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An oligodendrocyte-specific zinc-finger transcription regulator cooperates with Olig2 to promote oligodendrocyte differentiation

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Molecular mechanisms that control oligodendrocyte myelination during mammalian central nervous system (CNS) development are poorly understood. In this study, we identified Zfp488, an oligodendrocyte-specific zinc-finger transcription regulator, by screening for genes downregulated in the optic nerves of *Olig1*-null mice. The predicted primary structure of Zfp488 is evolutionarily conserved in vertebrates and invertebrates. In the developing CNS, *Zfp488* is specifically expressed in oligodendrocytes but not their precursors. Its expression increases in parallel with that of major myelin genes *Mbp* and *Plp1*. Zfp488 is a nuclear protein that possesses transcriptional repression activity. In the developing chick neural tube, Zfp488 can promote oligodendrocyte precursor formation upon Notch activation. In addition, Zfp488 can interact and cooperate with the bHLH transcription factor Olig2 to promote precocious and ectopic oligodendrocyte differentiation. Furthermore, knockdown of *Zfp488* via RNAi in an oligodendroglial cell line leads to the downregulation of myelin gene expression. Taken together, these data suggest that Zfp488 functions as an oligodendrocyte-specific transcription co-regulator important for oligodendrocyte maturation and that zinc-finger/bHLH cooperation can serve as a mechanism for oligodendroglial differentiation.

KEY WORDS: Oligodendrocyte myelination, Zinc finger protein, bHLH transcription factors, *Olig1*, *Olig2*, Mouse

INTRODUCTION

Myelin-producing oligodendrocytes play a crucial role in supporting normal neuronal function of the mammalian CNS. Formation of myelinating oligodendrocytes from their precursors requires activation and coordination of a set of stage-specific transcriptional regulators that are important for the biosynthesis of myelin components (Gokhan et al., 2005; Kagawa et al., 2001; Wegner, 2001). Although transcriptional regulation of oligodendrocyte precursor formation from neural progenitor cells is relatively well characterized in the developing CNS (Rowitch, 2004; Wegner, 2001), the molecular mechanisms governing oligodendrocyte maturation and myelinogenesis in postnatal CNS development are still poorly understood. The basic helix-loop-helix (bHLH) transcription factors *Olig1/2* play a crucial role in oligodendrocyte differentiation and myelination as well as remyelination (Arnett et al., 2004; Lu et al., 2002; Xin et al., 2005; Yue et al., 2006; Zhou and Anderson, 2002). Myelin formation in the postnatal CNS of *Olig1*-null (*Olig1ΔKO*) mice is severely compromised, despite the formation of oligodendrocyte precursor cells (OPCs) (Xin et al., 2005). At present, the downstream events mediated by *Olig1* in oligodendrocyte myelination remain elusive.

To identify *Olig1* downstream transcriptional regulators that may contribute to myelinating oligodendrocyte differentiation, we compared differential gene expression profiles between optic nerves from wild-type and *Olig1*-null mice (Xin et al., 2005). Optic nerves provide a naturally enriched source of myelinating oligodendrocytes as neural cell bodies in this tissue are exclusively glial, consisting mainly of those of myelinating oligodendrocytes and their precursors. By differential display analysis of genes downregulated in the optic nerves of *Olig1* mutant mice (Liang and Pardee, 1992), we identified a previously uncharacterized nuclear zinc-finger transcriptional regulator Zfp488 that is specifically expressed in differentiating oligodendrocytes but not in their precursors. Zfp488 can interact and cooperate with Olig2 to induce ectopic and precocious oligodendrocyte differentiation in the developing chick neural tube. Furthermore RNAi-mediated *Zfp488* knockdown leads to the downregulation of myelin genes in an oligodendroglial cell line. Thus, our studies suggest that Zfp488 is an oligodendrocyte-specific transcription modulator that cooperates with Olig2 to promote oligodendrocyte differentiation.

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MATERIALS AND METHODS

Tissue collection and RNA in situ hybridization

Brains, spinal cords and optic nerves from wild type and *Olig1* mutants at various embryonic and postnatal stages were harvested from ketamine/xylazine anesthetized mice, fixed in 4% paraformaldehyde at 4°C overnight, infused with 20% sucrose in PBS overnight, embedded in OCT and cryosectioned at 16 μm. Digoxigenin-labeled riboprobes were used to perform RNA in situ hybridization, as described previously (Lu et al., 2002), and the probes used were: murine and chick *Zfp488*, *Olig1*, *Olig2*, *Pdgfra*, *Plp1/Dm-20* (proteolipid protein) and *Mbp* (myelin basic protein). Detailed protocols are available upon request. Animal use and studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas.

RNA differential display and Northern blot:

Total RNA was harvested from the optic nerves from both wild-type and *Olig1* knockout (*Olig1ΔKO*) mice from postnatal week 2. mRNA differential display (Liang and Pardee, 1992) was performed to identify and isolate differentially expressed genes according to the manufacturer's instructions (GeneHunter, TN). Northern blot was performed as previously described (Lu et al., 2000).

