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Events at the transition between cell cycle exit and oligodendrocyte progenitor differentiation: the role of HDAC and YY1

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Abstract

The complexity of the adult brain is the result of an integrated series of developmental events that depends on appropriate timing of differentiation. The importance of transcriptional regulatory networks and epigenetic mechanisms of regulation of gene expression is becoming increasingly evident. Among these mechanisms, previous work has revealed the importance of histone deacetylation in oligodendrocyte differentiation. In this manuscript we define the region of interaction between transcription factor Yin-Yang 1 (YY1) and histone deacetylase 1, and characterize the functional consequences of YY1 overexpression on the differentiation of oligodendrocyte progenitors.

Keywords

chromatin; transcription; development; brain; myelin; epigenetic

INTRODUCTION

Oligodendrocytes, the myelin forming cells of the CNS, are generated from oligodendrocyte progenitor cells (OPCs) during early postnatal days (Sauvageot and Stiles, 2002). During this developmental window OPCs that reside throughout the neuraxis exit from the cell cycle and differentiate throughout the CNS (Baumann and Pham-Dinh, 2001). Although the mechanisms regulating proliferation in these cells have been studied extensively (Casaccia-Bonnel *et al.*, 1996; Casaccia-Bonnel *et al.*, 1999; Durand and Raff, 2000), the characterization of the molecular events at the interface between cell-cycle exit and the initiation of the complex program of differentiation remain elusive.

During the past decade, the role of epigenetic factors (post-translational modifications of the histone tails, histone variants switching, ATP-remodeling complexes and microRNAs) in the regulation of brain development (Seo *et al.*, 2005; Matsumoto *et al.*, 2006; Cao *et al.*, 2006), has been investigated extensively. Studies from our group (Marin-Hustegge *et al.* 2002; Shen *et al.* 2005) and others (Hsieh *et al.*, 2004; Kondo and Raff, 2004; Lyssiotis *et al.*, 2007) have identified global deacetylation of histone H₃ as one of the first epigenetic events occurring in

oligodendrocyte progenitors during the transition between cell-cycle exit and initiation of the transcriptional program of differentiation.

The removal of acetyl groups from lysine residues in the histone tails is performed by specific enzymes called histone deacetylases (HDACs). However these enzymes do not have the ability to directly bind to DNA. A recent study in our laboratory has identified the transcription factor Yin Yang 1 (YY1) as a recruiter of HDAC1 on the promoter of the transcriptional inhibitors *Tcf4* and *Id4* in order to decrease their transcription levels during oligodendrocyte progenitor differentiation (He *et al.*, 2007).

The transcription factor Yin Yang 1 (YY1) is a multi-functional protein that can either activate or repress gene expression depending on the binding partner, including HDAC1, p300, TBP, Rb and E1A. It has been shown that YY1 regulates the expression of diverse genes (e.g. p53, c-myc, IFN- β and CREB) that are important for cellular activity. However different studies describe distinct roles of YY1 in different aspects of cellular activities, including proliferation, apoptosis and differentiation (reviewed in Shi *et al.*, 1997). The role of yy1 in nervous system development, was suggested in studies in *Xenopus* embryo in which yy1 deletion caused neuronal defects (Kwon and Chung, 2003; Morgan *et al.*, 2004). The role of YY1 in oligodendrocyte differentiation has been indicated by in silico-analysis of the promoter sequences of genes that are downregulated by histone deacetylation during oligodendrocyte differentiation. It was noted that almost one third of the downregulated genes have YY1-binding sites on their promoter and conditional ablation of YY1 in the oligodendrocyte lineage significantly impairs myelination (He *et al.*, 2007). In this manuscript we have explored the role of YY1 as recruiter of HDAC activity by studying the consequences of complex disruption and investigated the effects of overexpressing YY1 in immortalized mouse OPCs (Jung *et al.*, 1995).

OBJECTIVES

METHODS

Animals—All the mice and rats used in this study were handled according to protocols approved by the Institutional IACUC committee. *Yy1^{flox/flox}* mice were previously generated by Dr Y. Shi (Affar *et al.*, 2006). *Cnp1-cre* mice in C57BL were provided by Dr K. Nave (Max Planck Institute, 2003). *Yy1* conditional knockout mice and control siblings were obtained from breeding *yy1^{flox/+}; cnp-cre^{+/-}* pairs or breeding *yy1^{flox/flox}* with *yy1^{flox/+}; cnp-cre^{-/-}* mice. Genotyping was performed as reported previously (He *et al.*, 2007).

