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# Epigenetic control of neural stem cell fate

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Unraveling the mechanisms by which neural stem cells generate distinct cell types remains a central challenge in central nervous system (CNS) biology. Recent studies have shown that epigenetic gene regulation plays an important role in the control of cell growth and differentiation. These epigenetic controls cover a wide spectrum, including the interaction of chromatin remodeling enzymes with neurogenic transcription factors, the maintenance of genome stability in neuronal cells and the involvement of noncoding RNAs in neural fate specification. Extracellular signaling systems that control the growth and differentiation of neural stem cells act, at least in part, by interfacing with these diverse epigenetic mechanisms.

## Addresses

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**Current Opinion in Genetics & Development** 2004, **14**:461–469

This review comes from a themed issue on  
Differentiation and gene regulation  
Edited by Michael G Rosenfeld and Christopher K Glass

Available online 20th August 2004

0959-437X/\$ – see front matter

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DOI 10.1016/j.gde.2004.07.006

## Abbreviations

|              |  |
|--------------|--|
| <b>BDNF</b>  | brain derived neurotrophic factor                  |
| <b>CBP</b>   | CREB-binding protein                               |
| <b>CNS</b>   | central nervous system                             |
| <b>CREB</b>  | Cre binding protein                                |
| <b>FGF-2</b> | basic fibroblast growth factor                     |
| <b>GFAP</b>  | glial fibrillary acidic protein                    |
| <b>HAT</b>   | histone acetyltransferase                          |
| <b>HDAC</b>  | histone deacetylase                                |
| <b>MBD</b>   | methyl-CpG binding protein                         |
| <b>MeCP2</b> | methyl DNA binding protein                         |
| <b>N-COR</b> | nuclear receptor corepressor                       |
| <b>Ngn1</b>  | neurogenin 1                                       |
| <b>NSC</b>   | neural stem cells                                  |
| <b>STAT3</b> | signal transducer and activator of transcription 3 |
| <b>TLX</b>   | Talless homolog                                    |

## Introduction

The term ‘epigenetic mechanisms’ refers to effects that promote cellular specification by imposing a specific and heritable pattern of gene expression on the progeny of differentiating cells, without altering the DNA sequence. Major epigenetic mechanisms include DNA methylation, histone modification, such as acetylation and methylation,

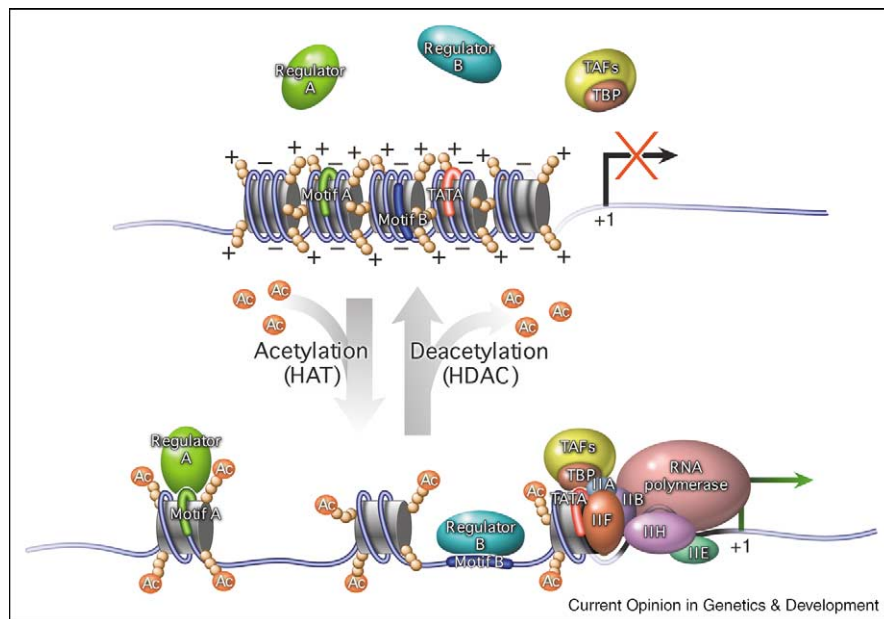
and regulatory noncoding RNAs. Neural stem cells (NSCs) in the mammalian CNS have provided a unique model system for understanding the molecular mechanisms that control cell-fate specification. NSCs are defined as cells that possess the ability to self-renew and maintain the ability to generate three major CNS cell types: neurons, astrocytes and oligodendrocytes (reviewed in [1]). During development, telencephalic neuroepithelial cells are thought to first go through limited cycles of expansion through symmetric divisions. These cells then undergo neurogenesis through mostly asymmetric divisions. In the cerebral cortex, during the neurogenic phase, the neuroepithelial cells have been found to take the form of radial glia [2,3]. Towards the end of neurogenesis, cortical progenitors switch back to symmetric divisions and give rise to astrocytes [4]. Oligodendrocytes tend to arise from specific locations within the CNS, in particular, the ventral region of the neural tube, and migrate into the white matter of the CNS to myelinate axons (reviewed in [5]). Adult NSCs have now been found in the two principal neurogenic regions, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ), and in some non-neurogenic regions, including the spinal cord [1,6]. These characteristics of embryonic, postnatal and adult NSCs can be recapitulated *in vitro*, either as free-floating neurospheres or as monolayer cultures, and have provided useful models for determining the contribution of epigenetic mechanisms.