Quantitative real time polymerase chain reaction (QRT-PCR)

QRT-PCR was performed using the ABI Prism 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems). RNA was extracted from cultured oligodendrocyte progenitor cells from neonatal rat forebrain and the CG4 oligodendroglial cell line (kindly provided by Dr Robin Miskimins) using Trizol (Invitrogen, Carlsbad, CA). cDNAs were generated using a first-strand cDNA synthesis kit (Amersham Bioscience, Piscataway, NJ). QRT-PCR was performed as previously described with *Gapdh* (glyceraldehyde-3-phosphatase dehydrogenase, TaqMan kit, Applied Biosystems) as an internal control (Xin et al., 2005). Primers used for expression analyses are as follows: *Mbp*, forward 5′-tcacagaagagaccctcaca-3′ and reverse 5′-gccgtagtgggtagttcttg-3′; *Cnp*, forward 5′-agctcaaggagaagaaccaat-3′ and reverse 5′-tgaagtgtcgaagctctttct-3′; *Zfp488*, forward, 5′-ctcgagaaaatgaccc-ttgt-3′ and reverse, 5′-catgacagctctgtgaaggt-3′; *Nkx2.2*, forward 5′-catc-gctacaagatgaaacg-3′ and reverse, 5′-ctgtactgggcgttgtattg-3′.

Oligodendroglial cell cultures and siRNA transfection

Cortical oligodendrocyte precursors were isolated from neonatal rat at P2 as previously described (McCarthy and de Vellis, 1980; Yang et al., 2005). Oligodendrocyte precursor cells and CG4 oligodendroglial cells were maintained in serum-free medium containing N2 supplement (Invitrogen), 20 ng/ml FGF2 and 10 ng/ml PDGF-AA (Kondo and Raff, 2000; Raff et al., 1983). The medium was then switched to a medium containing 400 ng/ml Triiodothyronine T3 and 20 ng/ml ciliary neurotrophic factor (CNTF) to promote oligodendrocyte differentiation (Barres and Raff, 1994; Gard and Pfeiffer, 1989). SMARTpool siRNA (Dharmacon Catalog Number: M-088215-00 and accession number: XM_224697) is against four regions of rat *Zfp488* mRNA with 5′ starting positions of duplexes at 25, 250, 730 and 1285. Transfection of the SMARTpool siRNA into CG4 cells was performed by using lipofectamine 2000 (Invitrogen) per manufacturer's instructions.

Transient transfection, luciferase assay and immunohistochemistry

COS-7 and NIH 3T3 cells were seeded and grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum one day before transfection. The cells reached 50-70% confluence on the day of transfection. Cells were transfected with LexA-Vp16, *Zfp488* and its variants using FuGENE6 according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN) and assayed 48-hour post-transfection for luciferase activities by using Promega luciferase assay kit. In addition, the pRSV-beta-Galactosidase plasmid was included to control for variable transfection efficiencies between different experiments. The immunohistochemical staining procedure using anti-Myc antibody (Sigma, MO) for detection of Myc-*Zfp488* and its derivatives for cellular localization was performed as described previously (Xin et al., 2005). Rabbit polyclonal antibody to Olig2 is kindly provided by Drs Chuck Stiles and John Alberta.

Co-immunoprecipitation and immunoblotting

COS7 cells were grown to about 60% confluence and then transiently transfected with 20 μg each of pCS2-MT:*Zfp488* and pFLAG-CMV-6b:*Olig2* by calcium phosphate precipitation. Whole cell lysates were prepared 48 hours after transfection by using 1× Passive Lysis Buffer (Promega, Madison, WI) supplemented with a protease inhibitor cocktail (1:200, Sigma, St Louis, MO). For immunoprecipitation, 600 μg of cell lysate proteins were incubated with 30 μg mouse anti-FLAG mAb in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% [w/v] Triton X-100, 1× protease inhibitor mixture, 1 mM DTT, 1 mM PMSF) at 4°C for 4 hours. The antigen-antibody complex was collected by adding 40 μl (bed volume) of protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and incubating at 4°C for 2 hours. After three washes with IP buffer, the complex-bound resin was suspended in 1× SDS buffer, boiled and resolved on a 12.5% SDS-PAGE gel. After Western blotting, proteins carrying the Myc epitope tag were detected with mouse anti-Myc mAb (Clontech Laboratories, Palo Alto, CA) by using chemiluminescence with the ECL kit (Pierce, Rockford, IL.) according to the manufacturer's instructions. Monoclonal antibody to Nkx2.2 was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa under the auspices of the National Institute of Child Health and Human Development.

Chick embryo in ovo electroporation

Chicken eggs were incubated at ~38°C. Approximately 1 μl (1.5 μg/μl) of expression vectors carrying pCMV-*GFP*, *Zfp488*, *Olig2*, *Nkx2.2*, *NICD* or combinations thereof were injected into a chicken embryo neural tube at stage HH13-15 (E2.5) with the aid of Picospritzer III (Parker Hannifin, Cleveland, OH). The subsequent electroporation was performed by using a square wave electroporator (CUY21, BEX, Japan) with five pulses of electrical shock (25V, 50 mseconds for each pulse). Embryos were harvested 3 days after electroporation. At this stage (E5.5), none of oligodendrocyte markers is normally expressed in the neural tube. The green fluorescent segment of neural tube was dissected, fixed for 1 hour in 4% paraformaldehyde-PBS on ice and embedded in OCT for sectioning on a cryostat for in situ hybridization or immunohistochemistry. At least five embryos with expression of each transgene or their combination were analyzed and characterized.

