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DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling

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Summary

DNA methylation is a major epigenetic factor that has been postulated to regulate cell lineage differentiation. We report here that conditional gene deletion of the maintenance DNA methyltransferase I (Dnmt1) in neural progenitor cells (NPCs) results in DNA hypomethylation and precocious astroglial differentiation. The developmentally regulated demethylation of astrocyte marker genes as well as genes encoding the crucial components of the gliogenic JAK-STAT pathway is accelerated in *Dnmt1*$^{-/-}$ NPCs. Through a chromatin remodeling process, demethylation of genes in the JAK-STAT pathway leads to an enhanced activation of STATs, which in turn triggers astrocyte differentiation. Our study suggests that during the neurogenic period, DNA methylation inhibits not only astroglial marker genes but also genes that are essential for JAK-STAT signaling. Thus, demethylation of these two groups of genes and subsequent elevation of STAT activity are key mechanisms that control the timing and magnitude of astroglial differentiation.

Key words: Dnmt1, CpG methylation, Neural differentiation, STAT1, Chromatin remodeling, MeCP2, Histone modification, Mouse

Introduction

CpG DNA methylation is one of the major epigenetic factors that influences gene expression in mammals (Jaenisch and Bird, 2003). DNA methylation is essential for embryogenesis as mice deficient in DNA methylation die at early to mid-gestation stages directly following gastrulation (Li et al., 1992; Okano et al., 1999). In addition to regulating development, DNA methylation has been implicated in tumorigenesis as changes of CpG methylation are frequently observed in cancer cells (Jones and Baylin, 2002). Moreover, alterations in DNA methylation and mutations of methyl-CpG binding protein 2 (MeCP2) have been linked to several mental retardation disorders such as Rett, ICF (immunodeficiency, centromere instability, facial anomaly), Fragile X and ATR-X syndromes, indicating that the central nervous system (CNS) is particularly sensitive to epigenetic abnormalities (Robertson and Wolffe, 2000). Thus, DNA methylation most probably plays crucial roles in the development and/or function of the CNS (Fan et al., 2001; Feng et al., 2005).

The mammalian CNS is established through a temporally and spatially well-organized sequence of events during development. Starting as a single layer of multipotent neural progenitor cells (NPCs), the developing CNS sequentially produces neurons, astrocytes, and oligodendrocytes at specific stages during development (Bayer, 1991; Qian et al., 2000; Sauvageot and Stiles, 2002). The sequential differentiation of neurons and glia from NPCs is not simply due to the sequential appearance of neuronal and glial inducing cues, because early CNS progenitors are not capable of immediately differentiating into glia, even when presented with strong glial-inducing factors (Sauvageot and Stiles, 2002; Takizawa et al., 2001). Therefore, during development, NPCs gradually acquire competence for gliogenesis. It is intriguing that when cells become gliogenic, they simultaneously lose their neurogenic potential, suggesting the existence of a neurogenic to gliogenic switch mechanism during CNS development.

Both cell intrinsic factors and extracellular cues have been postulated to influence the neuro- to gliogenic switch. For example, the presence of neurogenic factors such as the proneural basic helix-loop-helix (bHLH) genes has been shown to actively suppress the gliogenic state of NPCs during the period of neurogenesis (Nieto et al., 2001; Sun et al., 2001), contributing to the late onset of gliogenesis. In

addition, the transient rise of basic fibroblast growth factors (bFGFs) in the ventricular zone of the mouse cortex around embryonic day 14.5 (E14.5) may serve as a potential modulatory factor mediating the transition from neurogenesis to gliogenesis. Low levels of bFGF have been shown to increase neuronal differentiation, while high levels of bFGF expand the glial progenitor pool (Dono et al., 1998; Qian et al., 1997). In addition, bFGF regulates the expression of the epithelial growth factor receptor (EGFR), which is correlated with the appearance of astrogliogenic progenitors (Lillien and Raphael, 2000). The expression of EGFR in neural progenitors could also be influenced by other factors, such as bone morphogenetic proteins (BMPs), WNT proteins and sonic hedgehog (SHH) in vitro (Viti et al., 2003b). As oligodendrocytes are generated from the ventral part of the forebrain, gliogenesis in the developing cortex is primarily confined to astrocyte differentiation. Although it remains to be determined whether the aforementioned glia-inducing factors are either necessary or sufficient to act in vivo to regulate the neuro- to gliogenic switch, most of these factors have been shown to influence the essential astrogliogenic JAK-STAT pathway (Nakashima et al., 1999b; Song and Ghosh, 2004; Sun et al., 2001; He et al., 2005).

The JAK-STAT pathway, which is activated by cytokine leukemia inhibitory factor (LIF) through the heterodimeric receptor LIFRβ and gp130 can effectively induce astroglial differentiation (Bonni et al., 1997; Rajan and McKay, 1998). BMPs also use STATs to activate the expression of astrocyte marker genes via the association between STATs and the transactivating complex composed of the BMP-activated signaling factors Smad1 and P300/CBP (Nakashima et al., 1999b). Gene knockouts of LIF (Bugga et al., 1998), LIFRβ (Koblar et al., 1998), gp130 (Nakashima et al., 1999a) or STAT3 (He et al., 2005) all result in impaired astrocyte differentiation in vivo, further indicating that JAK-STAT signaling contributes to astrogliogenesis in the developing CNS.

Although LIF is a potent astroglial differentiation factor, it cannot immediately trigger astrogliogenesis in early cortical NPCs. The lack of an astrogliogenic response to LIF in early cortical NPCs has been primarily attributed to methylation of astrocyte marker genes such as *Gfap*, as it has been suggested that various components of the LIF-induced JAK-STAT pathway are present in both early and late NPCs (Molne et al., 2000; Song and Ghosh, 2004; Takizawa et al., 2001). Furthermore, methylation of the STAT binding element within the *Gfap* promoter was shown to inhibit the association of activated STATs with the glial promoter, thereby repressing transcription of the *Gfap* gene (Takizawa et al., 2001). However, we recently found that the overall activity of the JAK-STAT pathway is strongly suppressed during the neurogenic period and becomes robustly elevated during the gliogenic switch (He et al., 2005). In this report, we provide evidence that DNA methylation is one of the key mechanisms inhibiting JAK-STAT signaling in neurogenic NPCs. Global DNA hypomethylation in the developing CNS leads to precocious astrogliogenesis. However, hypomethylation-induced precocious astrogliogenesis is not simply due to demethylation of the STAT binding element within the GFAP promoter (Takizawa et al., 2001), but primarily due to the elevation of overall JAK-STAT signaling activity. Our findings suggest that DNA methylation regulates the timing and magnitude of astrogliogenesis through both modulation of JAK-STAT activity and its direct inhibition of glial marker genes via inactive chromatin remodeling.

Materials and methods

Transgenic mice, CNS precursor cell cultures, and immunocytochemistry

The generation of nestin-cre; Dnmt1 conditional knockout mice has been described by Fan et al. (Fan et al., 2001). The use of transgenic mice is approved by UCLA Institutional Animal Research Committee. Mouse neural precursor cells from E11.5 cortices or CNS (containing fore-, mid-, and hind-brain, and cervical spinal cord) were dissected, dissociated and cultured in DMEM/F12 chemically defined medium supplemented with B27 as described previously (Ge et al., 2002). FGF2 was added to the culture on the day of plating. After fixation (methanol:acetone, 1:1-v:v, for 2 minutes), cells were stained with antibodies against precursor, neuronal and astroglial markers, including Nestin (rabbit anti-Nestin, a kind gift from Ron McKay at NIH), MAP2 (rabbit anti-MAP2, Peninsula), S100β and GFAP (mouse anti-S100β, mouse anti-GFAP from Sigma and rabbit anti-GFAP from Accurate). Rabbit anti-Dnmt1 antibody (PATH52; 1:2000) was a gift from Dr Tim Bestor (Columbia University). Rabbit anti-β-gal antibody (1:500 dilution) was purchased from 5′-3′. Monoclonal antibody against 5′-methylcytosine (1:200) was from Calbiochem. Images were captured with an Olympus fluorescent microscope.

DNA methylation analysis

Bisulfite genomic sequencing analysis was performed essentially as described Clark et al. (Clark et al., 1994). Methylation-specific SNuPE assay was used to quantify the percentage of methylation at a particular CpG site as described in detail by Gonzalgo and Jones (Gonzalgo and Jones, 1997). The quantification of radioactive signals was performed with a phosphor-imager system (Molecular Dynamics).