Antibodies and plasmids—Acetylated H₃ (rabbit polyclonal; 1:1,000; Upstate Biotechnology), CC1/APC (mouse monoclonal; 1:50; Oncogene Research Products), Flag (mouse monoclonal; 1:1,000 for IHC and 1:2,000 for WB; Sigma) HDAC1 (rabbit polyclonal; 1:6,000; Affinity BioReagents, Inc.), NG2 chondroitin sulfate proteoglycan (mice monoclonal; 1:200; CHEMICON International), O₄ (mouse monoclonal; 1:10, gift from Dr Bansal, University of Farmington), Sox2 (rabbit polyclonal; 1:1,000; CHEMICON International), Tcf4 (rabbit polyclonal; 1:100; Santa Cruz), and YY1 (mouse monoclonal sc-7341; 1:100; Santa Cruz).

PCEP₄F-*yy1* encoding full-length, human YY1 fused with flag at the N terminal was a gift from Dr Seto of University of South Florida. To better visualize the transfected cells, the coding sequence of flag-YY1 was subcloned into pCX vector which expresses EGFP under the IRES element. To create a YY1 mutant that is defective for binding to HDAC1, six lysine residues within the central domain of the molecule (amino acids 173,174,178,179,182 and 183) were changed to arginine by mutating the AAG codon into AGG using the QuikChange II Site Directed Mutagenesis Kit (Stratagene). The mutations were confirmed by DNA sequencing.

Oli-Neu cell culture and transfections—Mouse oligodendrocyte progenitors immortalized with the neu antigen (Oli-Neu cell line) were a gift of Dr J. Trotter (University of Mainz, Germany) (Jung *et al.*, 1995). Cells were grown on poly-ornithine coated culture dishes and maintained proliferating in growth medium ODM plus 1% horse serum (HS) as described previously (He *et al.*, 2007). Proliferating Oli-Neu cells were transfected with different plasmids using FuGENE 6 (Roche) at a ratio of 3 μ l FuGENE6: 1 μ g DNA according to the manufacturer's manual. Two days later cells were immunostained for AcH₃.

Adenoviral infection of mouse OPCs—OPCs from *yy1^{flox/flox}* neonatal mice were isolated and infected with adenovirus-CMV-Cre as described previously (He *et al.*, 2007). Two days later the cells were processed for YY1 and AcH₃ immunocytochemistry.

Immunohistochemistry and immunocytochemistry—Immunocytochemistry of cultured cells with O₄ antibodies was performed live. Cells were rinsed gently in phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl) and incubated live with O₄ hybridoma supernatant (1:10) for 30 min at 37°C. Cells were then fixed with 4% PFA for 20 min at room temperature and stained with secondary antibodies as described. For immunohistochemistry, animals were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed, post-fixed overnight in the same solution at 4°C, and then cryopreserved in 30% sucrose in 0.1 M phosphate buffer and embedded in OTC. Frozen sections (20 μ m) were cut and incubated in blocking buffer (0.1 M phosphate buffer, 0.1% gelatin, 1% bovine serum albumin, 0.002% sodium azide) containing 10% normal goat serum (0.5% TritonX-100 was added for cytoplasmic and nuclear antigens) for 30 min and then incubated overnight with the primary antibody diluted in the same blocking buffer. After rinsing in PBS sections were incubated with the appropriate secondary antibodies conjugated to either fluorescein or rhodamine (Southern Biotechnologies, Amersham Biosciences, Jackson ImmunoResearch and Vector Laboratories). DAPI (1:1,000; Molecular Probes, Inc.) was used as nuclear counterstain.

Image acquisition, processing and quantification—Immunostained sections were analyzed using fluorescence microscopy (DM RA; Leica) followed by confocal microscopy analysis (LSM 510 Meta confocal laser scanning microscope; Carl Zeiss MicroImaging, Inc.). Confocal images of mouse brain sections were captured at 1 μ m intervals and stacks of six slices were typically used for the generation of the projections. The intensity of AcH₃ fluorescence staining was measured in arbitrary units using NIH ImageJ software. For the quantification of the *in vitro* experiments on transfected immortalized progenitors, a minimum 40 GFP⁺ cells were measured in each duplicate repeat of two independent experiments. For the quantification of the AcH₃ immunostaining in the cerebellum, 20 randomly selected CC1⁺ cells were measured in the similar area of each section, a minimum of three sections (corresponding to the levels of plates 127–136 from (Sidman *et al.*, 1997) per mouse and three mice each genotype were evaluated. Results were expressed as mean $\pm$ SD and statistically analyzed using two-tailed Student's *t* test, *P* < 0.05 was considered statistically significant.