RESULTS

Identification of an evolutionarily conserved zinc finger protein as a gene downstream of *Olig1*

To identify transcriptional regulators of myelinating oligodendrocyte formation representing potential downstream targets of *Olig1,* we screened for genes downregulated in the optic nerves of 14-day old *Olig1* null mice using differential display. In addition to previously identified genes known to encode myelin components or their regulators, a cohort of novel transcripts downregulated or undetectable in the *Olig1* mutant were identified (Fig. 1A, data not shown). Among them, a cDNA encoding a zinc-finger motif-containing protein was identified as Zfp488 (zinc-finger protein for oligodendrocyte differentiation). The predicted amino acid sequence of *Zfp488* (GenBank Accession Number XM_356719) is 52% identical and 64% homologous to that of a putative gene entered as zinc-finger protein 488 in the human genome database (GenBank Accession Number BC 051323). Northern blot analysis (Fig. 1B) indicates that *Zfp488* is a ~3.5 kb transcript absent from the brain of the *Olig1* mutant, consistent with the differential display result.

The *Zfp488* mRNA encodes a 337 amino acid peptide with two typical C2H2 zinc finger motifs at its C terminus. A bipartite nuclear localization signal (NLS) is located between them (Fig. 1C). To determine whether *Zfp488* has counterparts in other species, a homology search revealed that murine *Zfp488* appears to have an ortholog but no obvious paralogs in species, including human, rat, chicken and *Drosophila* (Fig. 1D), suggesting a conserved non-redundant function for Zfp488 during evolution. The structural feature with two zinc-finger motifs flanking an NLS is highly conserved across all species (Fig. 1D).

Zfp488 is specifically expressed in differentiated oligodendrocytes in the developing CNS

To determine the cell type(s) that express *Zfp488* in the CNS, we compared the expression pattern by performing in situ hybridization for *Zfp488* and stage-specific oligodendroglial lineage genes in the developing murine spinal cord, where oligodendrocyte development is relatively well characterized. Although the oligodendrocyte precursor marker *Pdgfra* is present in the ventral spinal cord at embryonic day 12.5 (E12.5) (Fig. 2B), expression of neither *Zfp488* nor the oligodendrocyte differentiation markers *Mbp* and *Plp1/DM20* is detected at this stage (Fig. 2A,C; data not shown). *Zfp488* is initially expressed as foci in the ventral domain of the spinal cord at E14.5 (Fig. 2D), which coincides with the expression of *Mbp* and *Plp1/DM20* (Fig. 2E,F). At the late embryonic stage E18.5, *Zfp488* expression appears to extend to the white matter of the spinal cord, concurrent with *Mbp* and *Plp1/DM20* expression in

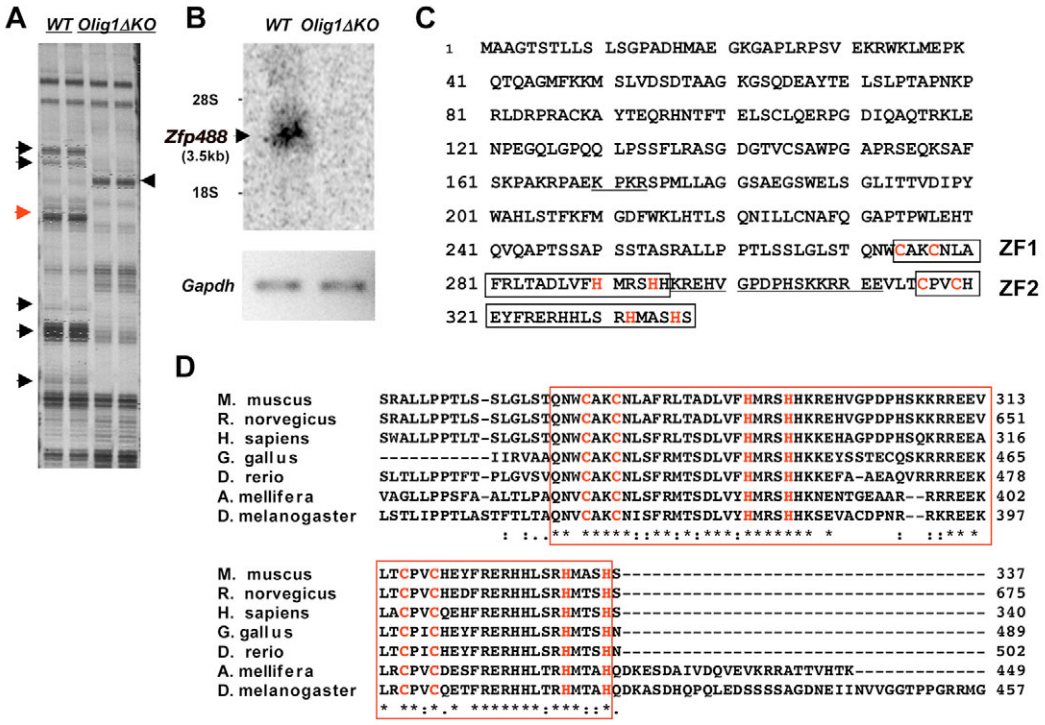

Fig. 1. Identification, predicted primary sequence, homology and expression of *Zfp488* mRNA transcript in the brain. (**A**) Differential gene expression between wild-type and *Olig1*-null (*ΔKO*) optic nerves was examined by mRNA differential display with duplicate samples. Arrows indicate the differentially expressed genes between wild-type and *Olig1*-null optic nerves. Red arrow indicates the position for *Zfp488*. (**B**) *Zfp488* expression in the brain of wild-type and *Olig1*-null mice at P14. Upper panel, a northern blot of RNA extracted from brain tissues of wild-type and *Olig1* mutant mice was probed with ^{32}P-labeled *Zfp488*, revealing a ~3.5 kb mRNA transcript in the wild-type brain and its absence in the *Olig1*-null brain. Lower panel shows expression of the housekeeping gene *Gapdh* examined by semi-quantitative RT-PCR (20 cycles) as a loading control. (**C**) Predicted amino acid sequence of mouse *Zfp488* protein, showing C2H2 type (boxed) zinc-finger domains in the C-terminal region. Potential nuclear localization signals are underlined. (**D**) Alignment of evolutionarily conserved zinc finger domains of *Zfp488* among invertebrates and vertebrates.

this region (Fig. 2G, compare with 2H,I). By contrast, *Zfp488* expression is not observed in peripheral myelinating Schwann cells in the dorsal root ganglion (drg) (Fig. 2D, compare with 2E, blue arrows).