In this review, we focus on recent reports investigating the role of epigenetics in neural fate specification in the mammalian CNS.

## Chromatin structure and the role of HDACs/HATs

An accumulation of studies in recent years has highlighted the active role that chromatin structure, particularly the covalent modifications that take place on histones, plays in the regulation of gene expression (reviewed in [7]). One of the best-characterized histone modifications to date is lysine acetylation, which is mediated by histone acetyltransferases (HATs) (Figure 1). Histone deacetylases (HDACs) catalyze the reverse reaction; in the deacetylated state, histones package the DNA into condensed chromatin, termed nucleosomes, which, in turn, prevent access of transcriptional activators to their target sites, thus resulting in transcriptional repression. Acetylation of the conserved N-terminal histone tails is thought to result in relaxation of the nucleosomes by decreasing the interaction of the positively charged histone tails with the negatively charged phosphate backbone of DNA. The nucleosomal relaxation facilitates access of transcriptional

Figure 1



Histone acetyltransferases (HATs) convert chromatin from a condensed nucleosomal structure to a more relaxed state by decreasing the affinity of histone proteins with the DNA backbone. The relaxation of chromatin allows cell-type specific regulator proteins and RNA polymerase complexes to access DNA and start transcription. Histone deacetylases (HDACs) catalyze the reverse reaction; this deacetylated state is often associated with gene repression. Ac, acetylation; TAF, TBP-associated factor; TBP, TATA-binding protein.

activators and allows gene activation. Furthermore, many transcriptional coactivators, such as p300 and CBP [CREB (Cre binding protein)-binding protein], display intrinsic HAT activity, which reinforces chromatin relaxation.

Conversely, hypo-acetylated chromatin is associated with transcriptionally silent genes, consistent with the discovery that HDACs are found in the same complex with several transcriptional repressors. There are at least eleven HDACs in humans, which fall into two classes, Class I and Class II, based on their homology with yeast HDACs Rpd3p and Hda1p, respectively (reviewed in [8]). Class III HDACs represent a distinct class altogether and are similar to yeast Sir2. Class I HDACs are expressed fairly ubiquitously, whereas Class II HDACs are enriched in heart, skeletal muscle and brain. Northern analysis of HDAC expression in embryonic rat hippocampal progenitors revealed that class II HDACs were specifically upregulated when cells were induced to differentiate, whereas class I HDAC expression remained unchanged, suggesting a regulatory role for class II HDACs during neural differentiation [9].

### HDAC and HAT regulatory complexes

Large repressor complexes have been purified and characterized functionally in yeast and mammals. As part of these large complexes, co-repressor complexes have been identified, including the mSin3A/B complex, which contains HDAC1 and 2 (of class I) [10], and the nuclear