Immunoprecipitation and Western blot—Proteins from the transfected Oli-Neu cells were extracted using a buffer containing 50 mM Hepes, pH 7.0, 250 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM EDTA, 0.01% PMSF, 1 mM aprotinin and 1 mM leupeptin for 15 min on ice. Equal amounts of protein (1 mg) were immunoprecipitated for 16–18 hours at 4°C using 2 μ g anti-flag antibody. Pcx-yy1 transfected cell lysate immunoprecipitated with normal mouse IgG (Santa Cruz) was used as negative control. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane, followed by Western blot probed with anti-flag and anti-HDAC1 antibody. Immunoreactive bands were visualized using HRP-conjugated

secondary antibodies followed by chemiluminescence using ECL Plus Western Blotting Detection System (Amersham Biosciences).

HDAC activity assay—HDAC activity was measured by using HDAC Activity Assay/Drug Discovery Kit (BIOMOL Research Laboratories, Inc.). Experimental procedures were designed and performed according to the kit protocol provided. In brief, tissue lysates from transfected Oli-Neu cells (prepared according to the same procedure described in Western blot analysis section) were used as sources for HDAC activity. Protein lysates (25 µg) were added to a 96-well plate in 25 µl HDAC assay buffer (BIOMOL Research Laboratories, Inc.). A fluorimetric acetylated substrate was added and the reaction allowed to proceed at room temperature for 1 hour, followed by incubation with the developer for 10–15 min. Enzymatic activity was evaluated in Perseptive Biosystems CytoFluor multi-well plate reader-4000 (excitation, 360 nm; detection of emitted light, 460 nm). The results were normalized to the amount of the protein.

Quantitative and semi-quantitative reverse transcriptase PCR—Mouse tissues of each age (embryonic day 18, postnatal days 2, 4, 9, 12, 15 and 22) or cell pellets were homogenized in Trizol® Reagent and RNA isolated following the manufacturer's instruction and cleaned using RNeasy Mini kit (Qiagen). Total RNA (2 µg) was used in a 40 µl reverse transcription (RT) reaction using the SuperScript RT-PCR kit (Invitrogen). Semi-quantitative PCR was performed in a 20 µl reaction mixture containing 2 µl cDNA as template and 0.1 µM specific oligonucleotide primer pairs using program denaturation at 94°C for 60 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec for 25 cycles. Actin was used as loading control. Quantitative RT-PCR was performed using Applied Biosystems SYBR green PCR master mix in 7900HT Sequence Detection PCR System. The melting curve of each sample was measured to ensure the specificity of the products. Data were normalized to the internal control *GAPDH* and analyzed using Pfaffl $\Delta\Delta C_t$ method. Primers sequences are given in Table 1. All the primers were designed for mouse genes except primers for *yy1* and *β -actin*, which recognize mouse, rat and human homology.

RESULTS

Because expression of the encoding myelin is regulated by a complex network of activators and inhibitors we reasoned that differentiation timing might be affected by the equilibrium between inhibitors and activators (Gokhan *et al.*, 2005). In support of this hypothesis, quantitative real-time PCR analysis of RNA isolated from the developing nervous system of mice between embryonic day 18 and postnatal day 22 revealed that myelin gene expression is preceded by downregulation of transcriptional inhibitors *Id4*, *Tcf4* and *Sox11* (Fig. 1). Our previous studies indicate that YY1 acts as lineage-specific repressor of these genes during oligodendrocyte progenitor differentiation by forming a protein complex with HDAC1 (He *et al.*, 2007). We now demonstrate that the activating function of YY1 depends on its ability to directly bind HDAC1. Briefly, either full-length or mutant YY1 containing six aminoacid substitutions (KR) in the central HDAC binding domain (Yao *et al.*, 2001), were subcloned into a pCX vector containing EGFP under the IRES element (Fig. 2A). The inability of mutant YY1 (KR) to bind to HDAC1 was confirmed by immunoprecipitation followed by Western blot analysis (Fig. 2B) of protein extracts from transfected Oli-neu cells (Jung *et al.*, 1995). The effect of YY1 overexpression on the enzymatic activity of HDAC1 was inferred by the decrease in global levels of acetylation of histone H₃ detected in transfected OPCs (Fig. 2). Transfected oligodendrocyte progenitors were identified by green fluorescence and NG2 immunoreactivity (Fig. 2C). The relative levels of acetylation of histone H₃ (AcH₃) in transfected cells were calculated by measuring the average fluorescence intensity of the AcH₃+ nuclei using NIH ImageJ (Fig. 2C,D). Although the AcH₃ intensity measured within each group varied (possibly reflecting either different levels of ectopic gene expression or

different stages of the cells), the average pixel intensity in the cells transfected with full-length YY1 was significantly lower than vector transfected controls (Fig. 2D). In contrast, the average intensity measured in cells expressing the mutant form of YY1 was equivalent to the intensity measured in vector transfected controls (Fig. 2D).

