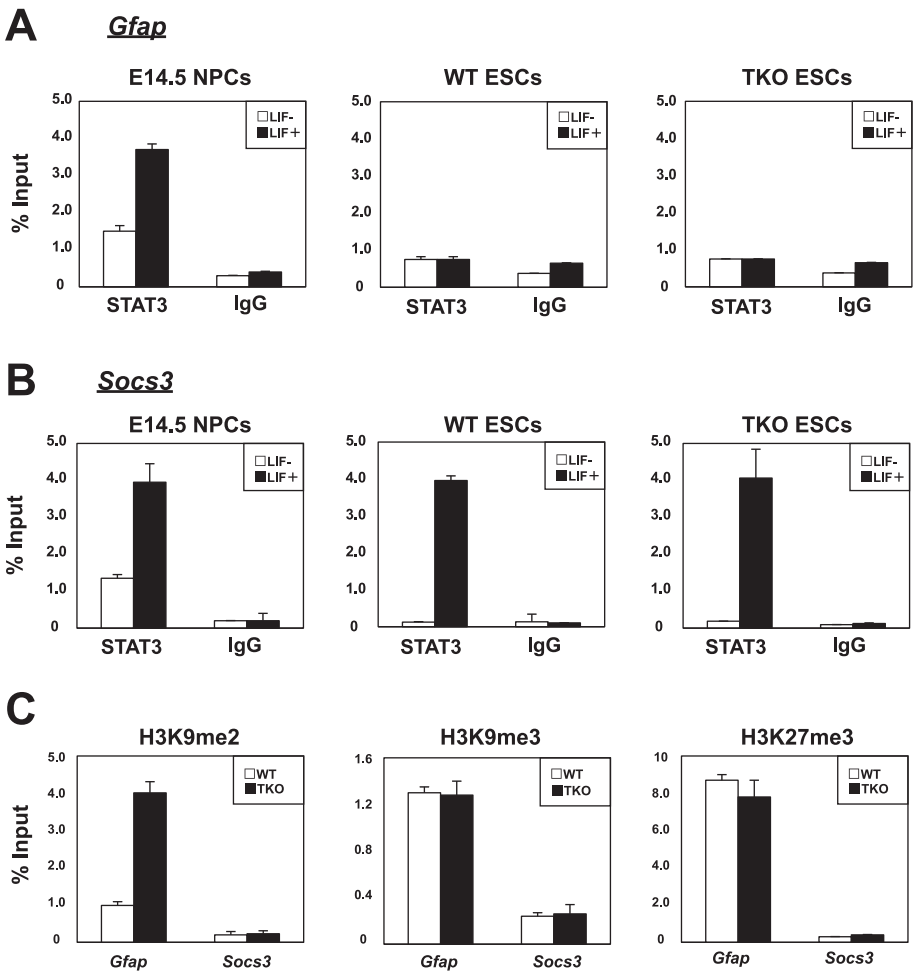

Fig. 2. STAT3 binding to *Gfap* and suppressor of cytokine signaling 3 (*Socs3*) and their histone modifications. (A and B) Chromatin immunoprecipitation (ChIP) assay using STAT3. The cells were stimulated with LIF for 20 min, fixed, and sonicated. Chromatin was precipitated with an antibody against STAT3, and enrichment of STAT3 on the promoter of *Gfap* (A) and *Socs3* (B) was studied with qPCR. Rabbit IgG was used as a control antibody. (C) Histone modifications in the promoters of *Gfap* and *Socs3*. The cells were fixed, sonicated, and precipitated with antibodies against H3K9me2, H3K9me3, or H3K27me. Enrichment of these modifications on STAT3-binding sites on the promoters of *Gfap* and *Socs3* was detected by qPCR. IgG, normal control rabbit antibody; WT-ESCs, wild-type ESCs (J1). Error bars represent standard deviation from mean of three independent experiments.

