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FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation

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The generation of distinct cell types during development depends on the competence of progenitor populations to differentiate along specific lineages. Here we investigate the mechanisms that regulate competence of rodent cortical progenitors to differentiate into astrocytes in response to ciliary neurotrophic factor (CNTF). We found that fibroblast growth factor 2 (FGF2), which by itself does not induce astrocyte-specific gene expression, regulates the ability of CNTF to induce expression of glial fibrillary acidic protein (GFAP). FGF2 facilitates access of the STAT/CBP (signal transducer and activator of transcription/CRE binding protein) complex to the GFAP promoter by inducing Lys4 methylation and suppressing Lys9 methylation of histone H3 at the STAT binding site. Histone methylation at this site is specific to the cell's state of differentiation. In progenitors, the promoter is bound by Lys9-methylated histones, and in astrocytes, it is bound by Lys4-methylated histones, indicating that astrocyte differentiation *in vivo* involves this switch in chromatin state. Our observations indicate that extracellular signals can regulate access of transcription factors to genomic promoters by local chromatin modification, and thereby regulate developmental competence.

During cortical development, neurons are typically generated before astrocytes and oligodendrocytes, and lineage studies indicate that they can be sequentially derived from individual progenitors[1–4]. There has been longstanding interest in understanding how this process by which progenitors differentiate into different cell types is regulated. Both *in vitro* and *in vivo* studies indicate that extrinsic cues such as bone morphogenic proteins (BMPs), epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) promote astrocytic differentiation in cortical progenitors[5–8].

Although much has been learned about differentiation-inducing signals and their biological effects, little is known about the molecular mechanisms by which the competence of cells to respond to differentiation signals is regulated. For example, CNTF, an astrocyte differentiation signal, induces STAT-dependent expression of astrocyte-specific genes (such as GFAP) in late cortical progenitors but not in early progenitors[9–11]. This is particularly striking given that CNTF receptors and STAT proteins are present in both early and late progenitors[9,10] and suggests that additional mechanisms may regulate the ability of CNTF to activate STAT-dependent gene expression.

One possibility is that the ability of CNTF to activate gene expression is regulated by a second extracellular signal. A potential candidate for such a signal is FGF2, which can influence the fate of cortical progenitors without inducing terminal differentiation[12,13]. Here we show that FGF2 regulates the competence of progenitors to respond to CNTF by positively regulating STAT access to the GFAP promoter. Our observations reveal an epigenetic regulatory mechanism of gene transcription that may be broadly used to control the timing of cell differentiation.

RESULTS

To determine whether FGF2 can regulate the competence of cortical progenitors to respond to CNTF, we examined GFAP expression in E18 rat cortical cultures. FGF2 (30 ng/ml) or CNTF (100 ng/ml) was added once after plating, and cells were grown for 6 d with no replacement of media. Whereas CNTF induced GFAP expression in a subset of cells in control cultures, co-treatment with FGF2 and CNTF led to a marked increase in the fraction of cells that express GFAP (**Fig. 1a–e**).

To obtain a more quantitative measure of this effect, we examined FGF2 and CNTF induction of GFAP expression by western blot analysis. At 3 days *in vitro* (3 d.i.v.), there was no detectable GFAP expression in cultures treated with FGF2, and only low levels with CNTF alone, but robust GFAP expression was detected in cultures treated with FGF2 and CNTF (**Fig. 1f**). To determine whether FGF2 and CNTF are required simultaneously or not, we exposed cells to FGF2 and CNTF sequentially. When cortical cultures were treated with FGF2 for 24 h and then switched to CNTF, there was a clear induction of GFAP expression (**Fig. 1f**). In contrast, treatment with CNTF followed by FGF2 was not effective in inducing GFAP expression (**Fig. 1f**). Thus, FGF2 alters the ability of CNTF to induce GFAP expression in cortical cells.