receptor co-repressor (N-CoR) complex, which contains HDAC3 [11]. The connection between chromatin regulation and neuron-specific gene expression was established with the discovery that recruitment of HDACs to the promoter of neuronal genes is essential for the repression of these genes in non-neuronal cells [12,13]. One common feature that is shared by these neuron-specific genes was a conserved 21–23-base pair DNA response element, known as RE-1 or NRSE (repressor element 1/neuron restrictive silencer element; referred to just as NRSE within this review). The NRSE is a binding site for the RE-1 silencing transcription factor/neuronal restricted silencing factor (REST–NRSF). REST–NRSF can mediate repression, through association with the mSin3A/B complex [14], with N-CoR [15] and with the novel co-repressor complex, CoREST/HDAC2 [16], suggesting the context-dependent nature of its repressor activities. CoREST can other silencing machinery to REST–NRSF target genes, including the methyl DNA binding protein MeCP2, heterochromatin protein 1 and the histone lysine methyltransferase, suppressor of variegation 39H1 [17]. In this model, some target genes (i.e. otoferlin) are HDAC-dependent and can be reactivated with treatment of the HDAC inhibitor trichostatin A (TSA), whereas other genes (i.e. SMARCCe) are silenced by HDAC- and DNA-methylation-dependent mechanisms and are only reactivated upon stimulation with both TSA and the DNA demethylating reagent 5-aza-cytidine.

Histone acetylation has been shown to be important for other aspects of neural development, including neuronal and oligodendrocyte lineage progression. In a recent study, the maintenance of histone acetylation was shown to be important for neuronal differentiation (J Hsieh, K Nakashima, T Kuwabara, E Mejia, FH Gage, unpublished). Adult multipotent neural progenitor cells differentiated predominantly into neurons in the presence of the HDAC inhibitor valproic acid (VPA). VPA treatment also actively suppressed glial differentiation, even in conditions that favored lineage-specific differentiation. Further analysis revealed that the VPA-mediated neuronal differentiation was correlated with the upregulation of REST/NRSF-regulated genes, including the neurogenic basic helix-loop-helix (bHLH) transcription factor, NeuroD. HDAC activity was shown to be important for oligodendrocyte progenitor cells (OPCs) to progress into mature oligodendrocytes [18]. Treatment of OPCs with TSA inhibits morphological branching as well as expression of mature oligodendrocyte markers. These studies highlight the diversity of epigenetic mechanisms that are involved in the regulation of neural gene expression.

### Chromatin remodeling and neuronal plasticity

In addition to effects on neural fate specification, changes in chromatin remodeling have also been associated with changes in neuronal plasticity. Recent studies have revealed that stimulation with different drugs — SKF82958 (a dopaminergic receptor agonist), pilocarpine (a muscarinic acetylcholine receptor agonist) and kainic acid (a kainate glutamate receptor agonist) — induced a rapid and transient phosphorylation at serine 10 and acetylation of lysine 14 of histone H3, as well as an upregulation of *c-fos* transcription in hippocampal neurons [19]. Phospho-H3 (ser10) has been associated with transcriptional activation of immediate-early genes [20,21]. In another study, a fascinating connection between long-term memory-related synaptic plasticity and chromatin regulation was observed in cultured *Aplysia* neurons. One significant feature of neurons is that they can regulate long-term memory storage bidirectionally; thus, the facilitatory transmitter 5-HT mediates long-term facilitation, while the inhibitory transmitter FMRF (Phe-Met-Arg-Phe)-amide(a) produces long-term depression [22]. Stimulation with 5-HT led to the recruitment of CREB with CBP, and increased histone H3 and H4 acetylation at the C/EBP (CCAAT/enhancer binding protein) promoter. Interestingly, exposure of neurons to FMRFa led to the replacement of CREB1, which induces early response genes, with CREB2, a repressor of long-term facilitation. In addition, exposure to FMRFa led to the recruitment of HDAC5, which correlated with a reduction of acetylated H4. Together, these studies suggest the importance of epigenetic mechanisms for the regulation of neuronal plasticity and memory storage.