Finally, we used a fluorimetric assay to compare the levels of HDAC enzymatic activity in cultured progenitors transfected with full-length YY1 (pCX-yy1-EGFP) with those in progenitors transfected with the control vector (pCX-EGFP) (Fig. 2E). The overall enzymatic activity of HDAC in cells that overexpress full-length YY1 (125.5 ± 6.1 AFU/ μ g protein) was 25% greater than the activity in cells transfected with vector alone (109.2 ± 6.2 AFU/ μ g protein). Together these results indicate that the ability of YY1 to modulate the acetylation of histone H₃ depends on the formation of a protein complex with HDAC1.

To confirm these data independently we tested the consequences of *in vitro* and *in vivo* ablation of *yy1* on the global levels of acetylated nuclear histone H₃ in oligodendrocyte progenitors. Ablation of *yy1* *in vitro* was achieved by infecting *yy1*^{flox/flox} oligodendrocyte progenitors with adenoviral vectors expressing the recombinase Cre as described previously (He *et al.*, 2007). Consistent with a crucial role of YY1 in modulating HDAC activity, the impaired removal of acetyl groups from the histones of YY1[−] cells resulted in increased acetyl H₃ levels compared to YY1⁺ cells (Fig. 3A). In *yy1* conditional knockout mice, a similar deficit of HDAC activity was inferred by the detection of higher levels (~1.5 fold increase) of acetylated histone H₃ in CC1+ oligodendrocyte lineage cells within white matter tracts of conditional mutants compared to wild type mice (Fig. 3B,C).

Our previous work indicates that loss of *yy1* prevents the progression of OPCs to terminal differentiation and arrests the cells at a stage characterized by simple morphology and O₄ immunoreactivity (He *et al.*, 2007). We therefore tested the effects of *yy1* overexpression on the progression of NG2⁺ cells along the lineage. Whereas transfection of the pCEP4F vector did not affect the overall expression of NG2 in Oli-Neu cells, transfection of pCEP4F-*yy1* decreased NG2 expression and increased O₄ immunoreactivity (Fig. 4A). A possible explanation for these results is that YY1 has a repressive role on the expression of differentiation inhibitors, but does not directly affect activators or modulate the expression of myelin genes. In agreement with this interpretation, RT-PCR of RNA samples isolated from *yy1*-overexpressing cultures revealed decreased levels of *Id4*, *Tcf4*, *Hes5* and *Sox2* and unchanged levels of the transcription activators (i.e. *Sox10*, *Olig1* and *Mash1*) and of myelin gene products (i.e. myelin basic protein and ceramide galactosyltransferase) compared to controls (Fig. 4B). The effect of YY1 on *Sox2* and *TCf4* expression was further validated by immunocytochemistry (Fig. 4C). Together these results indicate that YY1 is necessary but not sufficient to drive NG2 progenitors to a terminally differentiated phenotype.

CONCLUSIONS

- Although the transition between exit from the cell cycle and the onset of oligodendrocyte differentiation remains poorly characterized, the timing of oligodendrocyte progenitor differentiation is regulated by extrinsic and intrinsic signals.
- Crucial intrinsic factors in this transition are HDACs and YY1, which both initiate differentiation by repressing transcriptional inhibitors of myelin gene expression.
- However, extrinsic signals that regulate HDACs and YY1 in oligodendrocyte development are unknown and deserve further investigation.

- YY1 is part of the polycomb complex that catalyzes the methylation of histone tails, experiments are in progress to define whether either this modification or other epigenetic events contributes to the repression of inhibitory molecules.