Electrophoretic-mobility-shift assay (EMSA) and chromatin-immunoprecipitation (ChIP) analyses

Purification of nuclear extracts and EMSA assays were carried out as described (Bonni et al., 1997). For ChIP analysis, NPCs were left untreated or treated with LIF for 30 minutes and crosslinked with 1% formaldehyde for 20 minutes at room temperature. Experiments were performed as previously described (Martinowich et al., 2003). Normal rabbit serum or anti-β-gal antiserum was routinely used in ChIP assays for negative controls (data not always shown). Antibodies used for pull downs were anti-MeCP2 (Upstate), anti-H3 ^{dm}K9, anti-H3 $^{d/tm}$K4 (Upstate), anti-STAT1 (generated by Ke Shuai's laboratory at UCLA) (ten Hoeve et al., 2002) and anti-STAT3 (Santa Cruz). DNA samples obtained before (Input) and after (ChIP) immunoprecipitation were subjected to PCR amplification. PCR primer sequences for ChIP analyses are listed as follows. *Gfap* gene promoter encompassing the –1.5 kb STAT binding element: forward 5′ TAA GCT GAA GAC CTG GCA GTG 3′; reverse, 5′ GCT GAA TAG AGC CTT GTT CTC 3′. *Stat1* gene promoter set I (–1748 bp to –1478 bp encompassing one of the STAT binding elements, used for anti-STAT3 ChIP): forward, 5′ AAG TGG TGC TGT TCA AGG 3′; reverse, 5′ CAG AGG TAA GCT GAT TCC 3′. *Stat1* gene promoter set II (–670 bp to –449 bp encompassing the developmentally regulated CpG site, used for anti- MeCP2 ChIP): forward, 5′ GAC AGA GGG ATG TCC TGC 3′; reverse, 5′ CTT CGG ACC TCC ACT GAC 3′. S100β gene ChIP primers (–1142 bp to –817 bp encompassing both STAT binding and the developmentally regulated CpG site): forward, 5′ GGA ACA CGA GGG GCA AAG 3′; reserve, 5′ CGC TCT TGC CCA GAA ATG 3′.

DNA constructs, cell transfection, and promoter activity luciferase-reporter assay

The construction of the 1.9 kb rat GFAP-luciferase and β-gal reporter plasmids has been described before (Bonni et al., 1997). The mouse *Dnmt1* expression plasmid was kindly provided by Dr En Li (Chen et al., 2003). Plasmids were transfected into neural precursor cells using the Fugene-6 reagent as instructed by the manufacturer (Roche). For the promoter activity assay, fly-luciferase reporter plasmids were co-transfected into cells using Fugene-6 (Roche) along with a TK-renilla luciferase plasmid (Promega), which serves as a cell transfection control. Cultures were treated with or without LIF (50 ng/ml) for ~24 hours post-transfection and were lysed and subjected to dual luciferase assays (Promega). Statistical analysis of luciferase assay data and cell counting results was performed with StatView 5.0 software (SAS Institute)

TUNEL staining method

Cultured cells were fixed with 4% PFA/PBS and stained for TUNEL analysis using the Apoptag Fluoroscein In Situ Apoptosis Detection Kit (Chemicon) following the supplied manufacturer's protocol. TUNEL-positive cells were counted at 40× magnification (20 fields/coverslip) and reported as a ratio of total cell number as determined by DAPI staining.

Western and northern blot analysis

Proteins were extracted with NP40 lysis buffer and concentrations measured using the Bradford method. After denaturing with SDS lysis buffer, protein samples were fractionated on a SDS-PAGE gel (BioRad) and transferred to nitrocellulose membrane for immunoblotting. To measure phosphorylation of STAT1/3, cell cultures were harvested after a 20-minute stimulation with LIF and subjected to western blot analysis using antibodies specific for the phosphorylated form of STAT1/3 as described before (Bonni et al., 1997; Sun et al., 2001). RNA was extracted from brain samples with RNAzol and subjected to northern blot analysis as reported previously (Sun et al., 2001).

Retroviral lineage tracing assay

E11.5 NPCs from control and *Dnmt1*$^{-/-}$ CNS were infected with a replication-deficient retrovirus containing a β-galactosidase gene at the time of plating as described before (Bonni et al., 1997). At 4 DIV, cells were triple-labeled with antibodies against MAP2, GFAP and β-gal, and the percentage of MAP2/β-gal and GFAP/β-gal double-positive cells over the total number of β-gal positive cells was scored.

Results

In vivo and in vitro precocious astroglial differentiation induced by DNA hypomethylation in the developing CNS

NPCs sequentially give rise to neurons, astrocytes and oligodendrocytes in vivo during development. To determine whether DNA methylation influences astroglial differentiation in vivo, we examined transgenic mice bearing a cre-loxP conditional mutation in the maintenance DNA methyltransferase gene, *Dnmt1* (Fan et al., 2001). The Cre recombinase is driven by the *Nestin* enhancer, which allows for specific *cre* expression in the CNS ventricular/germinal zone nestin-positive NPCs (Fan et al., 2001). When the *Dnmt1* gene is deleted specifically in NPCs in the developing CNS at embryonic day 9-10 (E9-10), extensive DNA hypomethylation is detected in over 90% of the CNS cells, starting at E11-12 (Fan et al., 2001). We collected spinal cord and brain tissues from E12 to 18.5 mouse embryos and examined the expression of the astrocytic marker proteins GFAP and S100β. Through immunohistochemical analyses, we found that radially distributed S100β and GFAP fibers were apparent in E15.5 *Dnmt1*$^{-/-}$ (mut) cervical spinal cord, whereas both S100β and GFAP immunoreactivities were minimal in their wild-type counterparts (Fig. 1A). At E18, when radially distributed immature astrocytic fibers (S100β+, GFAP+) were detectable in the wild-type spinal cord, more stellate S100β-positive and GFAP-positive astrocytes were detectable in the mutant spinal cord, indicating the differentiation of more mature astrocytes (Fig. 1B). Using relatively more sensitive Western blot analyses, we detected GFAP protein in *Dnmt1*$^{-/-}$ spinal cord samples as early as E12. GFAP protein was below the detection threshold in samples from wild-type spinal cord until E14, at which point the level of this astrocyte marker in mutant samples was much higher (Fig. 1C). Consistent with the immunohistochemistry data, western blot analyses also indicated that the expression of both GFAP and S100β was enhanced in mutant spinal cords at E17-18 (Fig. 1C,D). To address whether precocious astroglial differentiation is widespread throughout the CNS in *Dnmt1*$^{-/-}$ animals, we examined GFAP levels in other regions of the CNS. Immunohistochemical analyses indicated that in mutant mice between E15-18, enhanced GFAP-positive fibers were present in multiple brain regions such as the cortical ventricular region and hippocampal primordial area, as shown in Fig. 1E,F. Consistent with these data, in E18 mutant embryos GFAP protein levels were increased in the olfactory bulb, cortex, striatum, thalamus and cerebellum when compared with control tissues (Fig. 1G). There was also an increase in GFAP mRNA in E18 *Dnmt1*$^{-/-}$ brains relative to controls (Fig. 1H). Together, these observations strongly support the notion that precocious activation of the astroglial differentiation program occurs in *Dnmt1*$^{-/-}$ NPCs. We noticed that GFAP expression was excluded from MAP2-positive neurons (data not shown), suggesting that DNA methylation does not interfere with lineage segregation of neurons and glia in vivo.

To uncover the underlying mechanism by which hypomethylation leads to precocious astrogliogenesis, we established an E11.5 mouse cortical NPC culture system, in which the neuro- to gliogenic switch can be recapitulated in vitro. Previous studies have demonstrated that cultured NPCs derived from early (E11.5) mouse cortices differentiate mainly down the neurogenic pathway, whereas NPCs derived from late (E14.5 and later) cortices have increased potential for glial differentiation (Qian et al., 2000). We found that when E11.5 mouse cortical NPCs were cultured for an extended period, they switched to producing GFAP-positive astrocytes after 4-5 days in vitro (DIV) (Fig. 2A). The generation of astrocytes in E11.5 cortical NPC cultures can be further promoted by treatment with LIF (Fig. 2A). However, even in the presence of LIF, no GFAP-positive astrocytes were observed in short-term (fewer than 4-5 DIV) mouse E11.5 cortical cultures, suggesting that there is an intrinsic mechanism blocking the initiation of astroglial differentiation in early NPCs.