GSBS in NPCs caused by bFGF has been implicated in the binding of activated STAT3 to GSBS and resultant *Gfap* transcription (Song and Ghosh, 2004). H3K27me3 is also known to repress tissue-specific genes (Cao *et al.*, 2002; Schwartz and Pirrotta, 2007; Hirabayashi *et al.*, 2009). Therefore, we reasoned that differences in histone modifications between *Gfap* and *Socs3* might play a role in the gene-specific binding of STAT3 in ESCs. The ChIP analysis of the STAT3-binding sites in the *Gfap* and *Socs3* promoters in WT and TKO ESCs showed that the levels of H3K9me3 and H3K27me3 modifications around GSBS were much higher than those around the *Socs3* STAT3-binding site (Fig. 2C). Unexpectedly, although a previous report showed no marked changes in global H3K9me2 modification levels in TKO ESCs (Tsumura *et al.*, 2006), H3K9me2 methylation levels around GSBS in WT ESCs were lower than that of TKO ESCs with yet unknown reasons. Nevertheless, the H3K9me2 methylation level around GSBS was still higher than that around *Socs3* STAT3-binding site in WT ESCs (Fig. 2C). The data implied that these histone modifications participate in the repression of *Gfap* in ESCs.

Reduction of H3K9me or H3K27me modification is not sufficient to induce Gfap expression in ESCs

In light of the above findings, we hypothesized that histone modifications, such as H3K9me2, H3K9me3 and H3K27me3, may impede STAT3 binding to the *Gfap* promoter in ESCs. To test this possibility, we first used ESCs deficient for G9a, a histone methyltransferase responsible

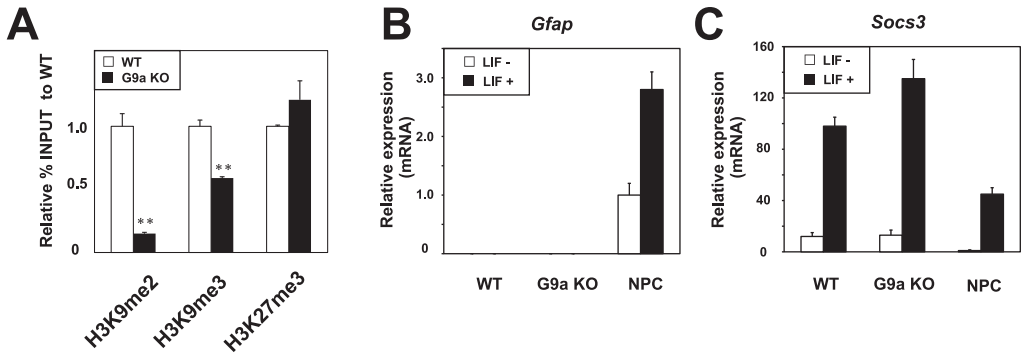

Fig. 3. H3K9 methylation and *Gfap* expression in G9a-KO ESCs. (A) The histone methylation status around GSBS in WT and *G9a*-KO ESCs was detected by ChIP assay using anti-H3K9me2, -H3K9me3, -H3K27me3 antibodies. (B and C) The *Gfap* (B) and *Socs3* (C) mRNA expressions were detected by qRT-PCR after LIF stimulation for 2 h. WT, wild-type ESCs (TT2). Error bars represent standard deviation from mean of three independent experiments. $^{**}P<0.01$.

for H3K9 methylation in euchromatin (Tachibana *et al.*, 2002). As expected, H3K9me2 and me3 in GSBS were significantly decreased in the absence of G9a, whereas K27me3 was not decreased (Fig. 3A). However, we did not observe *Gfap* expression in G9a-KO ESCs, even with LIF stimulation (Fig. 3B), suggesting that the reduction of H3K9me in the *Gfap* promoter was not sufficient for its expression and that H3K9me was dispensable in impeding binding of STAT3 to the DNA. On the other hand, *Socs3*, which was used as a positive control, was expressed at comparable levels in all cell types (Fig. 3C).