### The role of DNA methylation

Another class of epigenetic modifications that has been implicated in diverse gene regulatory processes, such as genomic imprinting and X-chromosome inactivation, is DNA cytosine methylation (reviewed in [23]). DNA methylation-mediated silencing occurs primarily through two mechanisms: inhibition of transcription factor binding to target genes by methylation at CpG sites, leading to transcriptional repression and binding of methyl-CpGs by methyl-CpG binding proteins (MBDs), which further recruit HDAC repressor complexes, resulting in a repression of chromatin. Several recent studies suggest that DNA methylation plays an extensive role in the CNS. MeCP2 (an MBD) was found highly enriched in post-mitotic neurons, and mutations in MeCP2 have been linked to the neurological disorder Rett syndrome (Rett syndrome patients are characterized by normal development until one year of age, followed by a rapid deterioration, involving loss of acquired speech and motor skills, microcephaly, seizures, autism, ataxia, intermitted hyper-ventilation and characteristic stereotypic movements) [24,25]. The enzymes that establish and maintain DNA methylation-specific patterns, including the *de novo* DNA methyltransferases 3a and 3b and the maintenance DNA methyltransferase 1 (Dnmt1), are expressed in neurons and appear to be necessary for their function [26,27]. Mice lacking Dnmt1, specifically in neural precursor cells at E9–E10, showed problems in neuronal function and died postnatally [28]. Other MBD proteins have also been suggested to play a role in adult neurogenesis. Mice deficient in MBD1 showed decreased neurogenesis, defects in spatial learning and a reduction in long-term potentiation in the dentate gyrus of the hippocampus [29]. MBD1<sup>-/-</sup> NSCs generated fewer neurons by comparison with wild-type cells, suggesting a role for MBD1 in neuronal fate commitment. The lack of MBD1 in NSCs also resulted in increased aneuploidy and an upregulation of ‘intracisternal A particle’ expression, a type of endogenous virus whose expression levels are frequently elevated in cancer cells with genomic instability [30]. One possibility is that genomic surveillance mechanisms, such as methylation of viral sequences, exist to maintain genome stability for CNS function. A recent study showed that NSCs, neurons and glia from the SVZ in postnatal mice are frequently aneuploid [31]. Whether the aneuploidy that is seen in neural cells is beneficial or harmful over time remains to be seen. It will be interesting to determine the connection with aneuploidy (and other types of genome-wide epigenetic events such as retrotransposition) and changes in neuronal function and plasticity.

A remarkable series of papers were published recently, describing the link between neuronal plasticity and DNA methylation-mediated transcriptional mechanisms [32,33]. One of the most extensively studied neural activity-dependent genes, brain derived neurotrophic factor (BDNF), is highly expressed in neurons, and its

transcription is upregulated by membrane depolarization [34]. The authors found that, in the absence of neuronal activity, MeCP2 binds to the BDNF promoter and silences gene expression. Furthermore, Chen and colleagues [32] showed that, in response to activity-dependent calcium influx, MeCP2 becomes phosphorylated and is released from the BDNF promoter, resulting in the activation of BDNF transcription. Martinowich and colleagues [33\*\*] demonstrated that depolarized neurons had decreased methylation within the BDNF promoter, leading to a decreased association with MeCP2 and other silencing proteins, such as HDAC1 and the co-repressor mSin3A, and more association with CREB, a transcriptional activator. Together, these reports reveal that neurons may use epigenetic mechanisms, such as DNA methylation, to reversibly modulate gene expression in response to environmental signals.