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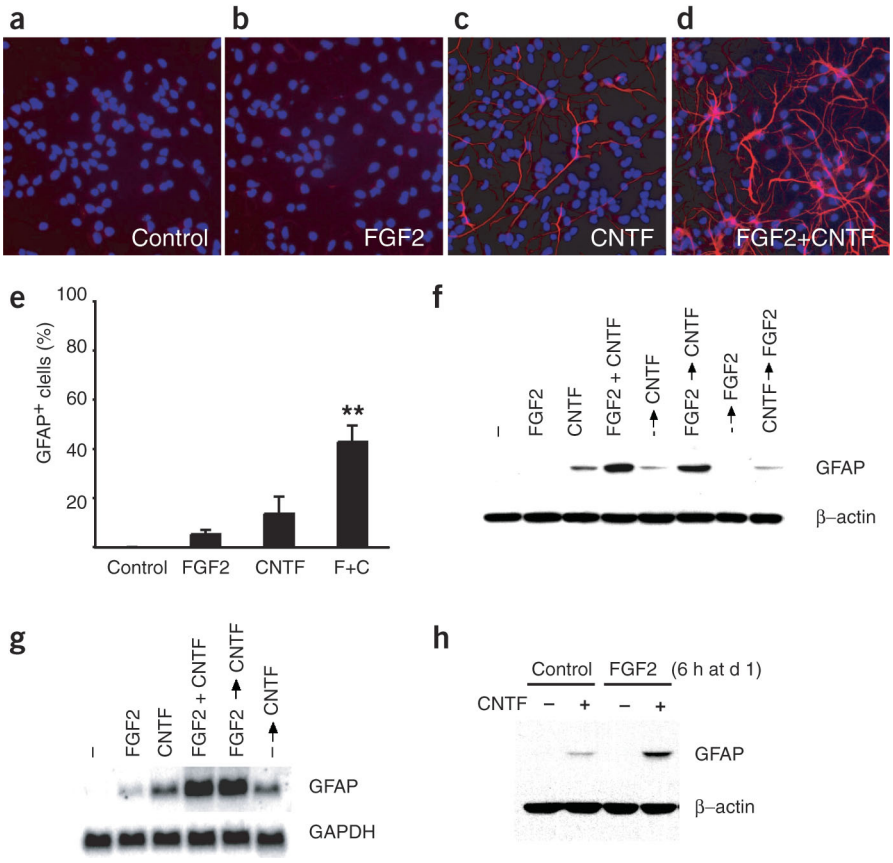

Figure 1 FGF2 induces CNTF responsiveness in cortical cells. (**a–d**) Examples of E18 rat cortical cells grown for 6 d under various conditions as indicated (30 ng/ml FGF2 and/or 100 ng/ml CNTF, a single administration after plating with no additional supplement) labeled by GFAP immunoflurescence (red) and Hoechst staining (blue). (**e**) Quantification of GFAP-expressing cells in cortical culture. Asterisks indicate statistically significant difference compared to control (**$P < 0.01$, paired *t*-test). (**f**) Western blot analysis showing GFAP and β-actin expression after treatments of FGF2 and CNTF for 3 d. For sequential addition experiments (indicated by arrows), cells were treated with FGF2 for 24 h first and then CNTF for 48 h, or vice versa. Several washes were done to remove the first factor before the next factor was added. (**g**) Effect of FGF2 and CNTF treatment on GFAP expression assessed by northern blot analysis. Experimental conditions were the same as in **f** except cells were treated with FGF2 for 48 h in sequential addition experiments. The blot was probed with GAPDH to confirm equal loading. (**h**) Effect of transient FGF2 stimulation on CNTF induction of GFAP expression by Western blot analysis. Cells were treated with FGF2 for 6 h at day 1, CNTF for the last 24 h, and analyzed at day 3.

To determine whether FGF2 regulates the ability of CNTF to induce expression of GFAP mRNA, we carried out northern blot analysis using total RNA isolated from cortical cultures. Stimulation with FGF2 or CNTF alone for 3 d only weakly induced GFAP expression (**Fig. 1g**). In contrast, co-stimulation with FGF2 and CNTF led to a robust increase in GFAP message. Treatment with FGF2 (48 h) followed by CNTF (24 h) led to a similar increase in GFAP mRNA levels. Thus, FGF2 regulates the ability of CNTF to induce GFAP expression.

Since FGF2 has a mitogenic effect on cortical progenitors, it is possible that FGF2 selectively stimulates proliferation of astrocyte precursor population, which may account for the increase in overall GFAP levels. To determine whether FGF2 could induce CNTF responsiveness independent of its mitogenic effect, we carried out experiments in which cultures were transiently exposed to FGF2 at day 1 (for 6 h), then stimulated with CNTF at day 2, and then analyzed at day 3. Unlike continuous exposure to FGF2 for 5 d, a transient exposure to FGF2 at day 1 did not induce proliferation (**Supplementary Fig. 1** online). However, subsequent exposure to CNTF was effective in inducing GFAP expression in cells exposed transiently to FGF2 (**Fig. 1h**). Thus, FGF2 can induce CNTF responsiveness independent of its mitogenic action.

Although these experiments indicate that FGF2 induces CNTF responsiveness in a population of cortical cells, they do not reveal whether or not FGF2 induces progenitors to acquire astrocytic fates at the expense of neuronal fates. To address this question, we used retroviral labeling of E18 rat cortical cultures for clonal analysis (**Fig. 2a–d**). E18 cortical cultures were transduced with the GFP-expressing replication-deficient retrovirus pBMN-IRES-GFP at low titer, and the fates of cells in individual clones were examined by immunofluorescence analysis using antibodies against green fluorescent protein (GFP), nestin (marking progenitors), β-tubulin III (marking neurons) and GFAP (marking astrocytes; **Fig. 2e**). As previously reported, a vast majority of cortical progenitors give rise to neuron-only clones under control conditions (**Fig. 2e**)[2,12,14]. FGF2 led to a marked reduction in both the absolute and relative number of neuron-only clones, indicating that FGF2 suppresses neuronal differentiation (**Fig. 2e,f**). CNTF alone did not notably affect clonal composition, but treatment with FGF2 and CNTF led to a marked increase in astrocytic ($GFAP^+$) clones compared to FGF2 alone (**Fig. 2e**). FGF2 effects were evident without any detectable change in the total number of clones or cell survival as assessed by a TdT-mediated dUTP nick end labeling (TUNEL) assay (**Fig. 2g,h**). The decrease in neuronal clones and the corresponding increase in astrocytic clones without change in total number of clones and cell survival suggest that these effects are not due to selective proliferation or cell survival. Thus FGF2 potentiates the ability of CNTF to induce cortical progenitors to adopt astrocytic fates.