In contrast to the lack of GFAP positive astrocytes in control (*Dnmt1*$^{+/+}$) cultures, GFAP-positive cells with astrocytic morphologies were observed in E11.5 *Dnmt1*$^{-/-}$ cultures at 2 DIV with LIF treatment (Fig. 2B). The precocious induction of GFAP expression as well as another astrocyte marker S100β in *Dnmt1*$^{-/-}$ NPC cultures was also evident (Fig. 2C,D). In

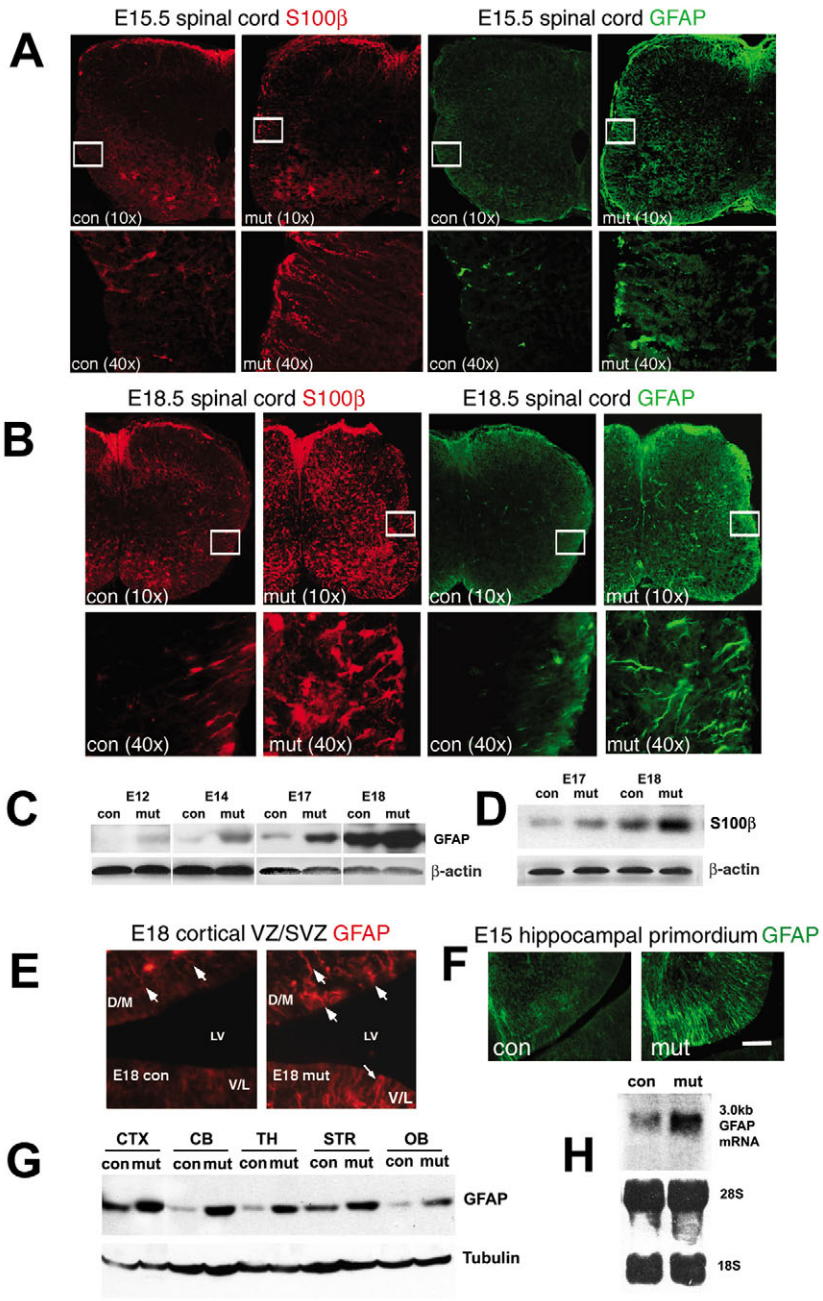

Fig. 1. Precocious astroglial differentiation in *Dnmt1*$^{-/-}$ CNS in vivo. (A,B) Immunohistochemistry studies indicate enhanced S100β- and GFAP-positive staining in E15.5 (A) and E18.5 (B) cervical spinal cords from littermate control (con) and Dnmt1$^{-/-}$mutant (mut) mice. Bottom rows show enlargements of the boxed areas in the tops rows. (C,D) Western blot analysis of GFAP (C) and S100β (D) proteins in E12-18 spinal cords at cervical/thoracic level. (E) GFAP immunostaining (red, arrows) of the E18.5 cortical VZ/SVZ in coronal sections (relatively caudal regions) (LV, lateral ventricle; D/M, dorsal/medial; V/L, ventral/lateral). (F) GFAP immunostaining of E15.5 hippocampal primordial areas. Scale bar: 157 μm. (G) Western blot analysis of GFAP protein in E18.5 brain samples. CTX, cortex; CB, cerebellum; TH, thalamus; STR, striatum; OB, olfactory bulb. Re-blotting with an antibody against βIII-tubulin serves as an internal control for loading. (H) Northern analysis of GFAP mRNA in E18.5 whole brain samples. con, control; mut, *Dnmt1*$^{-/-}$.

these cultures, virtually all of the cells from mutant mouse CNS lacked Dnmt1 (Fan et al., 2001). Using a monoclonal antibody against 5′-methylcytosine (5′meC), we found that the intensity of 5′meC staining was significantly weaker in *Dnmt1*$^{-/-}$ cells than that in control cells after three days in culture, which is consistent with decreased DNA methylation in *Dnmt1*$^{-/-}$ cells (Fig. 2E). However, immunostaining with the 5′-methylcytosine antibody is not quantitative enough to allow us to address whether newly generated GFAP-positive astroglial cells are exclusively derived from those *Dnmt1*$^{-/-}$ NPCs with a more extensively demethylated genome. Finally, we also carried out TUNEL staining and compared cell death phenotypes between cultured control and Dnmt1 mutant NPCs after 5 DIV. There was no apparent difference in the percentage of TUNEL-positive cells when mutant cultures were compared with control cultures [CON=2.7% (75/2728); MUT=3.9% (92/2347)], indicating that the increase in GFAP and S100β positive cells in *Dnmt1*$^{-/-}$ cultures did not result from changes in cell survival in vitro.

To determine whether the enhanced astrocyte differentiation detected in E11.5 *Dnmt1*$^{-/-}$ CNS progenitors was at the expense of neurogenesis, replication-deficient recombinant retrovirus carrying a transgene encoding the green fluorescent protein (GFP) was used to infect E11.5 wild-type and mutant CNS cultures at 1 DIV. The differentiation of the virally infected cells was analyzed 2 days later. These experiments demonstrated that the enhanced astrogliogenesis in *Dnmt1*$^{-/-}$ NPC cultures occurred at the expense of neurogenesis, suggesting that a precocious neuro- to gliogenic switch is achieved through DNA hypomethylation (Fig. 2F). Demethylation-induced GFAP expression persists into the gliogenic phase, as cortical cultures generated from E15 animals also show substantially higher numbers of GFAP-positive cells in mutant cultures than in control cultures with or without LIF treatment (Fig. 2G). Consistent with the in vivo observation, GFAP staining did not overlap with any MAP2 staining in both control and *Dnmt1*$^{-/-}$ E15 cortical cells (Fig. 2G). This finding indicates that DNA hypomethylation did not induce any aberrant/ectopic expression of glial marker genes in neurons. Thus, the effect of Dnmt1 deficiency on GFAP gene expression is restricted to the gliogenic NPCs that give rise to more GFAP-positive astrocytes.

Enhanced activation of the JAK/STAT astrogliogenic pathway due to DNA hypomethylation

Astroglial differentiation is regulated by LIF-induced activation of the JAK-STAT pathway. The major STAT proteins expressed in the nervous system are STAT1 and STAT3. Importantly, increased STAT1/3 expression and