We next investigated whether H3K27me3 is involved in the regulation of STAT3 binding to GSBS by using ESCs that lack this histone modification. H3K27me3 is a well-studied histone modification that participates in transcriptional repression of tissue-specific genes during cellular differentiation (Hirabayashi *et al.*, 2009). The Eed protein, which is a component of polycomb repressive complex (PRC) 2 and is necessary for the function of a PRC2 catalytic subunit, enhancer of zeste homolog 2, mediates H3K27 trimethylation, which in turn facilitates the recruitment of PRC1 to cause chromatin compaction and repress gene expression (Cao *et al.*, 2002; Schwartz and Pirrotta, 2007). To examine the effects of H3K27me3 on STAT3 binding, we used *Eed* conditional KO ESCs (1F1) (Ura *et al.*, 2008), which becomes null for *Eed* when cultured in the presence of tetracycline. In the absence of EED1, the amount of H3K27me3 in the *Gfap* promoter was markedly decreased, and this reduction was concomitant with the significant reduction in H3K9me as was reported previously for different genes (Czermin *et al.*, 2002; Kuzmichev *et al.*, 2002) (Fig. 4A). We observed that *Gfap* was repressed in the cells after LIF stimulation, while *Socs3* was expressed (Fig. 4C). Moreover, we treated *Eed* cKO ESCs with 5-aza-2-deoxycytidine (5-aza-dC) and tetracycline to reduce DNA methylation and simultaneously H3K27me3 on GSBS and then stimulated them with LIF (Fig. 4B). We observed that even in the absence of both DNA methylation and the repressive histone methylations, *Gfap* was still not expressed (Fig. 4C). Furthermore, the results of ChIP analysis showed that the *Gfap* promoter was not bound by STAT3 even after LIF stimulation, in contrast to that of *Socs3* (Fig. 4D). Taken together, these results suggest that simultaneous reduction of H3K27me, K9me, and DNA methylation is not yet sufficient to allow STAT3 binding to its target site.

Accessibility of the Gfap promoter to MNase correlates with a poised state of Gfap transcription

The observations that the removal of DNA methylation and repressive histone modifications were not sufficient for STAT3 binding to the target site have prompted us to seek for another mechanism underlying DNA binding by STAT3. Chromatin accessibility at *cis*-regulatory regions has been shown to play important roles in regulation of binding of transcription factors (Li *et al.*, 2011). Therefore, we hypothesized that chromatin accessibility at GSBS region in ESCs is low and thereby STAT3 is prevented from binding to GSBS; in contrast, GSBS may be easily accessible to STAT3 in E14.5 NPCs, which readily express *Gfap* upon LIF treatment.

To examine chromatin accessibility at GSBS, we performed a chromatin digestion assay using MNase (Lam *et al.*, 2008). The digestion efficiency was assessed via qPCR for GSBS and the transcription start site (TSS) of the *Gfap* promoter, which is immediately upstream of GSBS. The *H1foo* gene, which is silent in all cells except oocytes, was used as an internal control (Nimura *et al.*, 2009), because the region in the *H1foo* gene locus used here was completely resistant to MNase digestion in all the tested cell types (data not shown). We observed that GSBS was less protected from MNase in E14.5 NPCs and astrocytes than in other cell types (Fig. 5A and B), suggesting that chromatin