How might FGF2 regulate CNTF induction of GFAP expression? One possibility is that FGF2 regulates the expression of the CNTF receptors and influences the ability of CNTF to induce GFAP expression. To determine if this was the case, we examined CNTF receptor α (CNTFRα) levels by northern and western blot analyses. Both mRNA and protein levels of CNTFRα were not altered by 3 d of FGF2 stimulation (**Fig. 3a,b**). In addition, protein levels of LIF receptor (LIFR) and gp130, other major components of CNTF receptor signaling, were not changed by FGF2 (**Fig. 3b**). Although the possibility that FGF2 might affect CNTFRα expression in a small subpopulation of cells cannot be formally ruled out, the fact that we see no significant change in CNTFRα levels in a population of cells that shows a dramatic increase in GFAP levels suggests that the induction of GFAP expression is not due to a change in CNTFRα levels. Thus FGF2 appears to induce CNTF responsiveness by a mechanism other than regulation of expression of CNTF receptor components.

We also considered the possibility that FGF2 may regulate the ability of CNTF to activate downstream signaling. It has previously been

shown that CNTF stimulation leads to GFAP expression via activation of the JAK-STAT pathway[15,16]. In particular, STAT1 and 3 (STAT1/3) have been implicated in CNTF activation of the GFAP promoter[15]. Because tyrosine phosphorylation of STAT1/3 is necessary for nuclear translocation and transactivation[17], we examined whether FGF2 affects the ability of CNTF to induce STAT1/3 phosphorylation. CNTF acutely (within 15 min) induced tyrosine phosphorylation of both STAT1 and STAT3 (**Fig. 3c**). Acute stimulation with FGF2 did not induce STAT1/3 phosphorylation, nor did it affect the ability of CNTF to induce STAT1/3 phosphorylation. To determine whether prolonged exposure to FGF2 enhanced CNTF induction of STAT phosphorylation, we treated cultures with FGF2 for 24 h, and then stimulated the cells with CNTF for 15 min. Preincubation with FGF2 did not enhance CNTF-dependent STAT3 phosphorylation (**Fig. 3d**). Thus FGF2 apparently does not induce CNTF responsiveness by regulating the ability of CNTF to induce STAT phosphorylation.

With the knowledge that FGF2 enhances CNTF induction of GFAP expression (which is regulated by STAT proteins), but does not affect CNTF-induced STAT1/3 tyrosine phosphorylation, we decided to use chromatin immunoprecipitation (ChIP) to examine whether FGF2 influences the interaction of the STAT complex with the genomic GFAP promoter. E18 rat cortical cells were left unstimulated or stimulated with FGF2 for 5 d, and subsequently treated with CNTF for various durations. GFAP promoter fragments bound to STAT3 were identified by STAT3 immunoprecipitation followed by PCR amplification of a GFAP promoter fragment containing the STAT-binding site (between nucleotides –1510 and –1518 in the mouse GFAP promoter). In cultures not treated with FGF2, CNTF induced a slight increase in association of STAT3 with the GFAP promoter (**Fig. 3e**). In contrast, in FGF2-treated cultures, there was a much greater association of STAT3 with the GFAP promoter, suggesting that FGF2 stimulation may facilitate access of the STAT3 complex to the promoter (**Fig. 3e**).