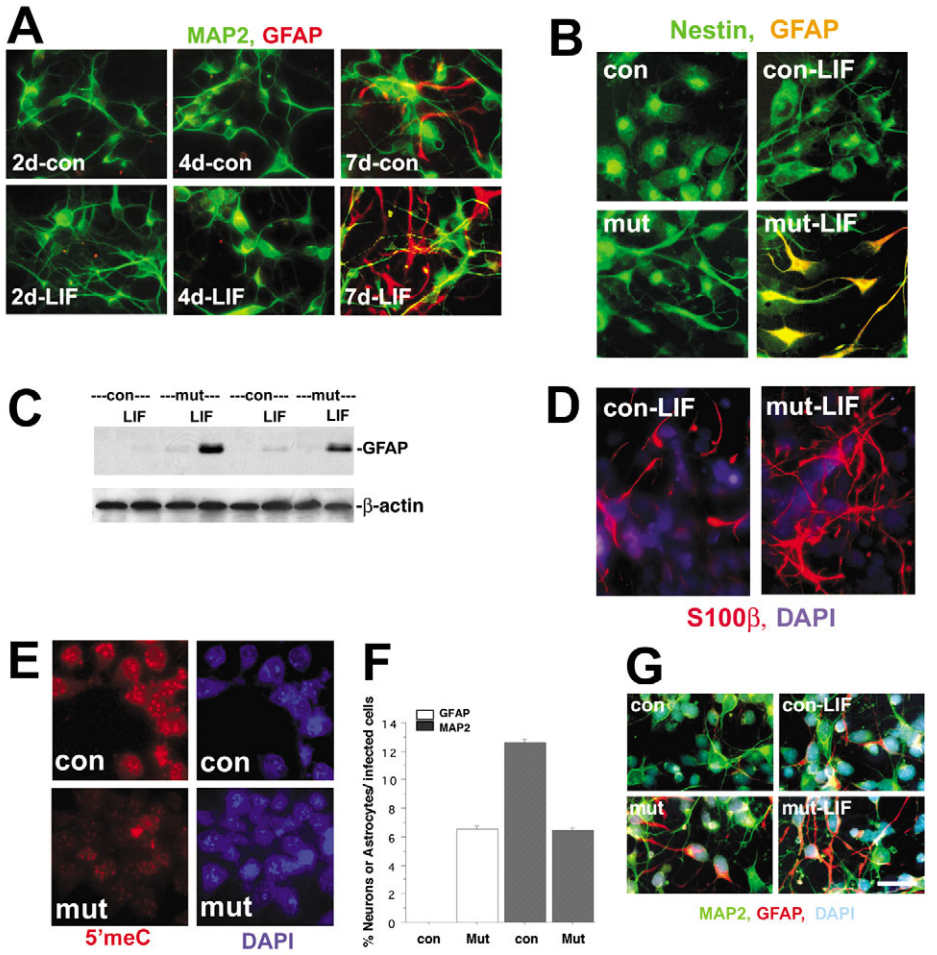

Fig. 2. Precocious astroglial differentiation in methylation-deficient E11.5 and E15.5 mouse CNS cultures. (A) Wild-type E11.5 mouse cortical precursor cells from Balb/c wild-type mice were dissociated and cultured for 2 days (2 d), 4 days (4 d) and 7 days (7 d) in the absence (con) and presence of LIF (50 ng/ml). Cells were stained with antibodies against a neuronal marker MAP2 (green) and GFAP (red). (B) E11.5 CNS cells from control (con) and *Dnmt1*$^{-/-}$ (mut) littermate embryos were cultured with or without LIF treatment for 2 days, and stained for GFAP (red) and a neural progenitor marker, nestin (green). Co-localization of nestin and GFAP (orange) in newly differentiated astrocytes in *Dnmt1*$^{-/-}$mutant cultures (mut-LIF) indicates precocious astrocyte differentiation. (C) Western blot analysis of GFAP protein in two pairs of 3-day-old E11.5 NPC cultures. β-actin serves as a sample loading control. (D) S100β staining (red) of E11.5 CNS cells that were cultured for 4 days with LIF treatment in the last 2 days. DAPI nuclear counterstaining (blue) indicates similar cell densities between control (con) and *Dnmt1*$^{-/-}$ (mut) cultures. (E) 5′-methylcytosine (5′meC) antibody staining (red) of 3-day-old E11.5 control (con) and *Dnmt1*$^{-/-}$ (mut) NPCs. Counterstaining with DAPI (blue). The nuclear staining pattern is distinct, with heterochromatic punctuates intensely positive for 5′meC. (F) NPCs were infected with a GFP-expressing retrovirus on the first day of culturing. Two- to 3 days later, the virally infected GFP cells were stained for GFAP or MAP2, and the percentage of cells differentiating into either neurons or glia was measured and plotted (n=4). (G) Four-day cultured E15.5 cortical cells from control (littermate) and *Dnmt1*$^{-/-}$ mice in the presence and absence of LIF, were triple labeled with MAP2 (green), GFAP (red) and DAPI (blue). Scale bar: 32 μm.

phosphorylation in E11.5 NPCs is correlated with the neuro- to gliogenic switch in vitro (Fig. 3A) (He et al., 2005). In addition, we noticed that genes encoding various components of the JAK-STAT pathway including STAT1, STAT3, gp130 receptor, as well as JAK1 contain STAT-binding elements in their promoters that can be activated by STATs, suggesting that an autoregulatory/positive feedback loop of this pathway exists. This positive-feedback loop may be important for the rapid and robust activation of JAK-STAT signaling during the neuro- to gliogenic transition (He et al., 2005).

We reasoned that the precocious onset of astroglial differentiation observed in the hypomethylated CNS may be mediated in part by increased activation of the JAK-STAT pathway. To test this hypothesis, we examined whether DNA hypomethylation induces hyperactivation of the JAK/STAT pathway. We found that upon transient LIF treatment the levels of activated/phosphorylated STAT1/3 (pSTAT1/3) were significantly higher in 4-day cultured E11.5 *Dnmt1*$^{-/-}$ cells than in control cells (Fig. 3B). The elevated levels of activated/phosphorylated STATs in *Dnmt1*$^{-/-}$ cultures could result from either increased STAT expression caused by DNA hypomethylation or from enhanced phosphorylation/decreased dephosphorylation of the STATs without alteration of their protein levels. To distinguish between these two possibilities, we analyzed levels of total STAT proteins in control and mutant cells. STAT1 protein levels were elevated in cultured *Dnmt1*$^{-/-}$ cells in comparison with those in control cells (Fig. 3B). The increase in STAT3 levels was moderate in mutant cells (Fig. 3B). All three splice variants of STAT1 mRNA were upregulated in E18.5 mutant brains (Fig. 3C), consistent with increased gene transcription of the STAT1 gene in hypomethylated cells. In addition, quantitative RT-PCR and western blot analyses indicated that both mRNA (data not shown) and protein levels of gp130 were elevated in *Dnmt1*$^{-/-}$ cells (Fig. 3D), which may contribute to the increase in STAT3 phosphorylation.

To examine whether the elevated STAT phosphorylation observed in *Dnmt1*$^{-/-}$ E11.5 CNS cultures had any functional consequences in the activation of astrocyte marker genes, we transfected a 1.9 kb GFAP promoter-luciferase reporter construct (Bonni et al., 1997) into 3 DIV cultures from either control or mutant E11.5 mouse CNS. The cells were either left untreated or treated with LIF (50 ng/ml) immediately following transfection and analyzed for luciferase activity 24 hours later. LIF treatment induced a stronger activation of the GFAP promoter in *Dnmt1*$^{-/-}$ cells than in *Dnmt1*$^{+/+}$ cells (Fig. 4A). To

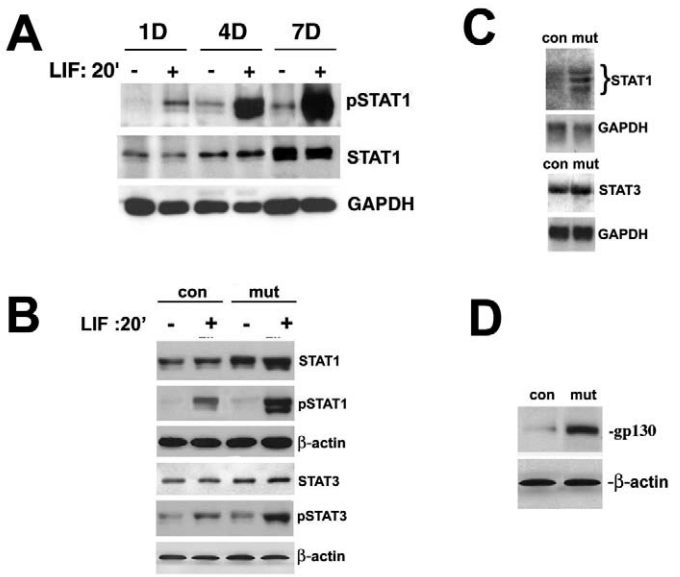

Fig. 3. Enhanced activation of JAK/STAT signaling in hypomethylated NPCs. (A) Western blot analysis of total STAT1 and pSTAT1 proteins in 1 day (1 D), 4 day (4 D) and 7 day (7 D) cultured E11.5 wild-type cortical cells with transient 20 minute LIF treatment. (B) Western blot analysis of total and phosphorylated STAT1/3 protein in 4-day-old cultured control (con) and *Dnmt1*$^{-/-}$ (mut) E11.5 CNS cells with 20 minutes LIF treatment. (C) Northern blot analysis of STAT1 and STAT3 mRNA in E18.5 CNS samples. (D) Western blot analysis of gp130 receptor protein in E18 cortices. con, control; mut, *Dnmt1*$^{-/-}$.

determine whether enhanced activation of the exogenous GFAP promoter resulted from the elevated activity of STAT1/3 or other transcription factors, we introduced a STAT-binding mutant of the GFAP promoter-luciferase reporter and measured luciferase activities in both control and methylation-deficient cells. When the canonical STAT1/3 binding element was mutated (from TTCCGAGAA to CCAAGAGAA), the LIF-induced GFAP promoter activation in both control and mutant cultures was abolished (Fig. 4A). This result indicated that the increase in GFAP promoter activity in *Dnmt1*$^{-/-}$ cells is caused by enhanced STAT function (Fig. 4A). Consistent with enhanced STAT1/3 activation in *Dnmt1*$^{-/-}$ cells, both EMSA and chromatin immunoprecipitation (ChIP) assays further indicated that *Dnmt1*$^{-/-}$ CNS cells contain more nuclear pSTAT1/3 and that more STAT1/3 were associated with the endogenous GFAP promoter in *Dnmt1*$^{-/-}$ CNS cells (Fig. 4B,C). Taken together, these data suggest that the enhanced activation of the JAK-STAT pathway in methylation-deficient CNS cells leads to stronger activation of astrocyte marker genes.