### Signaling molecules and repression of glial genes in non-glial cells

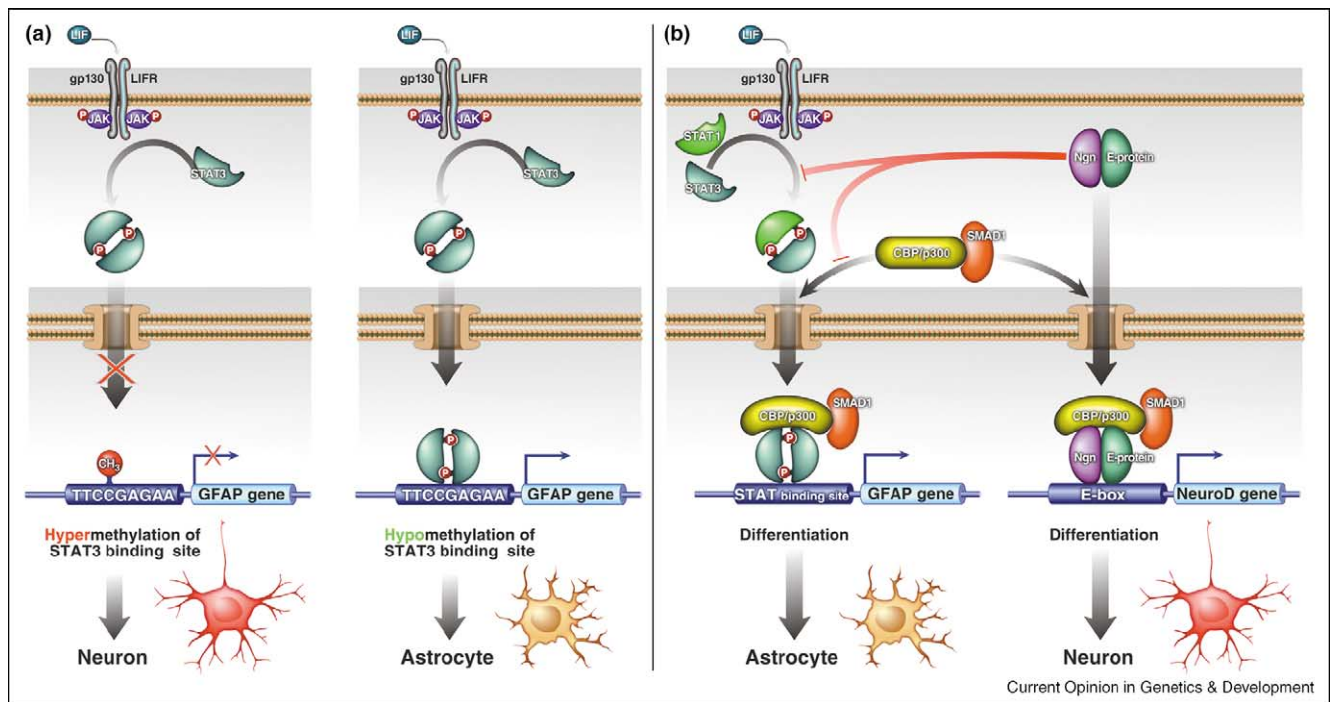
One important stem-cell biology question, regarding the specification of cell fate, is the connection between cell-extrinsic signals and cell-intrinsic programs (reviewed in [35]). Many extracellular molecules that play roles in neural stem-cell differentiation have been identified. These molecules transduce their signals into the nucleus via transcription factors that bind and activate target genes. There is increasing evidence that the transduction of these signals is regulated, at least in part, by epigenetic mechanisms at the level of histone modifications and/or DNA methylation (Figure 2a,b and Figure 3). As mentioned previously, neurons are generated before astrocytes in the developing telencephalon, and the question of how glial genes are restricted from developing neurons has long been of interest. One representative glial gene is *glial fibrillary acidic protein* (GFAP), a gene that is normally expressed in astrocytes. Takizawa and colleagues [36] described a mechanism in which the signal transducer and activator of transcription 3 (STAT3) activation of GFAP was blocked in early telencephalic neuroepithelial cells (E11.5), as well as in postmitotic neurons, by CpG methylation of the STAT3 binding site (Figure 2a). Through this mechanism, GFAP expression was repressed, even in the presence of leukemia inhibitor factor (LIF) signaling, one of the triggers of STAT3 phosphorylation and activation. Interestingly, E14.5 neuroepithelial cells became demethylated at the STAT3 binding site within the GFAP promoter and became responsive to LIF signaling, leading to GFAP activation and astrocyte differentiation. In addition to changes in DNA methylation, the regulation of GFAP expression appears to be controlled by several different transcriptional regulators, including N-CoR and the orphan nuclear receptor TLX (Tailless homolog) [37,38]. N-CoR<sup>-/-</sup> neural stem cells were found to preferentially differentiate into GFAP-positive cells, by comparison with wild-type cells. Furthermore, the brains of N-

CoR<sup>-/-</sup> mice displayed precocious GFAP expression, as early as E14.5, a time at which wild-type brains lack GFAP expression. This finding is consistent with the idea that N-CoR might be involved in the repression of GFAP and the regulation of lineage-specific differentiation. In addition, adult NSCs, isolated from TLX knockout mice, showed increased differentiation into GFAP-positive astrocytes, and an upregulation of GFAP and S100 $\beta$  expression (a different astrocyte marker) was observed in TLX<sup>-/-</sup> forebrains. The fact that there are so many different mechanisms to spatially and temporally restrict glial gene expression suggests the precision that is needed to control the neurogenic-to-gliogenic fate switch.