In the postnatal developing CNS, *Zfp488* expression is highly enriched in the white matter tracts of the spinal cord, the forebrain and the cerebellum (Fig. 3A-C). In addition, a population of *Zfp488*+ cells is also detected in the gray matter of the spinal cord and the forebrain (Fig. 3A,B; black arrows). In the optic nerve and the corpus callosum, *Zfp488*-expressing cells can form a linear array (Fig. 3D,E), a characteristic feature of interfascicular myelinating oligodendrocytes.

To further determine the identity of *Zfp488* expressing cells, we performed double in situ hybridization of *Zfp488* with markers for differentiated oligodendrocytes or their precursors in the brain at P14. The majority of *Zfp488*+ cells co-express *Plp1* in the corpus callosum (Fig. 3E). Similar co-expression of *Zfp488* and *Mbp* was also observed (data not shown). By contrast, *Zfp488* expression is essentially absent in oligodendrocyte precursors marked by *Pdgfra* expression (Fig. 3F), suggesting *Zfp488* expression is largely confined to differentiated oligodendrocytes.

To determine whether *Zfp488* is genetically downstream of *Olig1* throughout the CNS, we examined its expression in *Olig1* mutant mice. In this mutant, *Zfp488* expression is undetectable in the spinal cord and brain regions of both embryonic (Fig. 4A,C,E) and postnatal (Fig. 4B,D,F) stages. As *Olig1* mutant mice fail to form mature oligodendrocytes but do form OPCs, the loss of *Zfp488* expression in *Olig1*-null mice is consistent with the notion that *Zfp488* is mainly expressed in differentiated or mature oligodendrocytes.

To test whether *Olig1* can regulate *Zfp488* transcription, we co-transfected *Olig1* and a luciferase reporter driven by 3.2 kb *Zfp488* upstream regulatory region, which contains 22 consensus E-boxes potentially recognized by bHLH transcription factors. *Olig1* appears to transactivate the reporter driven by this candidate regulatory region approximately fourfold (Fig. 4G). Although *Olig2* activates the putative promoter to a lesser extent, the difference is not statistically significant (Fig. 4G). Thus, our data suggest that *Zfp488* expression can be directly or indirectly regulated by *Olig1*.

Zfp488 exhibits nuclear localization and has transcriptional repression activity

In silico analysis revealed that Zfp488 contains a possible NLS sequence: a bipartite NLS between its two zinc-finger motifs (Fig. 1C). To examine the subcellular localization of Zfp488, Myc-tagged *Zfp488* was cloned into a mammalian expression vector and transfected into NIH3T3 and COS7 cell lines. Myc-tagged Zfp488 expression was consistently observed in the nucleus defined by co-localization of DAPI and Myc immunoreactivity in these cell lines (Fig. 5A,B). To delineate the region responsible for nuclear localization, we performed a domain mapping experiment, wherein a series of truncation or deletion mutants were generated. The

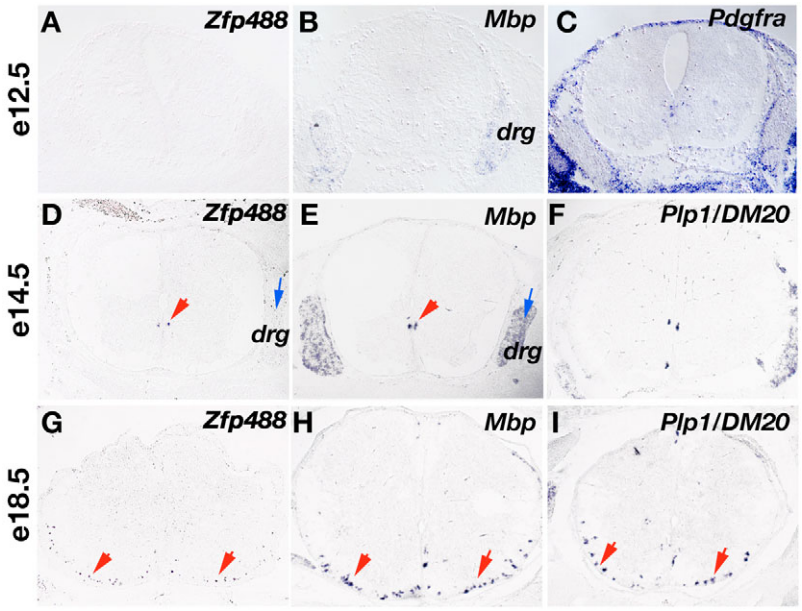

Fig. 2. Expression of *Zfp488* coincides with that of differentiated oligodendrocyte markers *Mbp* and *Plp1/DM20* in the embryonic spinal cord. In situ hybridization on transverse spinal cord sections from E12.5 (**A-C**), E14.5 (**D-F**) and E18.5 (**G-I**) with probes to murine *Zfp488*, *Pdgfra*, *Mbp* and *Plp1/DM20*. (A-C) At E12.5, *Pdgfra* is expressed in the ventral spinal cord (arrow) but *Zfp488* and *Mbp* are not. (D-F) Expression of *Zfp488*, *Mbp* and *Plp1/DM20* is initially detected at E14.5 in the ventral ventricular domain (D-F, red arrows) and *Zfp488* is absent in the dorsal root ganglia (blue arrows). (G-I) At E18.5, expression of *Zfp488, Mbp* and *Plp1/DM20* occurs in a similar distribution of cells in the ventral lateral white matter of the spinal cord (arrows).