It is conceivable that in hypomethylated *Dnmt1*$^{-/-}$ CNS, a number of genes are misregulated. To determine whether the precocious astrocyte differentiation observed in *Dnmt1*$^{-/-}$ CNS cells was predominantly mediated by elevated JAK-STAT activity, we transfected a dominant interfering form of STAT3, STAT3F, into the *Dnmt1*$^{-/-}$ cells. STAT3F harbors a mutation of tyrosine (Y) 705 into phenylalanine (F) within the STAT3 protein (Bonni et al., 1997). The mutant STAT3 (STAT3F) permanently binds to the STAT docking sites within the LIF receptors gp130 and LIFR, and therefore blocks endogenous STAT1/3 from being phosphorylated upon ligand stimulation. STAT3F significantly suppressed hypomethylation-induced astrogliogenesis (Fig. 4D,E), indicating a crucial role for elevated JAK-STAT phosphorylation, instead of other demethylation-induced pathways, in triggering precocious astroglial differentiation in *Dnmt1*$^{-/-}$ CNS.

Dnmt1 deficiency accelerates the developmentally regulated demethylation of glial differentiation-related genes

Multiple CpG sites within the rat *Gfap* promoter are methylated early on during CNS development and become less methylated during gliogenic stages in vivo (Teter et al., 1996). Takizawa et al. also reported that a single CpG site within the STAT-binding element in the mouse *Gfap* promoter undergoes demethylation during the neuro- to gliogenic switch in the developing CNS. To further examine the relationship between changes in DNA methylation and gliogenesis, we analyzed the methylation status of the mouse *Gfap*, *Stat1* and S100β genes in neurogenic and gliogenic NPCs. Within the *Gfap* promoter, we focused on a region from –1557 bp to –1280 bp, which is highly conserved across species of mouse, rat and human. In fact, this region in the rat *GFAP* gene has been designated as the neuroectoderm/astrocyte methylation domain (Fig. 5A), because it undergoes demethylation during the neurogenic to gliogenic phase transition (Teter et al., 1996). Importantly, the relative position and sequence of the STAT-binding element (5′TTCCGAGAA3′) within this neuroectoderm methylation region is 100% conserved among the three species. We performed bisulfite genomic sequencing analyses on 8 CpG sites within this promoter region. Our analysis showed that selective demethylation occurred at five out of the eight CpG sites in E11.5 cortical NPCs over 4 days in culture, including the CpG within the STAT-binding element (Fig. 5A). Similarly, selective CpG demethylation was also observed on the *Stat1* and S100β promoters in E11.5 cortical culture during the neuro- to gliogenic switch (Fig. 5B, see Fig. S1 in the supplementary material).

Loss of Dnmt1 activity would be predicted to cause accelerated demethylation of the *Gfap*, *Stat1* and S100β promoters in the developing CNS. To directly examine the methylation status of the STAT-binding element within the *Gfap* promoter in both control and Dnmt1 mutant cells, we performed bisulfite genomic sequencing analyses and the methylation-site-specific single nucleotide primer extension (SNuPE) assays (Gonzalgo and Jones, 1997). The SNuPE assay showed a significant decrease in methylation of the STAT-binding site in cultured E12.5 *Dnmt1*$^{-/-}$ CNS cells compared with control cells (Fig. 5C). The CpG site within the *Gfap* STAT-binding site is virtually completely unmethylated in E18.5 *Dnmt1*$^{-/-}$ brains in vivo (Fig. 5C), suggesting that demethylation of this CpG site also occurs in the neuronal population. However, neuronal *Gfap* expression was not detected, supporting the notion that demethylation of the CpG within the STAT binding element is not sufficient to induce GFAP expression. It is likely that additional mechanisms exist in neurons to actively suppress the demethylated GFAP gene promoter in *Dnmt1*$^{-/-}$ neurons. Substantial demethylation of the *Stat1* and *S100β* promoters was also observed in *Dnmt1*$^{-/-}$