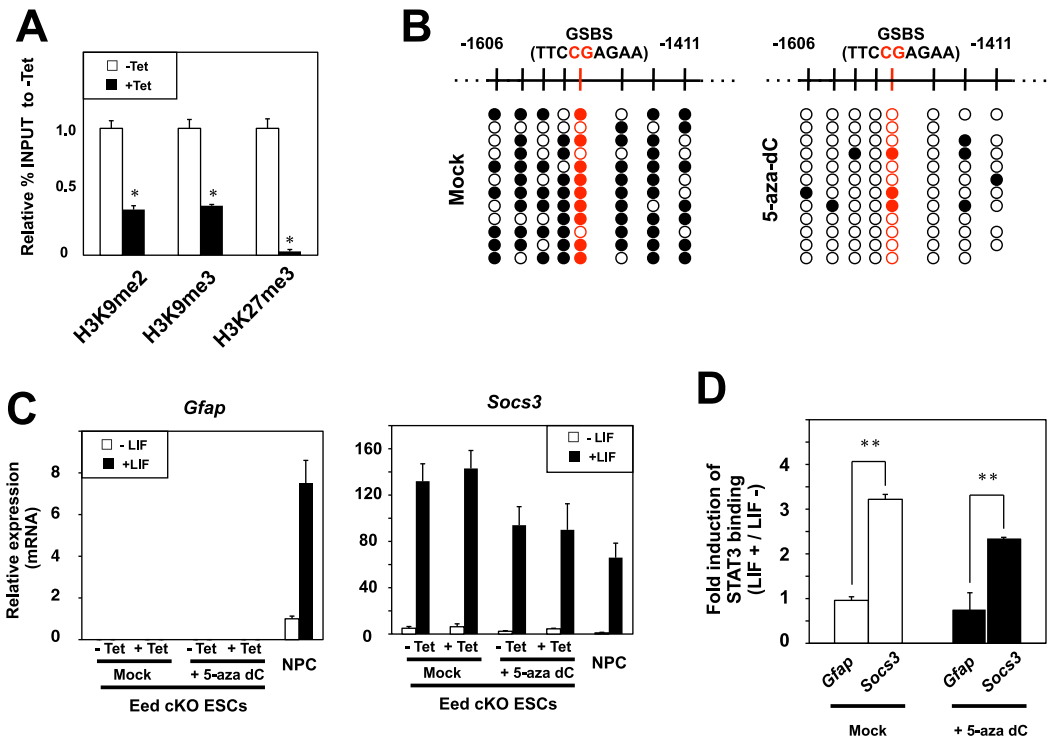

Fig. 4. Involvement of H3K27 methylation in *Gfap* repression in ESCs. (A) Histone modifications around GSBS in embryonic ectoderm development (EED) conditional KO (cKO) ESCs. After treatment with tetracycline for 1 day to inhibit EED1 expression, the ChIP assay was performed with anti-H3K9me2, anti-H3K9me3, and anti-H3K27me3 antibodies. Enrichment of the modifications on GSBS was detected by qPCR. (B) The DNA methylation status around GSBS in *Eed* cKO ESCs after mock or 5-aza-dC (10 μM) treatment for 4 days, after which bisulfite analysis was performed. Closed circle, methylated CpG; open circle, unmethylated CpG. (C) The expression of *Gfap* or *Socs3* mRNA in Eed cKO ESCs after 5-aza-dC treatment. Extracted RNA was subjected to qRT-PCR. (D) The STAT3 binding to GSBS in Eed cKO ESCs in presence of tetracycline after 5-aza-dC treatment analyzed by the ChIP assay with anti-STAT3 antibody. Data is presented as the fold induction to no LIF-treatment. Error bars represent standard deviation from the means of 3 independent experiments. $^{**}P<0.01$.

around GSBS in E14.5 NPCs is more accessible than that in the other types of cells. E14.5 NPCs can express *Gfap* in response to LIF stimulation as do most astrocytes, but not the other cells tested (Fig. 1C). Therefore, these results indicate a correlation between a poised state for *Gfap* expression and presence of accessible chromatin around GSBS. On the other hand, TSS in E14.5 NPCs and astrocytes was more protected than GSBS in those cells and furthermore was similar to that of TKO ESCs, neurons, and astrocytes (Fig. 5C). TSS in WT ESCs and E11.5 NPCs was somehow less protected than in other cell types (Fig. 5C), but the difference was much smaller compared to that seen with GSBS in E14.5 NPCs and astrocytes, and the others.