### Activation of neurogenesis and gliogenesis

Insights have also been gained recently into the mechanisms that actively promote neuronal and glial fate specification at the expense of other fates. Sun and colleagues [39] investigated the role of neurogenic bHLH transcription factor Neurogenin1 (Ngn1) in cortical precursor cells. They found that overexpression of Ngn1 could dramatically induce neuronal differentiation. Remarkably, in the presence of glial-inducing factors, such as LIF and ciliary neurotrophic factor, Ngn1 induced neuronal differentiation while suppressing glial differentiation. To explain the switch from neurogenesis to gliogenesis during development, they proposed a sequestration model (Figure 2b), where Ngn1 inhibits glial differentiation by sequestering activator complexes away from glial-specific genes and by directly inhibiting STAT3 activation when the cortical precursor cells are neurogenic. These activator complexes include CBP/p300 and Smad1, which is a downstream signaling protein that is activated by bone morphogenetic proteins (BMPs), known to cooperate with STATs to activate gene expression [40]. CBP/p300/Smad1 can now be targeted to neuronal promoters (such as NeuroD) to promote neurogenesis. As gestation proceeds, Ngn1 expression is downregulated and STAT3 can form a complex with Smad1, bridged by p300, to effectively induce astrogenesis. Recently, histone methylation has also been found to be implicated in the regulation of GFAP expression [41\*] (Figure 3). Lysine methylation has been directly linked to epigenetic inheritance; histone H3 methylation at lysine 4 (K4) leads to transcriptional activation, whereas histone H3 methylation at lysine 9 (K9) is associated with transcriptional silencing (reviewed in [42]). Using chromatin immunoprecipitation (ChIP) assays, Song and Ghosh [41] showed that there was more K9 methylation at the STAT3 binding site within the GFAP promoter in progenitor stages, when GFAP was repressed. Stimulation with basic fibroblast growth factor (FGF-2) potentiated the ability of CNTF (ciliary neurotrophic factor) to enhance astrocyte differentiation. Interestingly, FGF-2 induction caused K9 to become demethylated, but K4 to become hypermethylated, resulting in STAT3

Figure 2



Epigenetic control of lineage-specific gene expression. **(a)** Changes in DNA methylation at the Stat3 binding site control the switch from neurogenesis to gliogenesis in the developing telencephalon. In early telencephalic neuroepithelial cells (E11.5) and in postmitotic neurons, STAT3 activation of GFAP is blocked by CpG methylation. Later in E14.5 neuroepithelial cells, demethylation of the STAT3 binding state leads to GFAP activation and astrocyte differentiation. **(b)** In the sequestration model, neurogenic bHLH transcription factors, such as Ngn1, recruits activator complexes away from glial genes (i.e. GFAP), toward neuronal genes (i.e. NeuroD). The downregulation of Ngn1 allows activator complexes to return to glial genes, leading to gliogenesis. P, phosphorylation.

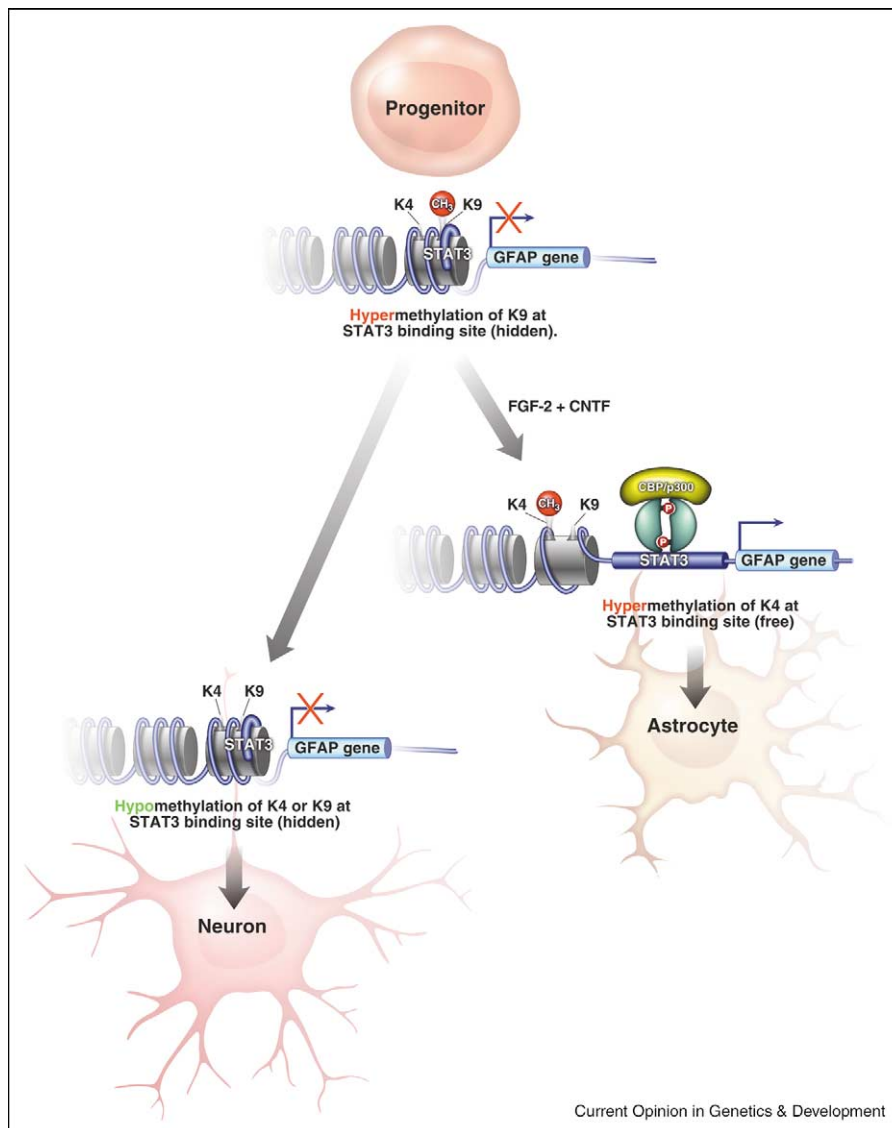
binding and activation of GFAP expression. By contrast, neurons showed low levels of both K9 and K4 methylation, leaving open the mystery of the exact mechanism that keeps GFAP repressed in neurons. These reports highlight the diverse epigenetic mechanisms that control lineage-specific gene expression for the generation of different neural cell types.