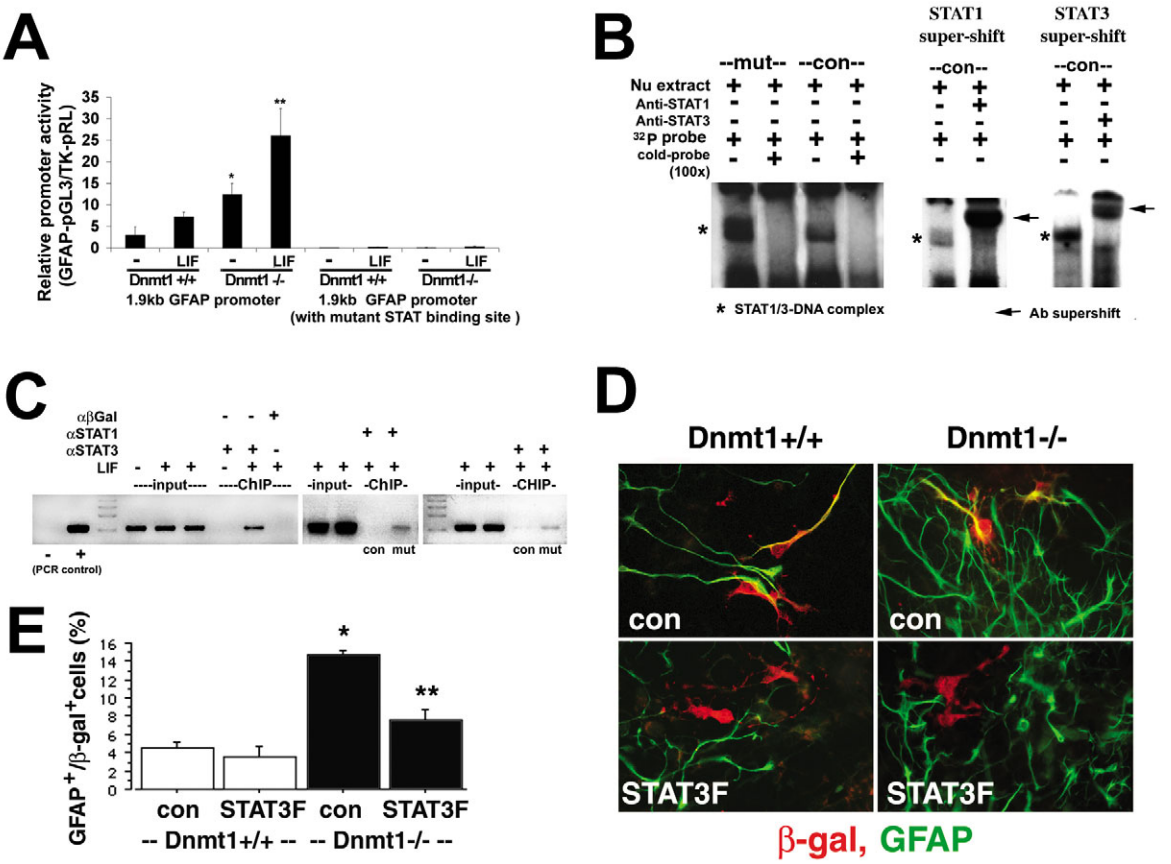

Fig. 4. Precocious astroglial differentiation is mediated by enhanced activation of JAK/STAT signaling in *Dnmt1*$^{-/-}$ NPCs. (A) Wild-type or a STAT-binding mutant form of the 1.9 kb rat GFAP promoter-luciferase reporter constructs were co-transfected with the renilla-TK control plasmid into 3 day cultured E11.5 control and *Dnmt1*$^{-/-}$ CNS NPC cultures. After 24 hours, cells were lysed and subjected to dual-luciferase assays (Promega). **P*<0.001 compared with the control group (*Dnmt1*$^{+/+}$) without LIF treatment. ***P*<0.01 compared with the group of *Dnmt1*$^{-/-}$ cells without LIF treatment (ANOVA with Post-hoc tests). (B) EMSA assay using a 25 bp unmethylated probe containing the STAT-binding element within the GFAP promoter with nuclear extracts from cultured control and *Dnmt1*$^{-/-}$ E11.5 CNS cells as in A. The identity of the DNA-protein complex (*) was characterized using anti-STAT1 and anti-STAT3 supershift assays (arrows). (C) Left panel, bFGF expanded (gliogenic) cortical progenitor cells were left untreated or treated with LIF for 30 minutes and subjected to chromatin immunoprecipitation (ChIP) assay with an antibody against STAT3 (Santa Cruz). A control antibody, anti-β-galactosidase, was used to control for ChIP assay specificity. In the right two panels, ChIP assays were performed on 3-day-old cultured control (con) and *Dnmt1*$^{-/-}$ (mut) E11.5 CNS cells using the STAT1 and STAT3 antibodies. (D,E) E11.5 control *Dnmt1*$^{+/+}$ and *Dnmt1*$^{-/-}$ CNS NPCs were cultured for 48 hours and co-transfected with a β-gal-expressing construct and a control plasmid (con) or a dominant-negative STAT3F plasmid (STAT3F). After another 48 hours, cells were fixed and double-stained with antibodies against GFAP (green) and β-gal (red), and counted for the percentage (mean±s.e.m.) of GFAP and β-gal double-positive cells over total β-gal positive cells. **P*<0.01 compared with the *Dnmt1*$^{+/+}$ (con) group. ***P*<0.01 compared with the group of *Dnmt1*$^{-/-}$ cells with β-gal transfection (con) (ANOVA with Post-hoc tests).

brains during development, which could contribute to the induction of STAT1 and S100β in hypomethylated NPCs and/or astroglia (Fig. 5D, see Fig. S1 in the supplementary material).

To examine whether re-introducing Dnmt1 gene expression in E11 *Dnmt1*$^{-/-}$ NPCs would abrogate the phenotype of precocious astroglial differentiation, we cultured E11 control and *Dnmt1*$^{-/-}$ NPCs and transfected these cells with either a β-gal expression vector as a control or a mouse Dnmt1 cDNA expression plasmid (Chen et al., 2003) on the first day of culturing. BrdU-labeling experiments confirmed that 80% of β-gal transfected cells were positive for BrdU incorporation when BrdU was applied at the time of plasmid transfection, confirming that a majority of the plasmid transfected cells were mitotic neural precursor cells. Cultures were treated with LIF (50 ng/ml) for an additional 4 days to increase the number of GFAP-positive cells. By double-labeling cultured cells with GFAP/β-gal or GFAP/Dnmt1 antibodies, we found that overexpression of Dnmt1 completely blocked an increase in the percentage of GFAP-positive astrocytes in *Dnmt1*$^{-/-}$ cells (Fig. 5E,F). This result supports the notion that demethylation acts through a cell-autonomous effect on the neurogenic to gliogenic switch of E11 NPCs.

It has previously been reported that methylation of the STAT cis-element within the *Gfap* promoter blocks STAT3 association (Takizawa et al., 2001). Using EMSA, we confirmed that methylation of the STAT-binding element attenuates phospho STAT1 (pSTAT1) and the STAT 1/3 complex from associating with the GFAP promoter (see Fig. S2 in the supplementary material). However, in contrast to the

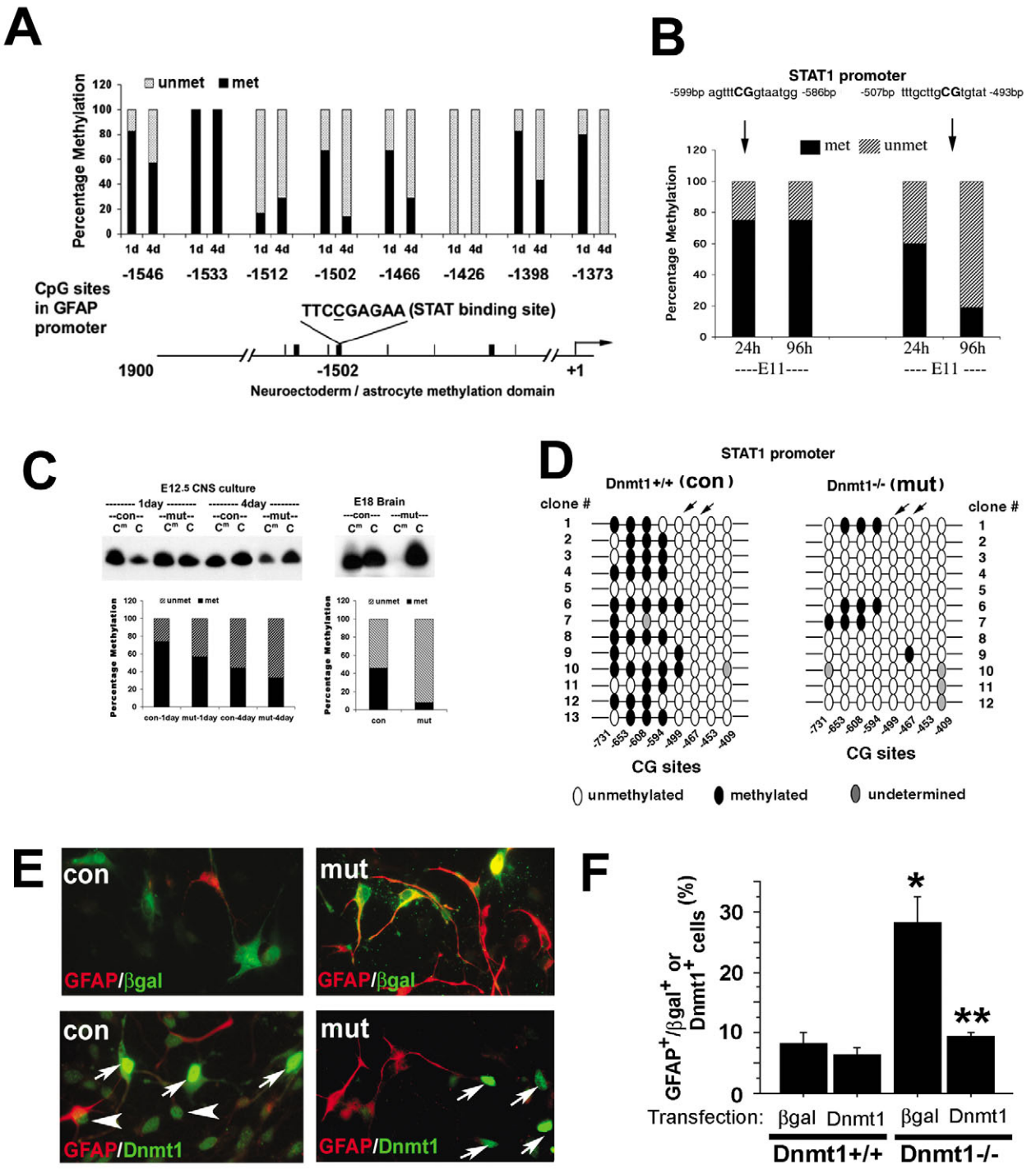

Fig. 5. Changes of DNA methylation on the GFAP and STAT1 promoters when NPCs become gliogenic in control and *Dnmt1*$^{-/-}$ cells. (A) Bisulfite sequencing analysis on eight CpG sites surrounding the STAT1/3 binding elements within the mouse *Gfap* promoter. The percentage of methylation at each of the 8 CpG sites was plotted. (B) Bisulfite sequencing analysis shows selective demethylation occurs at the –499 CpG site but not at the –594 CpG site during 24-96 hours of culturing period of wild-type E11.5 cortical cells. (C) Methylation-specific SNuPE assay was used to independently quantify the extent of methylation at the single CpG site lying within the STAT binding element in 1- and 4-day-old cultured E12.5 control (con) and *Dnmt1*$^{-/-}$ (mut) CNS cells and in E18.5 brain samples in vivo. (D) Bisulfite sequencing analysis of eight CpG sites within the *Stat1* promoter (between –731 bp and –409 bp promoter region of the gene) in E18 control and *Dnmt1*$^{-/-}$ CNS samples. (E,F). E11 control (con) or *Dnmt1*$^{-/-}$ (mut) NPCs were transfected with either a β-gal expression vector or a CAG-promoter-Dnmt1 expression plasmid (Chen et al., 2003) within the first 24 hours of cell culturing. After an additional 4 days of culturing in the presence of LIF (50 ng/ml) to promote glial differentiation, cells were double-labeled with GFAP/β-gal or GFAP/Dnmt1 (E) and quantified for the percentage of GFAP+/β-gal+ or GFAP+/Dnmt1+ cells as plotted in F. Dnmt1 overexpression cells can be easily detected by the strong Dnmt1 staining signals (arrows). Two arrowheads in the control culture indicate the typical nuclear staining pattern of the endogenous Dnmt1 protein. **P*<0.001 compared with control (*Dnmt1*$^{+/+}$) with β-gal plasmid transfection. ***P*<0.001 compared with *Dnmt1*$^{-/-}$ cells with β-gal plasmid transfection (ANOVA with Post-hoc tests).