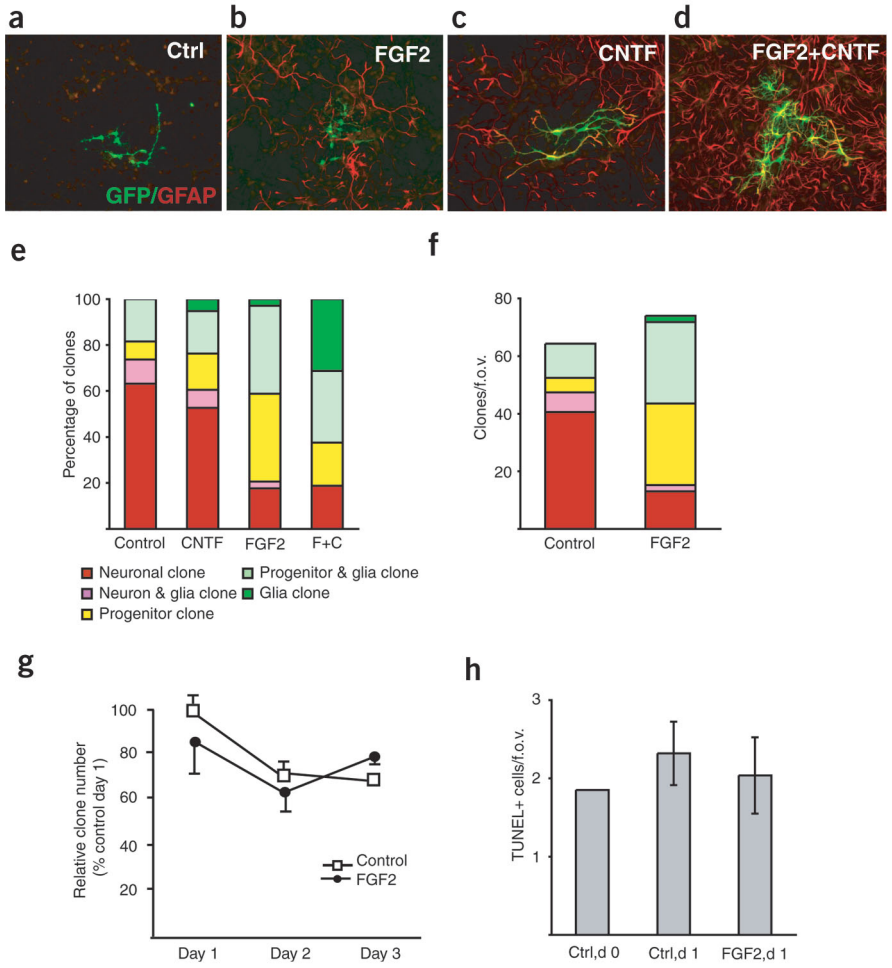

Figure 2 Effects of FGF2 and CNTF on cortical cell fates examined by clonal analysis. (**a–d**) Examples of clones from E18 rat cortical cells immunostained with anti-GFP (green) and GFAP (red) antibodies. Cells infected by GFP-expressing virus were grown with FGF2 (30 ng/ml) or CNTF (100 ng/ml) for 6 d. (**e**) Effect of FGF2 and CNTF treatments on clonal composition. Clones described above were classified as follows; neuronal clone (containing only β-tubulin-III$^+$ cells, red bars), neuron and glia clone (containing both β-tubulin-III$^+$ and GFAP$^+$ cells, pink bars), progenitor clone (containing only nestin$^+$ cells, yellow bars), progenitor and glia clone (containing both nestin$^+$ and GFAP$^+$ cells, light green bars) and glia clone (containing only GFAP$^+$ cells, green bars). (**f**) Comparison of the absolute number of clones between the control and FGF2 treated group at day 6. (**g**) Comparison of total number of clones between the control and FGF2 treated groups at various times in culture. (**h**) Numbers of apoptotic cells per field of view (f.o.v.) in the control and FGF2-treated groups as assessed by TUNEL assay 24 h after plating.

Given that STAT proteins activate transcription by recruiting the transcription co-activator CBP to the promoter, we asked whether FGF2 also facilitates the recruitment of CBP to the GFAP promoter[18,19]. FGF2 significantly enhanced CNTF-dependent CBP recruitment to the promoter, indicating that there was more efficient recruitment of the STAT-CBP complex to the GFAP promoter in FGF2-treated cells (**Fig. 3e**). Thus FGF2 facilitates CNTF-dependent recruitment of various transcription components to the GFAP promoter. Consistent with this, recruitment of RNA polymerase II (Pol II) to the GFAP promoter near transcription start site was also significantly enhanced by FGF2 (**Fig. 3e**).

The fact that FGF2 facilitates recruitment of various STAT transcription components to the GFAP promoter suggests that FGF2 may modify the chromatin structure of the genomic GFAP promoter. One mechanism by which FGF2 might modify chromatin structure at the GFAP promoter is by regulating DNA methylation. DNA methylation inhibits transcriptional activation and is developmentally regulated at the STAT-binding site of the GFAP promoter[9]. If FGF2 promotes STAT interactions with the GFAP promoter by regulating DNA methylation, one would expect FGF2 to reduce DNA methylation at that site. The percentage of cells in which the STAT-binding site was methylated decreased over time in culture, but was not further suppressed by FGF2 treatment (**Supplementary Fig. 2** online). This suggests that FGF2 does not regulate interaction of the STAT complex with the GFAP promoter by regulating DNA methylation.