Expression of NF-1A increases chromatin accessibility at GSBS

The transcriptional activation of *Gfap* by LIF in E14.5 NPCs did not affect the accessibility of GSBS to MNase (Fig. 6A); indicating that transcription *per se* does not affect the accessibility of GSBS. Next, we tested chromatin accessibility at the GSBS region during culture of E11.5 NPCs. The culture conditions recapitulate at least a part of *in vivo* changes, like that of the DNA methylation states, in the developmental process of NPCs (Takizawa *et al.*, 2001). As previously reported E11.5 NPCs changed their differentiation competency after 4 days of culture *in vitro* and expressed *Gfap* in response to LIF (data not shown). Interestingly, chromatin accessibility at GSBS in these cells was also increased significantly ($P<0.01$) (Fig. 6B).

We have recently found that a transcription factor NF-1A, which is induced by Notch-signal activation, is involved in the acquisition of astrocyte differentiation potential by NPCs (Namihira *et al.*, 2009). We have demonstrated that forced expression of NF-1A promoted DNA demethylation of GSBS in E11.5 NPCs and made the cells competent at LIF-dependent *Gfap* gene expression (Namihira *et al.*, 2009), and therefore, that the Notch-signaling pathway plays a central role in the acquisition of astrocyte differentiation potential. To examine whether NF-1A is also involved in the changes in accessibility of the chromatin at GSBS region, we studied the effect of NF-1A expression on