subcellular localization of these mutants was examined after transfection into NIH3T3 cells. All truncated Zfp488 mutants lacking the bipartite NLS motif were exclusively cytoplasmic (Fig. 5D,E). By contrast, the mutant carrying only two zinc finger motifs and the NLS can be detected in the nucleus (Fig. 5F), suggesting that this NLS is likely responsible for nuclear localization of Zfp488.

To determine the transcriptional activity of Zfp488, we used an in vitro Vp16 transcription activation reporter system carrying adjacent LexA and GAL4-binding sites (Fig. 5G) (Lu et al., 1999). Although the LexA-VP16 fusion protein activated reporter expression markedly, the GAL4-Zfp488 fusion protein alone did not alter the reporter activity. However, when *GAL4-Zfp488* was co-transfected with *LexA-VP16*, the binding of GAL4-Zfp488 adjacent to the LexA-VP16 activator led to an approximately eightfold reduction of VP16 activation (Fig. 5H), suggesting that Zfp488 possesses repressive transcriptional activity.

To further define the region responsible for this repressive activity, a series of truncation or deletion mutants were generated as GAL4 fusions. The repressive effects of these mutant forms of Zfp488 were examined and compared (Fig. 5H). Deletion of zinc-finger motifs (up to residue 184 in the C-terminal) did not abolish the repressive effects. By contrast, further deletion of amino acids 69-184 eliminates the ability of Zfp488 to inhibit VP16-mediated activation. In addition, expression of the zinc-finger domain alone did not affect Vp16 trans-activation (Fig. 5H). Thus, our results suggest that a potential repression domain resides in the segment spanning amino acids 69-184 of the Zfp488 protein outside the zinc-finger domains.

Zfp488 promotes oligodendrocyte precursor formation in the presence of Notch signaling activation

To examine whether ectopic expression of *Zfp488* in vivo could promote oligodendrocyte generation, we carried out a gain-of-function study in the developing chick neural tube (Sun et al., 2001; Zhou et al., 2001). A *Zfp488* expression vector was electroporated into the neural tube of E2.5 chick embryos. The embryos were then harvested 3 days later at E5.5, when the differentiation of endogenous oligodendrocytes has not yet occurred (Ono et al., 1995). Misexpression of *Zfp488* alone did not induce ectopic oligodendrocyte differentiation in the neural tube (Fig. 6A,B). One possibility is due to the endogenous and prevailing proneural activity at this stage (Zhou et al., 2001). To repress the neurogenic activity, we misexpressed *Zfp488* together with $Notch^{ICD}$ (*NICD*), a constitutively active form of Notch. NICD is known to repress a number of proneural genes while instructing and/or permitting gliogenesis in vertebrate systems (de la Pompa et al., 1997; Ma et al., 1996; Morrison et al., 2000; Park and Appel, 2003). As a control, overexpression of *NICD* itself did not induce oligodendrocyte precursor formation (Fig. 6C,D). However, misexpression of *Zfp488* with *NICD* in the chick neural tube was able to promote ectopic expression of committed oligodendrocyte precursor markers *Pdgfra* and *Sox10* on the electroporated side of the neural tube (Fig. 6E-G). The ectopic expression of OPC markers was mainly detected in the ventricular zone of both the dorsal and ventral neural tube (Fig. 6E-G, arrowheads). No mature oligodendrocyte markers such as *Mbp,* however, were detected (Fig. 6H). These data suggest that *Zfp488* is able to promote precocious oligodendrocyte precursor formation upon Notch signaling activation.

Co-misexpression of *Zfp488* and *Olig2* induces ectopic and precocious oligodendrocyte differentiation

The absence of ectopic *Mbp* expression in the *Zfp488/NICD* co-electroporation assay as described above might reflect the inhibitory effect of Notch signaling on oligodendrocyte maturation (Wang et al., 1998). Alternatively, *Zfp488* might need to cooperate with additional transcriptional regulators to promote terminal differentiation of OPCs. As *Olig2* and *Nkx2.2* are involved in promoting oligodendrocyte differentiation (Qi et al., 2001; Zhou et al., 2001), we therefore examined whether misexpression of *Zfp488* with these oligodendroglial regulators could promote ectopic oligodendrocyte differentiation.