Another mechanism by which FGF2 may facilitate STAT recruitment to the GFAP promoter is by regulating histone acetylation or

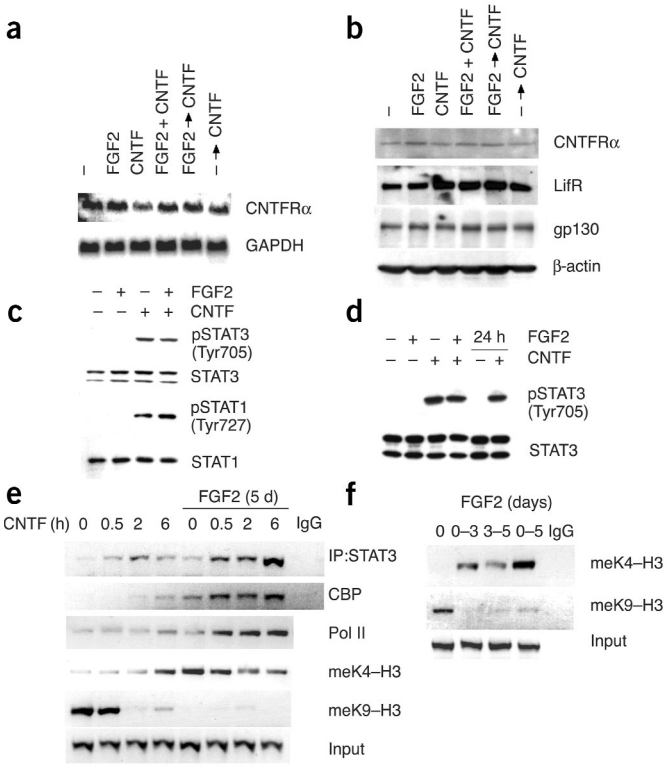

Figure 3 FGF2 regulates histone methylation and facilitates recruitment of the STAT/CBP complex to the GFAP promoter. (**a**) Effect of FGF2 and CNTF treatment on CNTF receptor α (CNTFRα) transcript levels. The blot was probed with GAPDH to confirm equal loading. Cells (in **a**,**b**) were treated as described in **Figure 1g**. (**b**) Protein levels of CNTFRα, LIFR and gp130 after FGF2 and CNTF treatments. (**c**) Effect of acute FGF2 treatment on STAT phosphorylation. Cells were stimulated with FGF2 and/or CNTF (15 min, day 3). Phospho-STAT1 and STAT3 were assessed using phospho-specific antibodies. (**d**) Effect of prolonged exposure of FGF2 on STAT phosphorylation. Cells were treated with FGF2 and/or CNTF for 15 min (lanes 2–4) or treated with FGF2 for 24 h followed by CNTF for 15 min (lane 6) at day 5. STAT3 phosphorylation was assessed using phospho-specific antibodies. (**e**) Effect of FGF2 treatment on histone methylation and the association of STAT3 and CBP with genomic GFAP promoter. Cells were grown with FGF2 for 5 d and stimulated with CNTF for indicated duration (hours). Histone methylation levels and recruitment of STAT3, CBP and RNA polymerase II (Pol II) to the GFAP promoter were analyzed by ChIP assays. Dimethyl-specific histone H3 antibodies to Lys4 (meK4-H3) and Lys9 (meK9-H3) were used. (**f**) Analysis of methylation state of histone H3 associated with genomic GFAP promoter, assessed by ChIP assays. Cells were treated with FGF2 for indicated period (days) and were analyzed at day 5.

methylation[20–22]. Histone acetylation and histone H3 methylation at Lys4 lead to transcriptional activation, whereas histone H3 methylation at Lys9 is associated with transcriptional silencing[20–23]. ChIP experiments using anti-acetylated histone antibodies indicated that histone acetylation around the STAT-binding site of the GFAP promoter increased in response to CNTF but was not further enhanced by FGF2 treatment (data not shown). However, FGF2 led to a striking increase in Lys4 methylation and completely suppressed Lys9 methylation around the STAT-binding site of the GFAP promoter (**Fig. 3e**,**f**). Western blot analysis indicated that FGF2 treatment did not lead to cell-wide changes in histone methylation, indicating that the FGF2-induced changes were due to alterations of histone methylation at specific promoters (**Supplementary Fig. 3** online). The switch from Lys9 to Lys4 methylation indicates that FGF2 alters histone H3 methylation at the GFAP promoter in ways that favor promoter activation.

If FGF2-induced changes in histone methylation at the GFAP promoter are involved in regulating astrocytic differentiation *in vivo*, one would expect a difference in the GFAP promoter histone methylation state among progenitors, neurons and glia. To examine this possibility, we purified progenitors by flow cytometry and obtained pure populations of neurons and astrocytes by controlling culture condition (see Methods). We checked the purity of each population by western blot analysis (**Fig. 4a**). The ability of the purified progenitors to differentiate into neurons and astrocytes was confirmed by clonal analysis. As in the case of retroviral labeling in mixed cortical cultures, FGF2 and CNTF led to a marked increase in GFAP$^+$ clones in progenitor cultures (**Fig. 4b**).