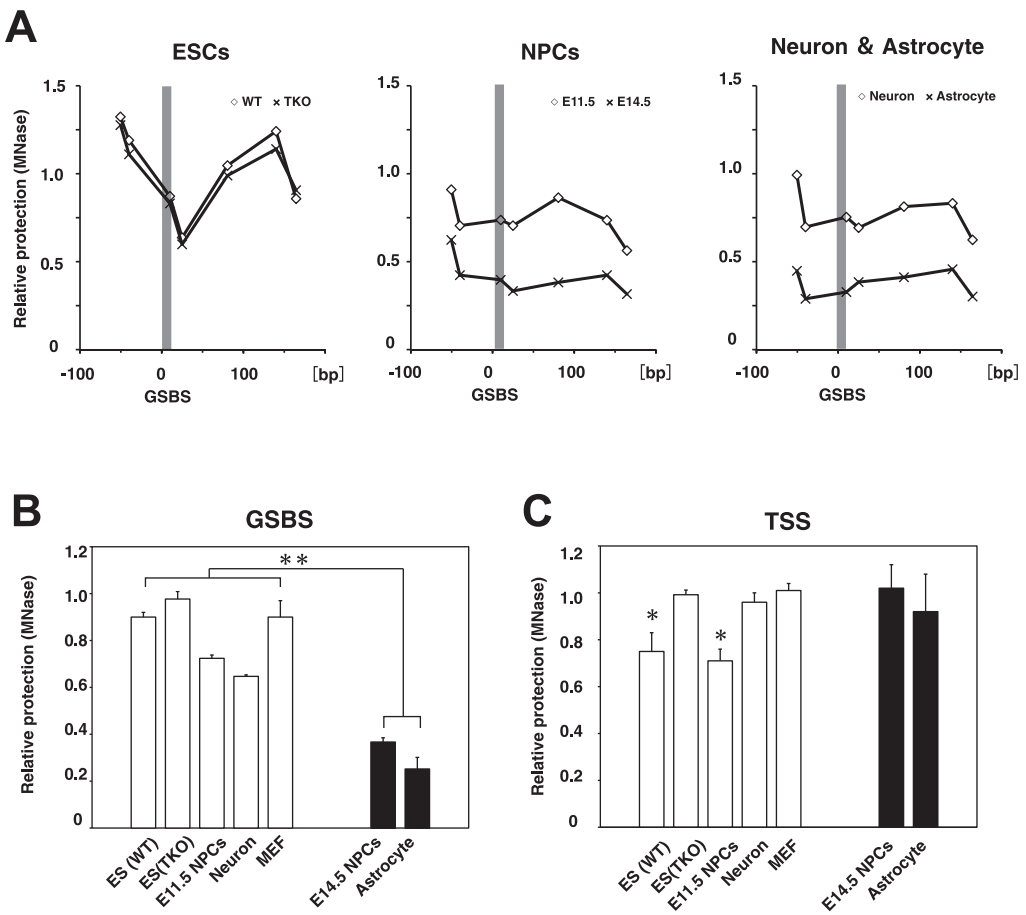

Fig. 5. Micrococcal nuclease (MNase) digestion around GSBS in different cell types. (A) MNase digestion status around GSBS (from –100 bp to +250 bp). Quantitative PCR against MNase products from WT ESCs (WT), TKO ESCs (TKO), E11.5 NPCs, E14.5 NPCs, Neuron, Astrocyte, was performed with various primer pairs tilling the entire length covering the GSBS. They were designed to yield 90–110 bp amplicons. Data was normalized to that of *H1foo*. Open square and cross mark denote a position that is at the center of each amplicon. (B and C) MNase digestion around GSBS (B) (primer set located in gray-shaded area of Fig. 5A) and the transcription start site (TSS) (C) of *Gfap* was determined in indicated cell types. Remaining amounts of genomic DNA after MNase digestion were determined by qPCR. The mean values of GSBS and TSS were normalized to that of *H1foo*. Error bars represent standard deviation from mean of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.

MNase digestion of GSBS. Forced expression of NF-1A in E11.5 NPCs significantly increased MNase digestion of GSBS but not that of TSS, whereas mock expression did not change the digestion efficacy (P<0.05) (Fig. 6C and D). This increase in chromatin accessibility indeed resulted in an increment in STAT3 binding to GSBS after LIF stimulation (Fig. 6E). These findings suggest that NF-1A is involved not only in DNA demethylation of GSBS (Namihira *et al.*, 2009) but also in its accessibility on the chromatin.

Discussion

In this study, we examined whether DNA demethylation around the STAT3-binding site in the *Gfap* promoter (GSBS) is sufficient to induce the *Gfap* gene expression. Using genetically manipulated ESCs, we clearly showed that depletion of DNA methylation or repressive histone modifications, such as H3K9me2 or H3K27me3, is not sufficient to allow *Gfap* expression. H3K9me3 around GSBS was not completely evacuated but significantly reduced both in G9a KO and Eed cKO ESCs. Therefore we concluded that reduction of H3K9me3 is not enough for *Gfap* to become transcriptionally competent. The MNase digestion assay demonstrated that GSBS in E14.5 NPCs that are poised for *Gfap* expression is more accessible than that in WT and TKO ESCs, and E11.5 NPCs. Furthermore and intriguingly, expression of NF-1A increased chromatin accessibility of GSBS in E11.5 NPCs. Therefore, we propose here that chromatin accessibility around GSBS also plays a crucial role in determining a poised state for *Gfap* expression and astrocyte differentiation.