DISCUSSION

Three major cell types define the structure and function of the brain in both adult and developing animals: neurons, astrocytes and oligodendrocytes. This complex architecture is achieved by precisely orchestrating timing of differentiation of distinct cell types in overlapping, but temporally distinct, waves, with the neurogenic phase occurring during early embryogenesis, followed by astrogliogenesis and, finally, by oligodendroglialogenesis (Sauvageot and Stiles, 2002). This sequential order of lineages is determined by the integration of extracellular signals and cell-intrinsic changes (Qian *et al.*, 2000; Shen *et al.*, 2006). During the last decade identification of the intrinsic mechanisms that underlie cellular differentiation in the developing CNS has been the subject of intense investigation. One line of investigation has focused on the role of chromatin modifiers as intrinsic modulators of fate choice during brain development (Ballas *et al.*, 2001; Marin-Husstege *et al.*, 2002; Hsieh and Gage, 2004; Ballas *et al.*, 2005; Fan *et al.*, 2005; Colvis *et al.*, 2005; Miller and Gauthier, 2007; Lessard *et al.*, 2007; He *et al.*, 2007; Liu *et al.*, 2007; Shen and Casaccia-Bonnet, 2007). Among the epigenetic factors that modulate brain development, different groups have focused on post-translational modification of the histone tails, switching histone variants, ATP-remodeling complexes and microRNAs (Seo *et al.*, 2005; Matsumoto *et al.*, 2006; Cao *et al.*, 2006).

The concept of the existence of an intrinsic timer in oligodendrocyte progenitors was suggested in a series of elegant experiments in clonal cultures of cells isolated from the optic nerve and studied using time-lapse video microscopy (Temple and Raff, 1986). It was proposed that in the presence of mitogens such as platelet-derived growth factor, progenitors undergo a certain number of cell divisions until they growth arrest. Differentiation would result from the progressive accumulation of intrinsic signals modulating the responsiveness of the cells to the differentiative effect of retinoic acid and thyroid hormone (Barres *et al.*, 1994). Because of the intriguing relationship between cell-cycle exit and differentiation, it has been proposed that the progressive accumulation of cell-cycle inhibitors such as p27Kip1, p18Ink4c and p57Kip2 (Durand and Raff, 2000; Tokumoto *et al.*, 2002; Dugas *et al.*, 2007) might be responsible for the timing of differentiation onset. However, it has been difficult to determine whether cell-cycle inhibitors modulate differentiation directly or indirectly, by affected the proliferative state of the cell (Casaccia-Bonnet *et al.*, 1997; Casaccia-Bonnet *et al.*, 1999; Ghiani and Gallo, 2001). Because overexpression of the cell-cycle inhibitors p27Kip1 or p18Ink4c did not induce differentiation of oligodendrocyte progenitors, even though cells were arrested at the G1/S transition, it has been proposed that cell-cycle exit is necessary but not sufficient to induce differentiation (Tikoo *et al.*, 1998; Tang *et al.*, 1999; Tokumoto *et al.*, 2002). An alternative possibility is that some cell-cycle genes might affect the transcriptional machinery after the cells have exited from the cell cycle. This hypothesis is supported by evidence that links p27Kip1 to transcriptional activation of myelin genes (Miskimins *et al.*, 2002) and p21Cip1 to oligodendrocyte differentiation independent of its role in cell-cycle regulation (Zezula *et al.*, 2001). More recent studies further support an alternative role for the cell-cycle related molecule p57Kip2 as a modulator of the transcriptional network regulating oligodendrocyte differentiation (Dugas *et al.*, 2007). Together these data indicate the existence of a complex series of events that links exit from the cell cycle to differentiation of oligodendrocyte progenitors.

Timing of differentiation caused by disinhibition of late differentiation genes: examples from the astrocytic and neuronal lineages

In the neuronal lineage, timing of differentiation of neuronal precursors has been linked to the expression of neuronal genes following a de-repression event linked to the activity of HDACs (Ballas *et al.*, 2001; Hsieh and Gage, 2004; Ballas *et al.*, 2005). It has been shown that activation of neuronal gene expression is achieved by the removal of multi-protein complexes containing co-repressors, HDACs and repressor element 1 (RE1)-silencing transcription factor (REST) from the promoters of differentiation-associated genes (Roopra *et al.*, 2000; Ballas *et al.*, 2005). In agreement with these findings, treating cells with HDAC inhibitors (Kondo, 2006; Balasubramanian *et al.*, 2006; Lyssiotis *et al.*, 2007; Siebzehntrubl *et al.*, 2007), and systemic administration of HDAC inhibitors to either neonatal (Liu *et al.*, 2007) or adult animals (Hsieh *et al.*, 2004) increases neuronal gene expression and favors neurogenesis. More recently, it has also been suggested that the assembly of specific SWI/SNF complexes participates in defining the timing of neurogenesis (Lessard *et al.*, 2007). Thus, these studies identify histone acetylation of neuronal gene promoters as a crucial event for differentiation.