ChIP assays indicated that in progenitors histone H3 at the GFAP promoter had high levels of Lys9 methylation and relatively low levels of Lys4 methylation (**Fig. 4c**). In contrast, neurons had low levels of Lys9 and Lys4 methylation. Interestingly, astrocytes had very little Lys9 methylation and high levels of Lys4 methylation at the GFAP promoter (**Fig. 4c**). These observations suggest that progenitors, neurons and astrocytes have distinct histone methylation profiles, and that astrocytic differentiation *in vivo* is associated with a dynamic change in the state of histone methylation at the STAT-binding site on the GFAP promoter. It is perhaps surprising that neurons, which do not express GFAP, do not have very high levels of Lys9 methylated H3 at the GFAP promoter. It may be that transcriptional competence of this promoter is based both on levels of Lys4 methylation and the ratio of Lys4- to Lys9-methylated H3.

To determine whether purified progenitors, which differentiate into astrocytes in response to FGF2 and CNTF (**Fig. 4b**), also undergo a change in histone methylation in response to FGF2, we examined Lys4 and Lys9 methylation at two regions on the GFAP promoter: the STAT-binding site and the transcription start site (TATA site). FGF2 led to an increase in Lys4 methylation and elimination of Lys9 methylation of histone H3 at the STAT-binding site in progenitor cultures (**Fig. 4d**). In contrast, histone methylation levels at the TATA site showed no significant difference between unstimulated and FGF2-stimulated cells, suggesting that FGF2-induced changes in histone methylation are specific to the STAT-binding site (**Fig. 4c**). Importantly, we did not detect any GFAP expression either by western blot analysis or immunofluorescence in response to FGF2 among purified progenitors, indicating that histone methylation changes at the STAT-binding site precede GFAP transcription, and are not consequences of transcriptional activation (**Fig. 4d** and data not shown).

To further explore the relationship between histone methylation and astrocyte differentiation, we examined whether expression of a histone methyltransferase (HMT) that regulates Lys4 methylation was sufficient to promote GFAP expression. Methylation of histone H3 at Lys4 is regulated by a methyltransferase called SET7/9 (refs. 24,25). The ability of CNTF to induce GFAP$^+$ clones was significantly enhanced by SET7/9 overexpression, consistent with our observation that Lys4 methylation is associated with CNTF-induced GFAP expression (**Fig. 4e**). Overexpression of SET7/9 did not affect the fraction of neuronal clones, suggesting that SET7/9 specifically affects the expression of glial genes (**Supplementary Fig. 4** online). To determine whether histone methylation was required for glial differentiation, we treated cortical cultures with 5′-methyl-thioadenosine (MTA), a general methyltransferase blocker, and examined the ability of CNTF to induce GFAP[26]. In the presence of MTA, a condition that effectively suppressed histone methylation at Lys4 (**Fig. 4f**), neither CNTF alone nor co-treatment of FGF2 and CNTF was effective in inducing GFAP

expression (**Fig. 4g**). These results indicate that FGF2 stimulation induces Lys4 methylation at the STAT site of the GFAP promoter, which facilitates CNTF-dependent recruitment of STAT proteins and CBP to the promoter to activate transcription.

DISCUSSION

Our observations show that FGF2 regulates the competence of progenitors to differentiate into astrocytes by regulating the ability of CNTF to induce GFAP expression. This is mediated by an FGF2-induced increase in Lys4 methylation and a decrease in Lys9 methylation at the STAT-binding site of the GFAP promoter. These alterations are relevant since there is a strong correlation between histone methylation patterns and transcriptional activation[21]. We find that misexpression of SET7/9, a Lys4-specific methyltransferase, is sufficient to induce endogenous GFAP expression, indicating that Lys4 methylation alone can facilitate GFAP transcription. It is reported that Lys4 methylation antagonizes Lys9 methylation by impairing Lys9 substrate recognition by Suv39h1, a Lys9-specific methyltransferase, and thereby prevents association of a negatively acting histone modifying complex[24,27]. Thus FGF2 may reverse the histone methylation pattern at the STAT-binding site. We also examined Ser10 phosphorylation at histone H3, which can influence Lys9 methylation[21], but we did not detect any change following stimulation (data not shown).

While our findings are in agreement with the general view that Lys4 methylation is associated with transcriptional activation, and Lys9 methylation is associated with transcriptional silencing, there is debate about the specific role of these modifications in transcriptional regulation. According to one model, RNA polymerase II is responsible for the recruitment of histone methyl transferases to the Pol II elongation machinery during transcriptional activation[28–30]. In such a model, histone methylation is not particularly promoter-specific, and acts at a relatively late step in transcriptional activation. An alternate view is that histone methyltransferases are recruited to specific promoters by transcription factors and are involved in the early steps of transcriptional activation, as reported for the Rb-E2F complex[31]. We find that FGF2 induces changes in histone methylation at the GFAP promoter well before Pol II recruitment, and at specific sites within a promoter, which supports an early role in transcriptional activation. It will be interesting to determine whether FGF2 regulated factors target histone methyl transferases to the GFAP promoter to regulate Lys4 and Lys9 methylation. Such mechanisms would likely exert an important influence on gene activation, and their identification will be a major goal of future studies.