Gfap promoter, the STAT-binding elements of the *Stat1* and S100β promoters do not contain any CpG sites. Therefore, the aforementioned mechanism, i.e. reduced binding affinity between STATs and the methylated STAT cis-element, cannot contribute to DNA methylation mediated suppression of *Stat1* and S100β genes. Therefore, we examined whether DNA methylation inhibits expression of *Stat1*, S100β and *Gfap* through a general gene silencing mechanism, i.e. inactive chromatin remodeling.

DNA methylation inhibits glial differentiation genes through a methyl-CpG binding protein mediated chromatin remodeling process

DNA methylation induced gene silencing is usually mediated through binding of methyl-CpG-binding proteins that recruit histone deacetylases and histone methyltransferases to trigger inactive chromatin remodeling. Using ChIP assays, we determined whether STAT3 and methyl-CpG binding proteins reciprocally associate with *Gfap*, *Stat1* and *S100β* promoters, based on the methylation status of these genes. We found that in early neural progenitors (E11.5, 1 DIV), a time at which glial genes are more methylated, the *Gfap*, *Stat1* and *S100β* promoters are more tightly associated with the methyl-CpG binding protein, MeCP2, but not the transcriptional activator STAT3 (Fig. 6A-E). However, in late, more gliogenic, cells (E11.5, 6-7 DIV), where glial genes are demethylated, all three glial differentiation-related genes are more closely associated with STAT3, but not with MeCP2. These findings are consistent with the notion that methylation of glial differentiation-related promoters may lead to gene silencing through methyl-CpG-binding protein mediated inactive chromatin remodeling. Using western analysis as well as immunostaining, we established that MeCP2 is expressed in E11.5 CNS progenitors (see Fig. S3 in the supplementary material). In addition, the level of MeCP2 expression in our E11.5 mouse cortical cultures does not appear to dramatically change over the culturing period (see Fig. S3 in the supplementary material). MeCP2 is known to recruit histone modification enzymes such as histone H3 lysine9 methyltransferases and histone deacetylases (Lunyak et al., 2002), which may function to modify histone tails, resulting in inactive chromatin remodeling. To assess whether the chromatin structure of glial genes is inactive or active during the neurogenic (E11.5, 1 DIV) and the gliogenic (E11.5, 6-7 DIV) phases of the CNS culture, we probed the status of histone codes such as dimethyl-lysine 9 of histone H3 (H3dmK9) for inactive chromatin and di- or tri-methyl-lysine4 of histone H3 (H3d/tmK4) as an indicator for active chromatin. As anticipated for neurogenic progenitors, the *Gfap*, *Stat1* and *S100β* promoters displayed more inactive chromatin modifications (Fig. 6F-H). Conversely, in gliogenic cells, the promoters are more associated with the active chromatin code (Fig. 6F-H).

To examine whether DNA hypomethylation leads to active chromatin remodeling in *Gfap*, *Stat1* and *S100β* promoters in the absence of Dnmt1 in vivo, we performed ChIP assays and compared the code of histone modifications in E16-18 control and *Dnmt1*$^{-/-}$ CNS. As shown in Fig. 6I,J), *Dnmt1*$^{-/-}$ cortical cells exhibited an increase in H3-K4 di/tri-methylation and a dramatic decrease in H3-K9-dimethylation in *Gfap* and *Stat1*

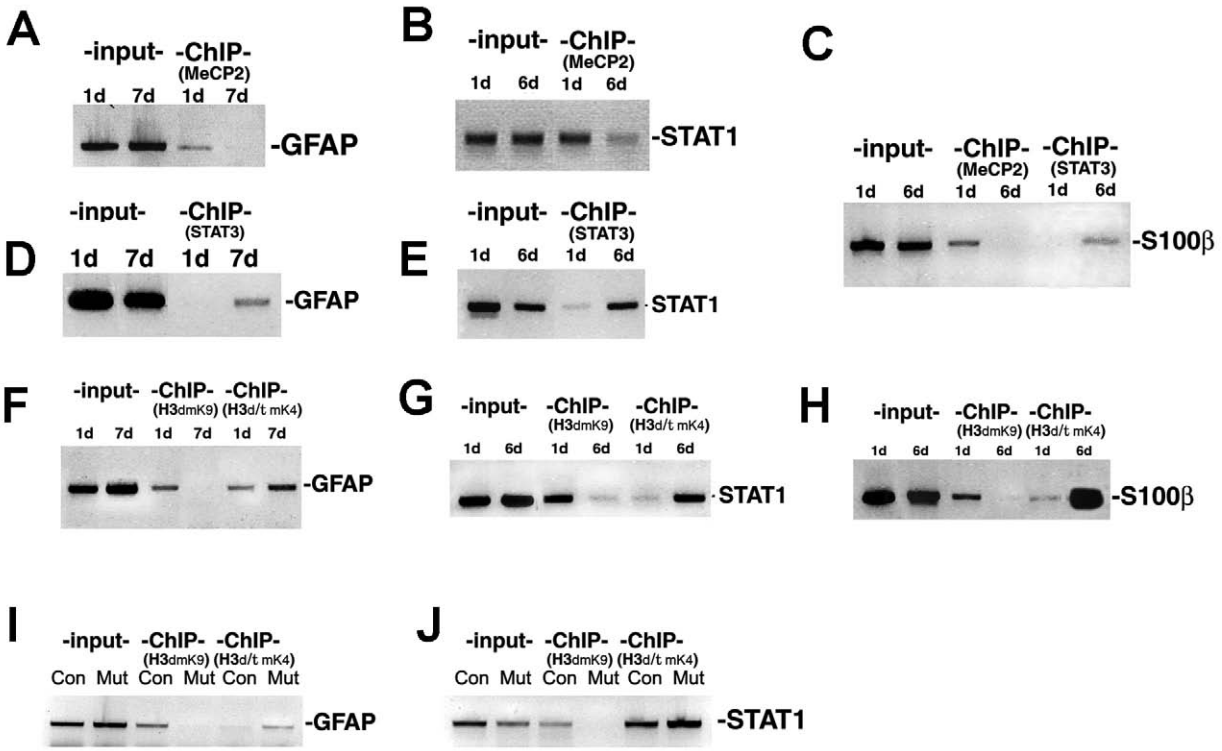

Fig. 6. Effect of DNA methylation on pSTAT association and activation of the *Gfap*, *Stat1* and *S100β* promoters. (A-E) ChIP assays to analyze the association of MeCP2 and STAT3 with the *Gfap, Stat1* and *S100β* promoters. (F-H) ChIP assays of histone H3 di-methyl lysine 9 (K9) and di- or tri-methyl lysine 4 (K4) within the *Gfap*, *Stat1* and *S100β* promoters. (I,J) E16 control and *Dnmt1*$^{-/-}$ cortical tissues were analyzed by ChIP assays for histone H3 di-methyl lysine 9 (K9) and di- or tri-methyl lysine 4 (K4) within the *Gfap* and *Stat1* promoters

promoters. A moderate increase in H3-K4 di/tri-methylation was also observed on the *S100β* promoter (data not shown). Taken together, these data indicate that enhanced active chromatin remodeling occurs on those genes involved in astroglial differentiation in *Dnmt1*$^{-/-}$ cells in vivo.

Discussion

Epigenetic modifications such as DNA methylation have been proposed to regulate cell differentiation during development (Paulsen and Ferguson-Smith, 2001); however, the molecular mechanism by which DNA methylation regulates specific cell differentiation programs have just begun to be characterized (Lunyak et al., 2002; Stancheva et al., 2003; Stancheva et al., 2002; Stancheva and Meehan, 2000). Here, we have shown that in the absence of Dnmt1 there is precocious onset of astrogliogenesis in the developing CNS as measured by the early appearance of GFAP- and S100β-positive glial cells. Our finding suggests that precocious astroglial differentiation in *Dnmt1*$^{-/-}$ NPCs is predominantly mediated by enhanced activation of the astrogliogenic JAK-STAT signals through accelerated demethylation of gene promoters associated with the JAK-STAT pathway. Mechanistically, accelerated demethylation promotes active chromatin remodeling/derepression of the JAK-STAT pathway genes as well as astroglial marker genes. When Dnmt1 expression is restored in *Dnmt1*$^{-/-}$ NPCs, precocious astroglial differentiation is prevented, suggesting a causal relationship between Dnmt1 expression and the control of the onset of astrogliogenesis in NPCs. Our data support a model in which DNA methylation, through inhibition of STAT activity, plays an important role in regulating the timing of astroglial differentiation in mammalian CNS progenitor cells both in vitro and in vivo (Fig. 7).