Similarly, studies of astrocytic cell differentiation have reported the gradual epigenetic de-repression of chromatin in specific promoter regions of the astrocytic lineage marker glial fibrillary acidic protein (*gfap*). This gene is repressed in neural precursor cell because DNA methylation of CpG islands in its promoter precludes the access of the crucial transcription factor signal transducer and activator of transcription (STAT) (Bonni *et al.*, 1997; Feng *et al.*, 2005). The increase in concentration of *gfap* transcripts correlates with a gradual decrease in DNA demethylation and concomitant increase in acetylation and methylation of crucial lysine residues on the histone tails of the chromatin region around the promoter, and with the appearance of astrocytes during development (Nakashima *et al.*, 1999; Takizawa *et al.*, 2001; Song and Gosh 2004). Thus astrogliogenesis is favored by histone acetylation and requires DNA demethylation.

In the oligodendrocyte lineage myelin genes, such as *mbp*, proteolipid protein (*plp*) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (*CNPase*), define the differentiated state of oligodendrocytes. Previous studies have reported the presence of repressive complexes containing HDACs and the transcriptional factors NKX2.2 or HES₅ on the *mbp* promoter (Wei and Miskimins, 2005; Liu *et al.*, 2006). Similar repressive complexes containing HDAC and the co-repressor SIN₃ have been identified on the proximal region of *plp* promoter (Romm *et al.*, 2005). By analogy to the astrocytic and neuronal lineages it has been suggested that timing of differentiation might be achieved by counteracting the enzymatic activities in the repressive complexes. However, pharmacological inhibition of HDACs *in vitro* prevents rather than facilitates the expression of myelin genes (Marin-Husstege *et al.*, 2002). In addition, systemic administration of HDAC inhibitors to developing rat pups alters the timing of oligodendrocyte differentiation and delays myelination (Shen *et al.*, 2005). Therefore, inhibition of repressive complexes containing HDAC on myelin genes is not sufficient to initiate oligodendrocyte differentiation.

Because myelin gene expression is regulated by a complex network of activators and inhibitors, we reasoned that differentiation timing might be affected by the equilibrium between inhibitors and activators (Gokhan *et al.*, 2005). In this study we present evidence of a temporal correlation between decreased levels of transcriptional inhibitors and the onset of myelin gene expression. It is important to underline the role of these inhibitory basic-loop-helix factors such as members of the ID and HES family, and additional inhibitory molecules such as ATF5 for preventing oligodendrocyte differentiation (Kondo and Raff, 2000a; Kondo and Raff, 2000b; Samanta and Kessler, 2004; Mason *et al.*, 2005; Marin-Husstege *et al.*, 2006) and inhibiting myelin gene expression (Liu *et al.*, 2006). Because decreased expression of transcriptional inhibitors is important for the timing of progenitor differentiation and for myelin gene expression, we

searched for a mechanism that regulates the expression levels of the inhibitors themselves. Studies from our group have identified that global deacetylation of histone H₃ is crucial for the decrease in transcriptional inhibitors of differentiation and that this occurs during the transition between cell-cycle exit and differentiation onset (Marin-Husstege *et al.*, 2002; Shen *et al.*, 2005). Therefore we proposed that global histone deacetylation is responsible for the decreased transcription of differentiation inhibitors.

Histone deacetylation is mediated by the catalytic activity of HDACs, enzymes that do not have the ability to bind directly to DNA. We therefore sought to identify DNA-binding proteins that bind to the promoter of the differentiation inhibitors and recruit HDACs in order to decrease their transcript levels. The transcription factor YY1 is a recruiter of HDAC1 on the promoter of the transcriptional inhibitors *Tcf4* and *Id4* (He *et al.*, 2007). The transcription factor Yin Yang 1 (YY1) is a multifunctional protein that can either activate or repress gene expression depending on the binding partner. Its role in the developing nervous system was originally suggested from studies in *Xenopus* embryos (Kwon and Chung, 2003; Morgan *et al.*, 2004). However the analysis of HDAC1 function in mammals was limited by the fact that *yy1*-null mice do not reach gastrulation (Donohoe *et al.*, 1999). To overcome this, we have generated conditional-knockout mice by crossing *yy1^{lox/flox}* with the *cnp-cre* line to specifically delete *yy1* in the oligodendrocytic lineage.

The *yy1* conditional-knockout mice have a severe hypomyelinating phenotype that is characterized by tremor, ataxia and a shaking. This dramatic phenotype is consequent to impaired differentiation of oligodendrocyte progenitors, which are arrested at the transition between cell cycle exit and initiation of the differentiation program due to high levels of inhibitory molecules such as *Id4*, *Tcf4* and *Sox11* (He *et al.*, 2007). These phenotypes resemble the one detected in animals treated with HDAC inhibitors (Shen *et al.*, 2005; Liu *et al.*, 2007).