In summary, our experiments reveal a novel mechanism by which FGF2 regulates astrocytic differentiation. FGF2 induces CNTF responsiveness in cortical progenitors by facilitating recruitment of the STAT3-CBP complex to the GFAP promoter. We find that FGF2 induces Lys4 methylation and suppresses Lys9 methylation at the STAT site in the GFAP promoter, which suggests that FGF2 promotes recruitment of transcription components to the promoter by regulating chromatin structure. Assessment of histone methylation status in progenitors, neurons and astrocytes reveals that astrocytic differentiation *in vivo* is associated with a switch from Lys9 to Lys4 methylation. FGF2 stimulation of progenitors shows that this switch precedes GFAP expression, indicating that it is likely to be a key regulatory event in GFAP expression. In preliminary studies, we have found that other astrocyte-specific genes, such as S100-β, show a similar modification in histone methylation in response to FGF2, suggesting that regulated histone methylation may represent a general mechanism for epigenetic regulation of cell-type specific gene expression during development.

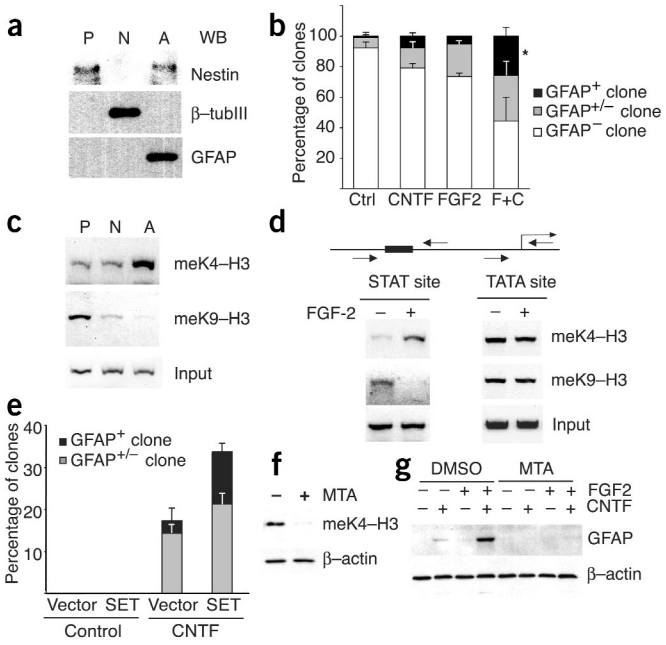

Figure 4 Comparison of histone methylation levels among progenitors, neurons and astrocytes. (**a**) Purity of progenitors (P), neurons (N) and astrocytes (A) assessed by western blot analysis using antibodies against nestin, β-tubulin III and GFAP. Each population was isolated as described in Methods. (**b**) Effect of FGF2 and CNTF on clonal composition in progenitor culture. Purified E17 cortical progenitors were infected by GFP virus and were grown with FGF2 and CNTF for 6 d. GFP clones were classified (for example, $GFAP^+$ clones contain only $GFAP^+$ cells, and $GFAP^{+/-}$ have both $GFAP^+$ and $GFAP^-$ cells). Asterisks indicate statistically significant differences by paired *t*-test ($^*P < 0.05$) between the control and the indicated experimental group. (**c**) Difference in histone methylation levels among progenitors (P), neurons (N) and astrocytes (A) assessed by ChIP assays. Each sample is normalized based on cell number. (**d**) Changes in histone methylation levels in progenitors in response to FGF2. E17 cortical progenitors were stimulated with FGF2 for 2 d. (**e**) Effect of SET7/9 on clonal composition. SET7/9 was retrovirally expressed and cells were grown for 3 d with CNTF for the last 24 h. (**f**) Effect of 5′-methylthioadenosine (MTA) on histone methylation at Lys4. Cells were preincubated with 0.3 mM MTA for 2 h and lysates were immunoblotted with antibodies against dimethyl-histone H3 (Lys4) or β-actin. (**g**) Effect of MTA on GFAP expression. Cells were preincubated with MTA (0.3 mM, 2 h) and stimulated with FGF2 and CNTF for 4 d. GFAP and β-actin levels were assessed by western blot analysis.