Expression vectors carrying *Zfp488*, *Olig2* or both were electroporated into E2.5 chick embryos harvested and analyzed 3 days later at E5.5. Misexpression of *Olig2* alone did not lead to ectopic oligodendrocyte formation in the dorsal spinal cord (Fig. 7A,B) despite the appearance of a small population of cells expressing Sox10 in the ventral domain. By contrast, co-electroporation of *Zfp488* and *Olig2* resulted in robust induction of

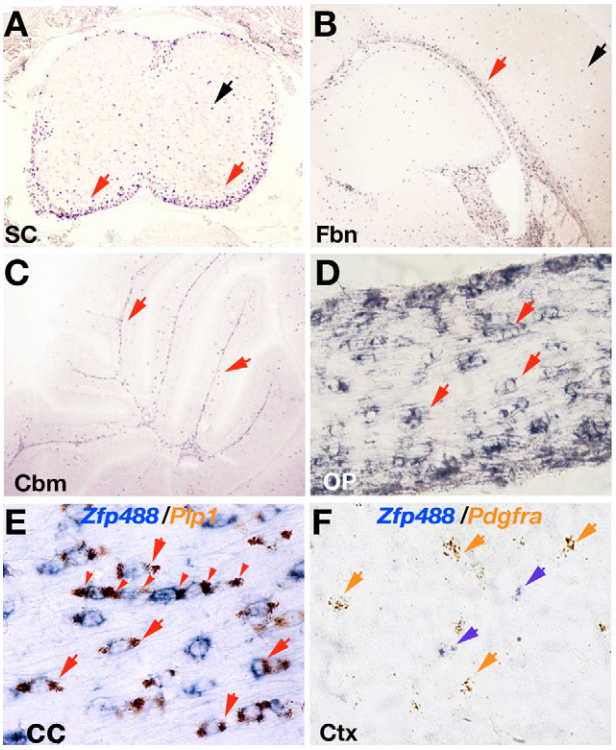

Fig. 3. *Zfp488* expression in oligodendrocytes of the postnatal CNS. In situ hybridization of transverse sections of postnatal cerebral cortex, cerebellum and longitudinal sections of optic nerves. (**A**) At the neonatal stage P7, *Zfp488* expression (red arrows) is mainly confined to the spinal white matter. (**B-D**) Expression of *Zfp488* (red arrows) is highly enriched in the white matter of the cerebral cortex (B), and the cerebellum (C) and the optic nerve (D) at P14. A small population of *Zfp488*-expressing cells in the gray matter of the spinal cord and the forebrain is also evident (A and B, black arrows). (**E**) Double in situ hybridization for *Zfp488* (blue color) and *Plp1* (brown color) shows that *Zfp488*-expressing cells (arrows) are co-labeled with *Plp1* in the corpus callosum. Small arrowheads indicate a linear array of interfascicular oligodendrocytes expressing both *Zfp488* and *Plp1*. (**F**) Double in situ hybridization for *Zfp488* and *Pdgfra* in the P14 cerebral cortex shows that *Zfp488*-expressing cells (purple) are not co-labeled with *Pdgfra* (brown) as indicated by purple and brown arrows, respectively. SC, spinal cord; Fbn, forebrain; Cbm, cerebellum; OP, optic nerve; CC, corpus callosum; Ctx, cortex.

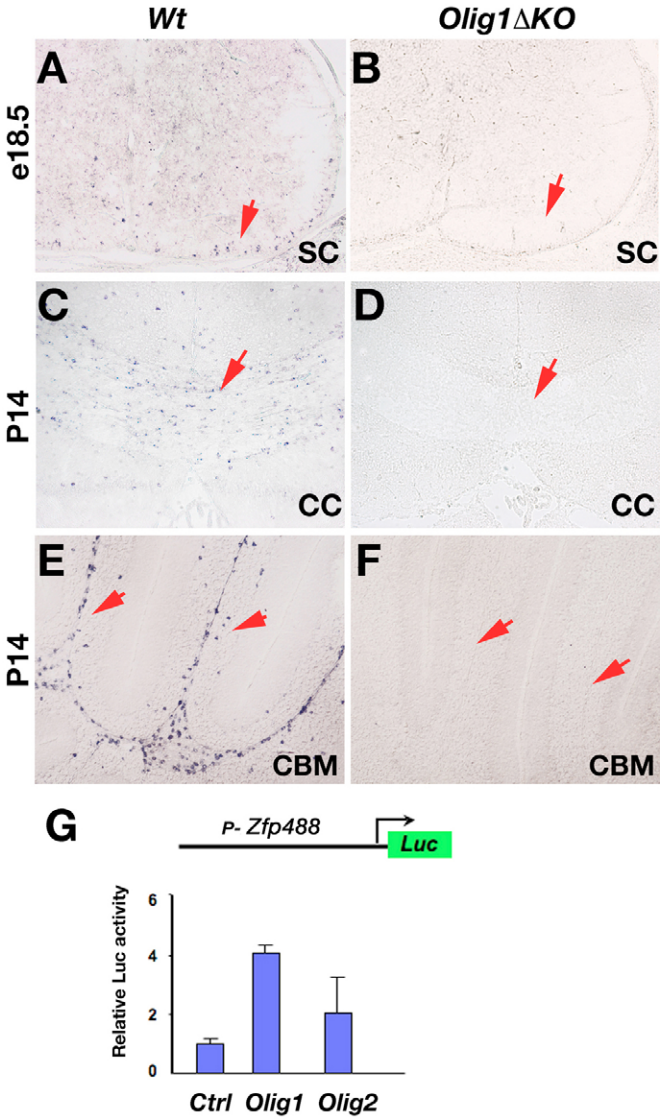

Fig. 4. *Zfp488* expression is absent throughout the CNS in the *Olig1*-null mice. (**A-F**) Expression of *Zfp488* mRNA was analyzed in situ on the spinal cord, the corpus callosum and the cerebellum taken from wild-type (A,C,E) or *Olig1ΔKO* (B,D,E) mice at E18.5 and P14 as indicated. (B,D,F) Absence of *Zfp488* expression in the spinal cord (SC), the corpus callosum (CC) and cerebellum (CBM) was observed, indicating that *Zfp488* expression requires *Olig1*. Arrows indicates *Zfp488* expression in the white matter of SC (A), CC (C) and CBM (E), respectively. (**G**) Luciferase reporter activity driven by a 3.2 kb *Zfp488* upstream regulatory region in the pGL3 vector indicates that transfection of Olig1 activates *Zfp488* expression by approximately fourfold ($P<0.01$). By contrast, Olig2 activity on the putative promoter is statistically insignificant ($P>0.05$) using the Student's *t*-test. Data are derived from at least three independent experiments and presented as the mean±standard deviation.