YY1 binds to the promoter region of oligodendrocyte differentiation inhibitors (i.e. *Tcf4* and *Id4*) and recruits HDAC1 into repressive complexes. In this manuscript, we provide additional experimental evidence to support the role of YY1 as crucial activator of HDAC activity in differentiating oligodendrocyte progenitors, because mutation in the interaction domain of HDAC1 caused severe consequences on the global levels of histone deacetylation. We also report that overexpressing YY1 in immortalized OPCs is sufficient to decrease the levels of multiple inhibitory molecules and to promote the progression of NG2 cells to the O₄⁺ stage. However, we did not detect precocious myelin gene expression, possibly because YY1 did not increase the levels of transcriptional activators that are necessary to reach a specific threshold in order to activate myelin gene expression.

A better understanding of the mechanisms that underlie the differentiation of oligodendrocyte progenitors will provide the basis for the development of potential therapies of demyelinating disorders by stimulating either endogenous or transplanted oligodendrocyte progenitors.

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A. myelin genes **B. inhibitors**

Figure 1. Timing of oligodendrocyte differentiation is characterized by a decrease in inhibitor levels before activation of myelin gene expression

(A) Time course of myelin gene expression during development from embryonic day 18 (E18) to postnatal day 22 (P22). RNA levels of myelin genes *CNPase* and *plp* were measured by quantitative PCR of brain stem and cortical areas of individual mice, harvested at the indicated time points ($n = 3$ mice at each time point). The values were normalized to *GAPDH* and referred as relative fold increase of the values measured in mice at E18. Data are mean $\pm$ SD. (B) Transcript levels of oligodendrocyte differentiation inhibitors (*Id4*, *tcf4* and *sox11*) were analyzed by qPCR during development in the brain stem and cortex. The values were

normalized to *GAPDH* and referred as relative fold increase of the values measured in mice at E18.

Figure 2. YY1 modulates global histone acetylation through interacting with HDAC1

(A) The functional domains of YY1, including the DNA binding domain and the HDAC-interacting region. The arrows indicate the position of the amino acid substitutions in the HDAC1 binding-deficient mutant of YY1, in which the six lysine residues in the central HDAC-binding domain are replaced by arginines. (B) Oli-Neu cells were transfected with empty vector, Flag-full-length *yy1* (YY1) or Flag-mutant *yy1* (YY1mut). Protein lysates were immunoprecipitated with anti-Flag mouse monoclonal antibody. After SDS-PAGE separation and transfer, the blots were processed for Western blot analysis using antibodies specific for HDAC1 and Flag. Immunoprecipitation with normal mouse IgG was used as negative control. Note that HDAC1 co-immunoprecipitates wild-type but not mutant YY1. (C) Immortalized

murine oligodendrocyte progenitors (i.e. Oli-neu cells) were transfected (green) with pCX-GFP (vector), full-length *yy1*-GFP (*yy1*) or mutant *yy1*-GFP (*yy1mut*) and stained for acetylated histone H₃ (AcH₃, red). Note the decreased AcH₃ immunoreactivity in cells transfected with full-length YY1 but not with the mutant YY1 compared to the vector. Cells were stained for progenitor marker NG2 to define their morphology. Scale bars, 25 μ m. (D) Scatter plot of fluorescence intensity of AcH₃ in the nuclei of GFP⁺ transfected cells, measured by NIH ImageJ. The average values from 40, randomly chosen cells in each group are presented. Note that even though the AcH₃ level varies in individual cells, only the full-length YY1, but not the mutant form of YY1 had the overall effect of decreasing AcH₃ level. (E) Total HDAC enzymatic activity in whole cell extracts of control (vector) and YY1-transfected cultures, measured by a fluorimetric assay, reveals that increased levels of wild-type YY1 but not the mutant form increases HDAC activity. Data are mean $\pm$ SD. * P <0.05, * P <0.01 (Student's t -test).

Figure 3. Defective oligodendrocyte differentiation in *yy1* conditional-knockout (cko) mice is associated with persistent global histone acetylation

(A) Oligodendrocyte progenitors generated from neonatal *yy1^{flox/flox}* mice were infected with adenovirus-CMV-*Cre* and 48-hours later stained for YY1 (green) and AcH₃ (red). Note that YY1-deleted cells (arrowhead) have higher AcH₃ immunoreactivity compared to uninfected cells (arrow) in the same field. Scale bar, 20 μ m. (B) Sagittal sections of the cerebellum of control and *yy1*-cko mice stained for oligodendrocyte marker CC1 (green) AcH₃ (red) shows a higher level of AcH₃ in *yy1*-cko mice compared to sibling controls at P18. Scale bar, 20 μ m. (C) Fluorescence intensity of AcH₃ in CC1⁺ cells in control mice ($n = 3$) and *yy1*-cko mice ($n = 3$) was calculated using NIH ImageJ on acquired confocal images. The average

fluorescence intensity in the control siblings is set arbitrarily at 100. Data are mean $\pm$ SD.
* $P < 0.05$, Student's t -test.