METHODS

Cell culture and reagents. The neocortex from E18 rat embryos was dissected in HBSS and dissociated using papain[12]. Cells were plated on culture plates coated with poly-L-lysine and laminin and were grown for 5 d in neurobasal medium supplemented with glutamine, penicillin-streptomycin and B27 (Invitrogen). FGF2 (30 ng/ml, Amgen and Invitrogen) and CNTF (100 ng/ml, Upstate Biotechnology) were prepared in neurobasal media and were added to cells once after plating. For transient application, a factor was added for the indicated duration and replaced with fresh media after several washes. For sequential application experiments, the first factor was applied and removed by several washes before the next factor was added. For MTA treatment, cells were pretreated with either MTA (0.3 mM, 2 h, Sigma) or DMSO (for the control group) before stimulation[26]. Each day, half of the medium was replaced with fresh medium containing the drug. Antibodies used were antibodies specific to STAT1, STAT3, CNTF receptor α, LIF receptor, gp 130 (Santa Cruz Biotechnology), phospho-STAT1, phospho-STAT3 (Cell Signal Technology) and β-actin (Sigma). TUNEL assays were done using the Apotag kit (Oncor). All animal procedures were approved by the Johns Hopkins Animal Care and Use committee.

Isolation of purified cell populations. To isolate cortical progenitors, acutely dissociated E14 rat cortical cells were labeled with anti-MMA antibody (Becton Dickinson) and were sorted using a DakoCytomation MoFlo high-speed cell sorter[32]. The gates for sorting were established by forward and side scatter patterns and green fluorescence at 520–540 nm to separate positive from negative cells. The sorted cells were characterized as Nestin$^+$, β-tubulin III$^-$ and GFAP$^-$. To obtain pure neuronal cultures, E18 rat cortical cells were grown with AraC for 5 d. These cells were Nestin$^-$, β-tubulin III$^+$ and GFAP$^-$. Astrocytes were purified from newborn pups as described previously[4]. These cells were Nestin$^+$, β-tubulin III$^-$ and GFAP$^+$.

Immunostaining and clonal analysis. Cells on glass coverslips were fixed with 4% paraformaldehyde (PFA) and processed for immunofluorescence as described previously[12]. The primary antibodies used were anti-GFP (Molecular Probes), anti-GFAP, Cy3-conjugated anti-GFAP, anti-β-tubulin III (Sigma) and anti-Nestin (Pharmingen) antibodies. Clones were classified into three types depending on double immunoreactivity with criteria described in each figure. More than 50 clones were analyzed in each group, and at least three separate experiments were performed.

Retroviral infection. Phoenix ecotrophic cells (DSHB) were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine and penicillin-streptomycin (Invitrogen)[33]. Cells were transfected with retroviral vector pBMN-IRES-EGFP by the calcium phosphate method. 48 h after transfection, supernatants were harvested, titered, and frozen at –80 °C until infection. For retroviral infection, viral supernatants were applied to E18 rat cortical cells once after plating.

Chromatin immunoprecipitation assays. ChIP assays were performed as originally described with minor modifications[34]. E18 rat cortical cells were grown and treated with FGF2 (30 ng/ml) and CNTF (100 ng/ml) for indicated durations. After discarding media, cells were fixed with freshly made 1% formaldehyde (Sigma) in PBS at room temperature for 20 min. Cells were rinsed with ice-cold PBS and harvested with harvesting buffer (100 mM Tris (pH 9.4), 10 mM DTT). After ice-cold PBS wash, cells were resuspended in lysis buffer (20 mM Tris (pH 8.1), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS and protease inhibitor cocktail (Roche)). Cell lysates were sonicated six times for 20 s at setting 1.2 using microtip (Misonix) and centrifuged at 10,000*g* for 10 min at 4 °C. Collected supernatants were precleared by incubating with Salmon Sperm DNA/Protein A agarose slurry (1 h, 4 °C, Upstate Biotechnology). Immunoprecipitation was performed overnight at 4 °C using anti-STAT3, anti-CBP, anti-pol II (Santa Cruz) and anti-dimethyl histone H3 (Lys4 or 9, Upstate Biotechnology) followed by additional incubation with protein A agarose slurry for 1 h at 4 °C. Because of the sequence similarity, some antibodies recognize both Lys9 and Lys27 methylation on histone H3 (ref. 35). Although the antibody we use has been used to examine Lys9 methylation, and there are no reports of Lys27 recognition[36–39], the possibility of Lys27 recognition has not formally been ruled out. For a negative control, an equal amount of rabbit IgG was incubated with lysates. Immune complexes were washed twice with lysis buffer and wash buffer (20 mM Tris (pH 8.1), 2 mM EDTA, 500 mM NaCl, 0.5% Triton X-100, 0.1% SDS), once with LiCl wash buffer (0.25 M LiCl, 10 mM Tris (pH 8.1), 1 mM EDTA, 1% NP-40, 1% deoxycholate), and twice with TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) at 4 °C. Immune complexes were eluted twice with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Crosslinks were reversed by incubation with 0.3M NaCl at 65 °C for 6 h. DNA was purified with desalting column (Qiagen) and used for PCR with following primers: GSS (taagctggaagtctggcagtg), GSAS (gctgaatagagccttgttctc), PolS (ggaaacccttgactctgggtac) and PolAS (caggtgtctggtaggaccatg).

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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