ectopic and precocious expression of *Sox10* and *Pdgfra* (Fig. 7C-E) on the electroporated side of the chick neural tube. Significantly, co-expression of *Zfp488* and *Olig2* promotes ectopic expression of *Mbp* in this region (Fig. 7F). Many of these ectopic oligodendrocytes appeared to be in the dorsal gray matter away from the ventricular zone, suggesting that they differentiated. By contrast, co-misexpression of *Zfp488* and *Nkx2.2* did not induce ectopic oligodendrocyte formation (Fig. 7G,H).

To avoid ectopic oligodendrocyte formation owing to interaction of *Olig2* with endogenous *Nkx2.2* in the ventral spinal cord, dorsal expression of *Olig2* and *Zfp488* was achieved by dorsally oriented in ovo electroporation (Fig. 7I). Under this circumstance, ectopic *Sox10* was detected ectopically in the dorsal region of the spinal cord (Fig. 7J, red arrows), although there was no ectopic expansion of this oligodendroglial marker into the ventral domain of the spinal cord (Fig. 7J, black arrow). Thus, Zfp488 appears to cooperate with Olig2 to promote ectopic and precocious oligodendrocyte differentiation.

Zfp488 is co-expressed and physically interacts with Olig2

To examine whether *Zfp488* and Olig2 are colocalized in the same cells, we performed double in situ hybridization and immunohistochemistry on the chick spinal cord at the stage E18. Expression of endogenous chick *Zfp488* is mainly confined to the white matter of the spinal cord (Fig. 7K). Essentially all *Zfp488*-expressing cells are Olig2 positive (Fig. 7L, arrows), although not