### The emerging role of noncoding RNAs

An emerging class of molecules with epigenetic effects on gene regulation consists of noncoding RNAs (reviewed in [43]). For example, noncoding RNAs play an important role in X-inactivation in mammals, transcriptional and post-transcriptional gene silencing in plants, and regulation of developmental timing in worms (reviewed in [44]). One class of noncoding RNAs, microRNAs, is thought to play roles in translational repression and has been isolated from many different organisms, including animals, plants and yeast [45]. In terms of mammalian development, a recent study isolated micro (mi)RNAs from mouse bone marrow and showed that specific miRNAs play a role in lineage-specific differentiation in hematopoietic stem cells [46]. Most of the small, noncoding RNAs that have been characterized to date play roles in gene silencing.

However, Kuwabara and colleagues [47] made the surprising discovery that noncoding RNAs could function in activating gene expression (Figure 4). The sequence of this novel, small, noncoding RNA matched the NRSE (as described earlier), which is the binding site for REST-NRSF. The authors went on to show that the RNA formed double-stranded (ds) molecules and was specifically expressed in adult multipotent neural progenitor cells early in neuronal differentiation. Gain-of-function studies demonstrated that the NRSE dsRNA could promote neuronal differentiation, whereas loss-of-function of the NRSE dsRNA resulted in an inhibition of neuronal differentiation. How then would REST-NRSF, whose usual function is to act as a transcriptional repressor complex, go on to activate neuron-specific gene expression? Through reporter luciferase and CHIP assays, the authors postulated a model wherein the NRSE dsRNA interacted with REST-NRSF and converted it from a repressor to an activator complex, leading to the activation of neuron-specific genes. These emerging studies highlight the diverse regulatory functions that small, noncoding RNAs and other types of epigenetic mechanisms might play in the control of mammalian development and stem-cell biology.