We hypothesize that during CNS development, the generation of neurons in the absence of glia relies on the suppression of glial differentiation programs during the neurogenic period. Many glial inducing factors, including LIF, BMP, NOTCH and the oligodendrogliogenic bHLH factor, OLIG2, are unable to induce glial differentiation during the neurogenic period (Ge et al., 2002; Sauvageot and Stiles, 2002; Sun et al., 2003; Viti et al., 2003a). The ability of these factors to regulate cell fate is thought to depend on the intrinsic state of the precursor cell. Our study suggests that the JAK/STAT pathway is a crucial control point for regulating the intrinsic responsiveness of neural progenitors to astrogliogenic factors. We previously demonstrated that the expression of a proneural bHLH factor, NGN1, which is exclusively expressed in progenitor cells during the neurogenic period, inhibits STAT1/3 phosphorylation and prevents pSTATs from activating glial gene transcription by sequestering the transcription co-activator, p300/CBP, away from glial specific promoters (Sun et al., 2001). This present study indicates that DNA methylation is another key mechanism inhibiting the expression of the various components of the astrogliogenic JAK-STAT pathway. Thus, JAK-STAT activation in neural precursor cells is under the tight control of several independent mechanisms.

Recently, other investigators have challenged the importance of JAK/STAT activation in the regulation of gliogenesis by emphasizing that pSTATs can be detected in the neurogenic CNS (Molne et al., 2000; Takizawa et al., 2001). However, our study, as well as those of others, indicate that the pSTAT1/3 signals detected in the neurogenic culture are minimal (Viti et al., 2003a; He et al., 2005). Weakly phosphorylated STATs in early neural progenitors cannot effectively activate glial gene transcription, even if the glial promoter is not methylated, as demonstrated in luciferase reporter assay (He et al., 2005). However, using a constitutively dimerized and nuclear localized form of STAT3, STAT3C, in combination with LIF stimulation, we observed precocious astrocyte differentiation in 2 DIV E11.5 and E12 cortical cells (He et al., 2005). Taken together, these data suggest that robust activation of STATs may either override the methylation inhibition of glial genes or accelerate the demethylation process of glial genes.

CpG methylation can directly inhibit gene transcription when methylation blocks the association of transcription factor to the cis-element, as demonstrated for the association of the GFAP promoter with STAT3 (Takizawa et al., 2001) and STAT1 (see Fig. S2 in the supplementary material). However, the canonical STAT-binding elements in many other genes

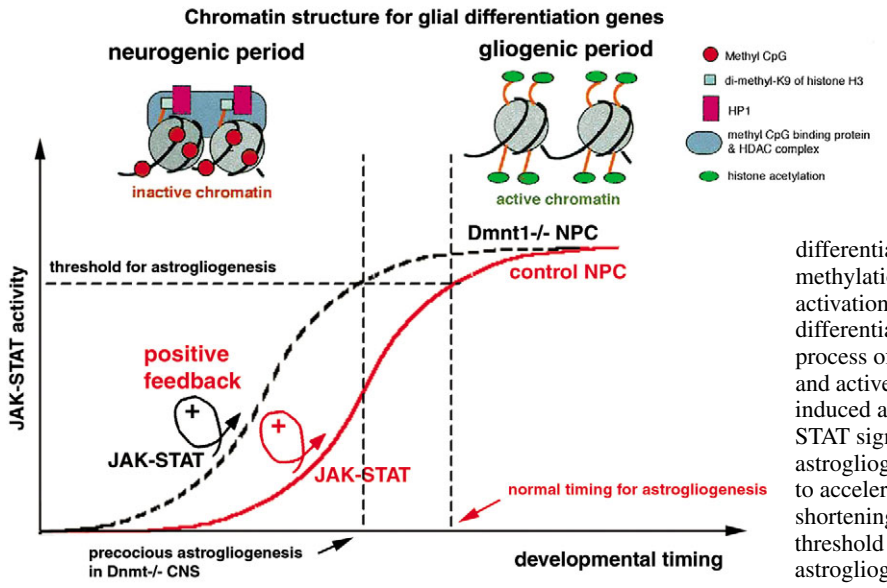

Fig. 7. A model for DNA methylation-related glial gene chromatin remodeling during the switch from neurogenesis to gliogenesis. We have previously demonstrated that a positive-feedback loop for the JAK-STAT pathway allows for rapid activation of this pathway once it is derepressed (He et al., 2005). The time it takes to reach the threshold STAT activity for astroglial differentiation marks the onset of astrogliogenesis. DNA methylation serves as one of the key mechanisms blocking activation of the JAK-STAT pathway and glial cell lineage differentiation during the neurogenic period. Through a process of developmentally regulated DNA demethylation and active chromatin-remodeling, the JAK-STAT pathway is induced and astrocytic marker genes become responsive to STAT signaling, which marks the initiation of astrogliogenesis. In *Dnmt1*$^{-/-}$ NPCs, hypomethylation leads to accelerated activation of the JAK-STAT pathway, shortening the time required to reach the STAT activity threshold for astrocyte differentiation, leading to precocious astrogliogenesis.

involved in astroglial differentiation such as STAT1, STAT3, gp130, JAK1 and S100β do not contain a CpG site (He et al., 2005), suggesting that the direct effect of CpG methylation on the binding of STATs is not a general mechanism in the inhibition of JAK-STAT signaling. Instead, our data (Fig. 6) demonstrate that DNA methylation suppresses the gliogenic pathway genes via methyl-CpG-binding protein-mediated inactive chromatin remodeling. Indeed, the association of methyl-CpG-binding proteins such as MeCP2, which recruit histone modification enzymes associated with inactive chromatin remodeling, could be involved in glial gene silencing (Bird and Wolffe, 1999; Jones et al., 1998; Martinowich et al., 2003). Our ChIP study showed that MeCP2 was associated with methylated glial lineage genes in neurogenic cells. However, we cannot rule out the possible involvement of other methyl binding proteins (e.g. MBD1-3) in the nervous system for their potential role in glial gene silencing during early CNS development (Heinrich and Bird, 1998) (reviewed by Fan and Hutnick, 2005).

DNA methylation may be one of the many repressive mechanisms to prevent superfluous transcription to achieve cell-specific gene expression during differentiation. In this regard, methylation of the GFAP promoter is proposed to be one of the key silencing mechanisms for inhibiting GFAP expression in peripheral non-neural cell types such as fibroblasts (Condorelli et al., 1997). In the current study, we discovered that demethylation in the GFAP promoter in E18 *Dnmt1*$^{-/-}$ CNS neurons does not lead to ectopic expression of GFAP in neuronal cells (Fig. 1 and Fig. 5B), arguing for the existence of additional repression mechanism(s) in neurons that blocks ectopic GFAP gene transcription.

In this study, we have demonstrated that nestin-cre-mediated *Dnmt1* gene deletion in mitotic E11 NPCs leads to rapid demethylation in daughter cells in culture (Fig. 2E), indicating the essential role for Dnmt1 in maintaining DNA methylation in embryonic CNS cells (Fan et al., 2001). The observed phenotype of precocious activation and active chromatin remodeling of gliogenic genes is consistent with the demethylation phenotype in *Dnmt1*$^{-/-}$ NPCs. However, it is worth noting that Dnmt1 molecule has also been shown to directly inhibit gene transcription with its N-terminal transcription repression domain, which interacts with other transcription repressor components, including histone deacetylases and histone lysine-methyltransferases for inactive chromatin remodeling (Rountree et al., 2000; Fuks et al., 2000). To determine whether Dnmt1 molecule itself would repress any astrogliogenic genes such as GFAP, STAT1 and S100β in vivo, one potential experiment is to generate a new conditional *Dnmt1* mutant allele that introduces mutations in the catalytic domain and examine astrogliogenesis phenotype in E11 NPCs that would contain a full-length mutant form of Dnmt1 without methylation activity. If we do not observe precocious astroglial differentiation with this line of mutant mice, it will argue for the direct role of Dnmt1 in repressing astrogliogenic genes. An alternative result with no rescue of the precocious glial differentiation phenotype would favor the idea that demethylation of gene promoters is the key mechanism that mediates the effect of Dnmt1 mutant phenotype.

In summary, our study has provided evidence demonstrating that disruption of DNA methylation of GFAP and other glial differentiation-related genes (e.g. genes involved in the JAK-STAT pathway) alter the timing of glial cell lineage differentiation in vivo. We speculate that such a timing control mechanism may also regulate the differentiation of other cell type lineages. It is possible that the wave of de novo DNA methylation that occurs between the blastula and gastrula stages serves as a general mechanism to block differentiation of late cell lineages effectively such that a developmentally regulated gene-specific demethylation process controls sequential cell lineage differentiation.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/15/3345/DC1

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