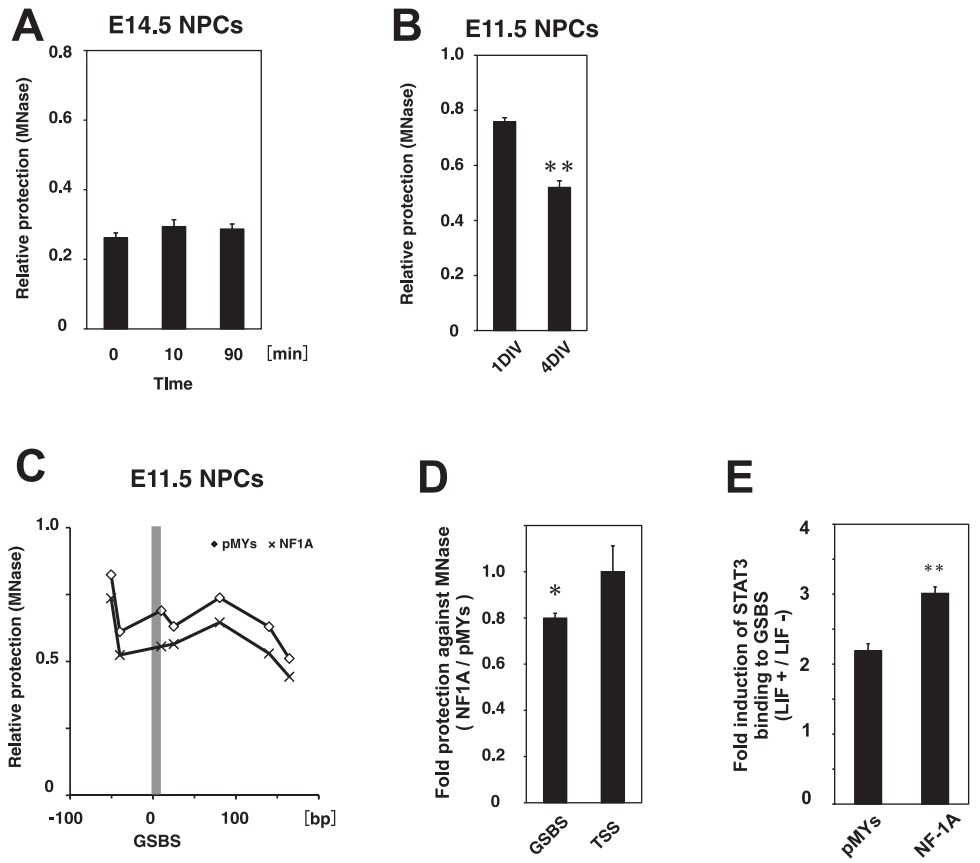

Fig. 6. NF-1A enhanced MNase accessibility to GSBS in E11.5 NPCs. (A) MNase digestion of GSBS in E14.5 NPCs after LIF stimulation. (B) MNase digestion around GSBS in 1DIV and 4DIV E11.5 NPCs. (C) MNase digestion status around GSBS with the NF-1A expression. Before MNase digestion, the cells were infected with a retrovirus expressing NF-1A or a vehicle (pMYs) for 24 h. Data normalized to *H1foo*. (D) Changes in MNase protection of GSBS with the NF-1A expression. Data were normalized to *H1foo* and represents the fold change in protection by NF-1A, compared to pMYs. (E) STAT3 binding to GSBS in LIF-stimulated E11.5 NPCs with or without NF-1A expression. Data is presented as the fold induction to no LIF-treatment . Error bars represent standard deviation from mean of 3 independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.

DNA methylation is involved in a wide range of cellular functions such as gene imprinting, lineage-specific gene expression, and tumorigenesis. At least 2 different mechanisms have been proposed for the repression of gene expression based on the DNA methylation status. One mechanism is the recognition of methylated sequences by DNA methyl-binding proteins, which form transcriptionally repressive complexes containing histone deacetylases (Jaenisch and Bird, 2003; Li *et al.*, 2007; Asano *et al.*, 2009). Another mechanism is explained by the existence of DNA methylation-sensitive transcription factors. For instance, STAT1 and 3 are incapable of binding to their cognate sequences when methylated (Takizawa *et al.*, 2001; Fan *et al.*, 2005). We previously showed that both types of regulation are involved in the repression of *Gfap* expression and that DNA demethylation is essential for the expression of astrocyte-specific genes (Takizawa *et al.*, 2001; Setoguchi *et al.*, 2006). In spite of the importance of DNA methylation in gene regulation, TKO ESCs, which lack most DNA methylation on their genomes, can maintain their undifferentiated state; indeed, we showed that the cells did not ectopically express *Gfap* and that STAT3 was unable to access its target site in the *Gfap* promoter. These results indicated that DNA demethylation is not the sole mechanism that controls the accessibility of transcription factors to GSBS and prevent its ectopic expression.