Figure 3



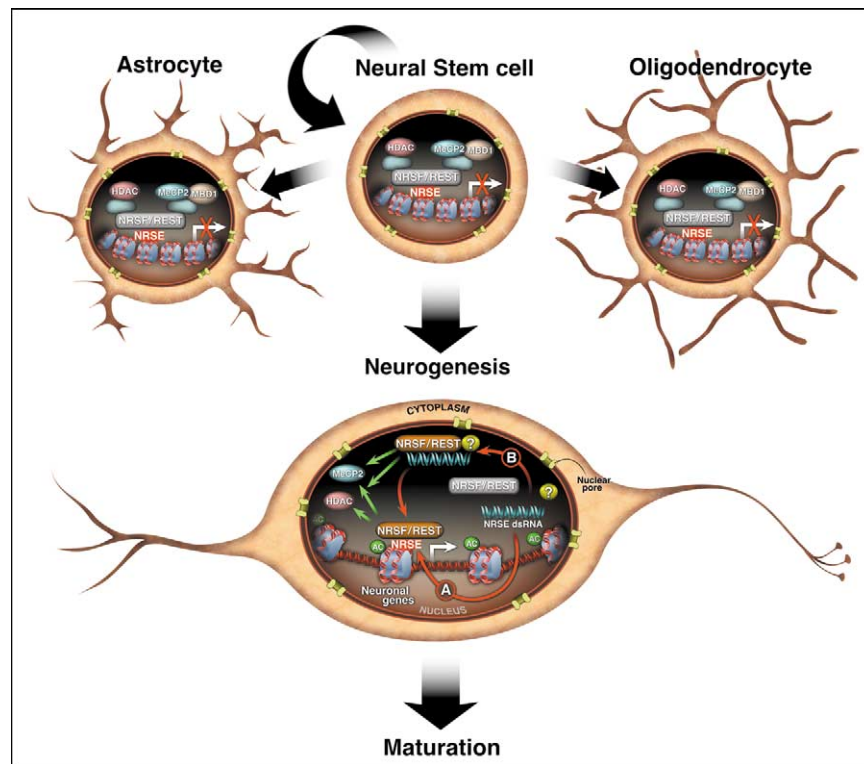
Regulation of lineage-specific gene expression by histone methylation. Hypermethylation of K9 keeps the Stat3 binding site in repressive chromatin and blocks the activation of GFAP in progenitor stages. Fgf-2 stimulation converts K9 methylation to K4 methylation, resulting in the relaxation of chromatin. Now Stat3 can bind to its binding site and GFAP is upregulated. Together with CNTF stimulation, cells differentiate into astrocytes. Although neurons are hypomethylated at K9, they are also hypomethylated at K4, within the Stat3 binding site, which may be the reason why there is no GFAP expression in neurons.

### Conclusions and perspectives

Why study stem cell biology using epigenetic mechanisms? First, the regulation of cell-fate specification and differentiation, and the maintenance of the undifferentiated state are not likely to be controlled by single genes, but presumably require the actions of many genes, sequentially and/or simultaneously. Epigenetic mechanisms, such as changes in chromatin structure and function, provide a means for coordinately activating and repressing arrays of genes at specific steps in the differentiation pathway. Second, epigenetic mechanisms are

usually associated with heritable changes in gene expression that are often reversible, a feature that may explain changes in stem-cell plasticity (such as the ability of a cell to transdifferentiate, dedifferentiate or become reprogrammed under various conditions). Finally, one important question in the study of stem-cell plasticity is whether the multipotential cell that is isolated in culture is reflective of its normal behavior *in vivo*. It is conceivable that in extracting a cell from its environmental niche and disrupting its normal cell-cell contacts, the epigenetic controls that preserve stable patterns of gene

Figure 4



Model for gene activation events by the NRSE dsRNA. The NRSE dsRNA promotes neuronal differentiation and activates neuron-specific genes through an interaction with the REST–NRSF transcriptional machinery. This interaction results in a displacement of HDACs and MBDs (including MeCP2) from the REST–NRSF complex. (a) and (b) describe two hypothetical models of how the NRSE dsRNA functions. Model (a) postulates that there is a physical interaction between the NRSE dsRNA and REST–NRSF protein. NRSE-containing neuronal genes are actively repressed by the REST–NRSF complex (through the association of HDACs and methyl-DNA binding proteins). At the onset of neuronal differentiation, the dsRNA directly interacts with NRSE dsDNA–REST–NRSF complex within the genome and triggers an organizational change, resulting in transcriptional activation. Alternatively, model (b) postulates that NRSE dsRNAs interact with the REST–NRSF complex and induce a conformational change, which prevents its association with co-repressor proteins, such as HDACs and methyl-DNA binding proteins. Reprinted with permission from [47\*\*]. AC, acetylation.

expression might be lost. Understanding the normal epigenetic changes that are associated with stem-cell growth and differentiation may help to clarify whether stem-cell plasticity is mostly due to experimental manipulation or whether it actually represents *in vivo* biology with untapped therapeutic potential.

### Acknowledgements

We thank K Nakashima and T Kuwabara for stimulating discussions and helpful comments on this manuscript, ML Gage for editorial assistance and J Simon for graphics.

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