Figure 4. Overexpression of *yy1* decreases the concentration of oligodendrocyte differentiation inhibitors but does not promote the differentiation

(A) Oli-Neu cells were transfected with either pCEP4F vector or pCEP4F-yy1 plasmid in proliferating medium. After 24 hours the cells were induced to differentiate by adding cAMP. At 1 day later, the cultures were stained for YY1 to confirm overexpression and were double immunostained for progenitor marker NG2 and early differentiated marker O4. Scale bar, 50 μ m. (B) Oli-Neu cells were transfected with either pCEP4F vector or pCEP4F-yy1 plasmid in proliferating medium. After 24 hours the cells were induced to differentiate and 48 hours later the total mRNA was extracted and semi-quantitative PCR performed. Note the decreased levels of *NG2*, *TCF4*, *Id4*, *Hes5* and *Sox2*. However the concentration of myelin genes did not increase,

probably because the levels of transcriptional activators such as *Sox10*, *Olig1* and *Mash1* is not upregulated. (C) Oli-Neu cells were transfected with either pCX vector or pCX-yy1 plasmid and maintained in proliferating medium. After 2 days the cells were fixed and subjected to immunostaining for TCF₄ and Sox2. The concentration of both proteins decreased in cells that overexpress *yy1* compared to either untransfected cells in the same field or vector-transfected cells. Scale bar, 20 μ m.

Table 1

PCR primers Semi-quantitative PCR

Gene	Forward primer	Reverse primer
<i>Semi-quantitative PCR primers</i>		
<i>β-actin</i>	5'-TGGAATCCTGTGGCATCC-3'	5'-TCGTACTCCTGCTTGCTG-3'
<i>CGT</i>	5'-TGCCAACGTATCCTTCTTCC-3'	5'-CATTGTCCCATGTCAAGCAC-3'
<i>Hes₅</i>	5'-CAAGGAGAAAAACCGACTGC-3'	5'-GCTGGAAGTGGTAAAGCAGC-3'
<i>Id₄</i>	5'-GATGAAGGCGGTGAGCCCGGTG-3'	5'-GACAGCATTCTCTGCCGCTGA-3'
<i>Mash1</i>	5'-CTTCCTTAAGGCCTCTGGCT-3'	5'-GAACCCGCCATAGAGTTCAA-3'
<i>MBP</i>	5'-ATGGCATCACAGAAGAGACC-3'	5'-CATGGGAGATCCAGAGCGGC-3'
<i>NG2</i>	5'-GAACGCATCAGCCACCGTAA-3'	5'-GGACGCTTCTTCCTGGTTTC-3'
<i>Olig1</i>	5'-ATGAGCTGGTGGGTACAGG-3'	5'-CACCAGCTGGGAGAGAGAAC-3'
<i>Sox2</i>	5'-ATGATGGAGACGGAGCTGAA-3'	5'-CTCCGGAAGCGTGTACTTA-3'
<i>Sox10</i>	5'-GACCAGTACCCTCACCTCCA-3'	5'-CCCCTCTAAGGTCGGGATAG-3'
<i>Tcf₄</i>	5'-CTCACGCCTCTCATCACGTA-3'	5'-TGAATGCATTAAGGGGCTTC-3'
<i>yy1</i>	5'-GGTGCAGATCAAGACCCTGGA-3'	5'-GTGTGCGCAAATTGAAGTCCA-3'
<i>Quantitative PCR primers</i>		
<i>GAPDH</i>	5'-ACCCAGAAGACTGTGGATGG-3'	5'-CACATTGGGGGTAGGAACAC-3'
<i>plp</i>	5'-CCCACCCCTATCCGCTAGTT-3'	5'-CAGGAAAAAAGCACCATTGTG-3'
<i>Id₄</i>	5'-GCCCAACAAGAAAGTCAGCAA-3'	5'-CCAGCTGCAGGTCCAGGAT-3'
<i>Sox11</i>	5'-GACGACCTCATGTTCGACCT-3'	5'-TCCAGGTCCTTATCCACCAG-3'
<i>Tcf₄</i>	5'-GTCCTCGCTGGTCAATGAAT-3'	5'-CCCTTAAAGAGCCCTCCATC-3'