Histone modifications play essential roles in the regulation of gene expression and cell differentiation. Both H3K9me and H3K27me confer transcriptional repression by forming condensed chromatin (Cao *et al.*, 2002; Tachibana *et al.*, 2002; Schwartz and Pirrotta, 2007). H3K9me is recognized by heterochromatin protein 1, and H3K27me is bound by PRC1, both of which lead to compaction of chromatin (Zeng *et al.*, 2010; Schwartz and Pirrotta, 2007). It is generally considered that condensed chromatin functions as a blockade to prevent transcription factors from accessing their recognition sequences. However, in the current study, we failed to demonstrate that reduction of these histone

modifications increases STAT3 binding to GSBS in ESCs.

What mechanism underlies the inhibition of STAT3 access to GSBS other than DNA methylation? To address this question, we studied MNase accessibility to chromatin and found that E14.5 NPCs, which are capable of expressing *Gfap* in response to LIF stimulation, have greater chromatin accessibility around GSBS than other cell types (Fig. 5A). Our results suggested that chromatin structure around GSBS is implicated in the regulation of STAT3 accessibility and therefore *Gfap* expression. As mentioned above, higher-order chromatin structure is regulated via histone modifications such as H3K9me2/3 and HK27me3, but in the current case, they do not seem to be critical. Because MNase specifically digests linker but not nucleosomal DNA (Bai and Morozov, 2010), changes in nucleosome positioning around GSBS is most likely responsible for alterations in chromatin accessibility (Fig. 5A and B). Currently, we cannot exclude the considerable possibility of existence of co-factors that exclusively impede binding of STAT3 to GSBS in ESCs or E11.5 NPCs, or on the other hand specifically facilitate it in E14.5 NPCs and astrocytes. Nevertheless, the results of MNase digestion assay supported the hypothesis that chromatin accessibility, at least in part plays a role in controlling STAT3 binding.

The question how chromatin accessibility at GSBS changes during brain development remains open; however, several lines of evidence provide vague possibilities. In this study, we determined that the activation of STAT3 did not increase accessibility of chromatin at the GSBS region (Fig. 6A). Demethylation of DNA at GSBS was not facilitated by STAT3 activation either (Takizawa *et al.* 2001). Furthermore, changes in nucleosome positioning or chromatin accessibility take place at a time frame during development, which is similar to that of DNA demethylation in GSBS (Fig. 6C and Takizawa *et al.*, 2001). Chodavarapu and colleagues (2010) showed, in both plants and humans, via whole-genome analysis of MNase digested DNA, that nucleosomal DNA is highly methylated compared to the flanking or nucleosome-free DNA. Taken together with our current findings, regulation of DNA demethylation could be considered to associate with nucleosomal remodeling at GSBS. In fact, forced expression of NF-1A in E11.5 NPCs, which we have recently shown could induce demethylation in GSBS, increased chromatin accessibility and increase STAT3 binding to GSBS after stimulated with LIF (Fig. 6D and E), suggesting that DNA demethylation and nucleosome remodeling of GSBS might be under the control of the same molecule(s) during brain development.

The STAT transcription factors play central roles in cell differentiation and growth in many tissues. An increasing number of reports showed that the activation of STATs is associated with the pathogenesis of many cancers (Chiarle *et al.*, 2005; Yu *et al.*, 2009) and DNA methylation of their cognate sequences have also been implicated in cancer growth (Chen *et al.*, 2000). Our results in this study indicate that not only DNA methylation but also accessibility around their cognate sequences to STATs are of great importance in the modulation of their transcriptional activities. It will be interesting to investigate transcription factor accessibility around the target sites in other cell types, such as cancerous cells.

In summary, the expression of *Gfap* gene is completely repressed in ESCs, which is not released even by the removal of DNA methylation and/or repressive histone marks, and this correlates with chromatin remodeling, presumably nucleosome positioning around GSBS. This suggests that Gfap, a cell-type–specific gene, is suppressed by multiple mechanisms in ESCs. Based on our results, we propose a novel mechanism, chromatin accessibility, which regulates the binding of STATs to their target sites.

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