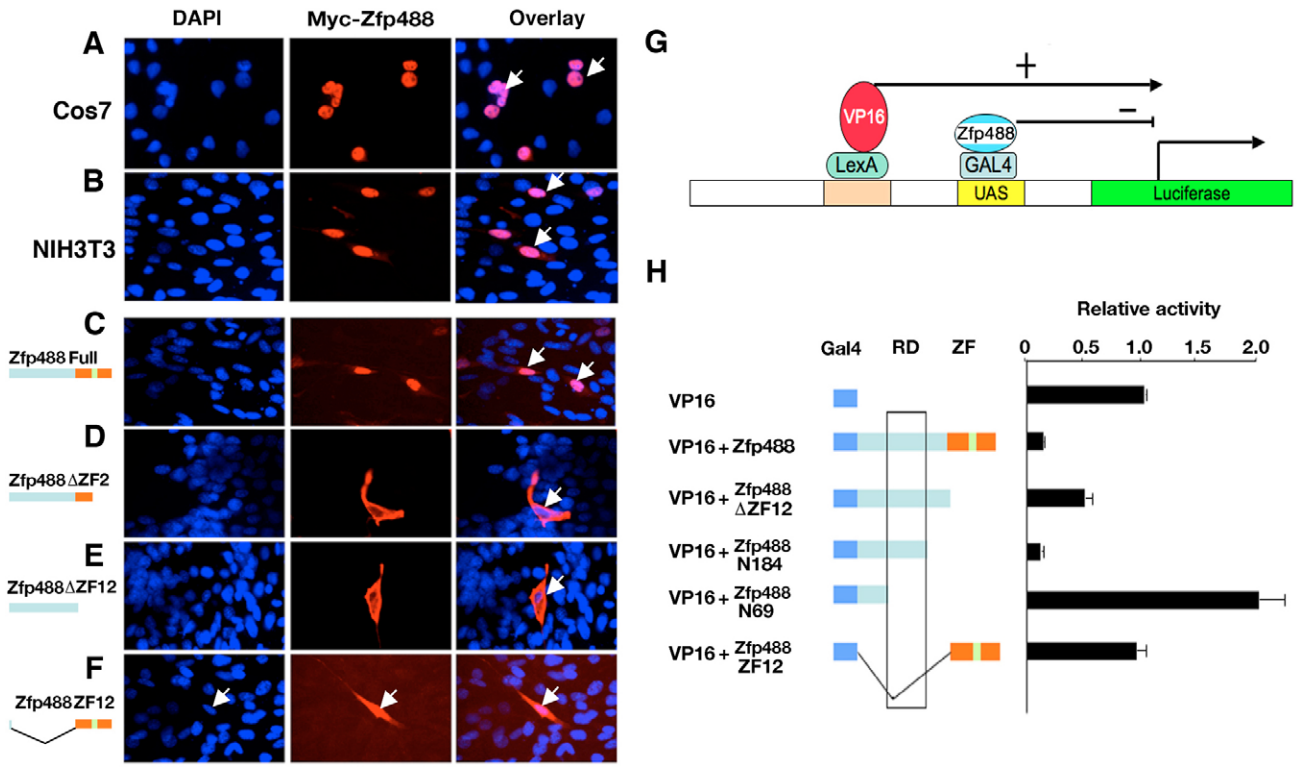

Fig. 5. Zfp488 exhibits nuclear localization and has transcriptional repression activity. Cells transfected with expression vectors (pCS2) for Myc-tagged Zfp488 and its various mutants were assessed for nuclear localization defined by DAPI staining and for Zfp488 transcription activity in an in vitro assay. (**A**,**B**) Nuclear localization of Myc-tag staining (arrows) is detected in both COS7 and NIH3T3 cells when transfected with Myc-Zfp488 and analyzed by indirect immunofluorescence using anti-Myc antibody (red) together with DAPI (blue) to delineate the nucleus. (**C-F**) COS7 cells transfected with expression vectors for Myc-tagged proteins of Zfp488 full-length (C), Zfp488ΔF2 (D), Zfp488ΔF12 (E) and ZF12 (F) domain only were examined for Myc (red) and DAPI (blue) immunofluorescence. Arrows indicate expression of Myc-Zfp488 and its derivatives. (**G**) A schematic diagram shows the in vitro assay for Zfp488 transcription activity. (**H**) NIH3T3 cells were transiently transfected with L8G5-luc reporter (Lu et al., 1999) and expression vectors encoding LexA-VP16, GAL4-Zfp488 or its truncated forms, as indicated. Transfection of *GAL4*-Zfp488 resulted in repression of luciferase reporter activity induced by LexA-VP16 in this assay. The Zfp488 construct lacking both zinc-finger motifs (Zfp488ΔZF12) and its N-terminal fragments 1-184 (Zfp488N184) and 1-69 (Zfp488N69), as well as its zinc-finger domain only segment (Zfp488ZF12), were fused in-frame with GAL4 in an expression vector. The relative activity of GAL4-Zfp488 truncated derivatives was normalized to that of LexA-VP16 activation. At least three independent transfection experiments were performed and data are presented as the mean±s.d.

all Olig2+ cells express *Zfp488* (Fig. 7L). Thus, co-expression of *Zfp488* with Olig2 is consistent with the cooperation of Zfp488 and Olig2 in promoting oligodendrocyte differentiation.

As *Olig2* and *Zfp488* are co-expressed in oligodendrocytes and cooperate to promote oligodendrocyte differentiation, we then examined whether these two factors can associate with each other. *Zfp488*, *Olig2* or both expression vectors were transfected into COS7 cells. Co-immunoprecipitation (IP) was performed from the cell lysates 48 hours after transfection. Olig2 was detected in the immunoprecipitated complex containing Zfp488 (Fig. 7M), suggesting that Zfp488 can either directly interact with Olig2 or that they are components of a protein complex. By contrast, Zfp488 did not appear to interact with Nkx2.2 in this assay (Fig. 7N).

Zfp488 expression is upregulated as oligodendroglial cells become differentiated

Our data suggest that Zfp488 is involved in the late stage of oligodendrocyte differentiation and maturation by interacting with Olig2, while a previous study indicates that Nkx2.2 interacts with Olig2 at an early stage for oligodendrocyte fate determination (Zhou et al., 2001). To define the relation between *Zfp488*, *Nkx2.2* and myelin gene expression, we examined expression of these genes during the progression of oligodendrocyte differentiation in primary culture. OPCs were isolated from the neonatal rat brain and cultured in growth medium in the presence of PDGFAA and bFGF (Kondo and Raff, 2000; Raff et al., 1983). Differentiation was induced by switching to oligodendrocyte differentiation medium containing T3 and CNTF (Barres and Raff, 1994; Gard and Pfeiffer, 1989). Total RNA from these cultures was isolated at different time points before and after the induction of oligodendrocyte differentiation and subjected to quantitative real-time PCR analysis. Upon OPC differentiation, *Zfp488* expression was upregulated rapidly in conjunction with increased expression of oligodendrocyte differentiation markers *Mbp* and *Cnp* (2′,3′-cyclic nucleotide 3′-phosphohydrolase) (Lappe-Siefke et al., 2003) (Fig. 8A). This is in contrast to the downregulation of *Nkx2.2* during oligodendrocyte differentiation (Fig. 8A) (Qi et al., 2001; Wei et al., 2005). Thus, the level of *Zfp488* expression accumulates in parallel with that of myelin genes, and is inversely correlated with that of *Nkx2.2* during oligodendrocyte maturation.

RNAi-mediated *Zfp488* knockdown results in a decrease in myelin gene expression

To determine the effects of *Zfp488* knockdown on myelin gene expression, we used RNAi targeting *Zfp488* in CG4 cells, a cell line with properties of rat immature oligodendrocytes (Espinosa de los

Monteros et al., 1997; Tontsch et al., 1994; Wei et al., 2005). *Zfp488* expression was rapidly upregulated when CG4 cells were in differentiation medium. This is correlated with the expression of *Mbp* and *Cnp* but negatively related to *Nkx2.2* expression (Fig. 8B). This observation recapitulates the pattern of *Zfp488* expression seen during primary oligodendrocyte differentiation, suggesting that CG4 cells exhibit normal overall oligodendroglial gene regulation. To knock down *Zfp488* mRNA, short interfering RNA (siRNA) duplexes were designed against rat *Zfp488*. Transfection of CG4 cells with *Zfp488* siRNAs led to a reduction of ~60% of *Zfp488* expression examined by quantitative real time-PCR analysis (Fig. 8C). The down-regulation of *Zfp488* caused a decrease in the expression level of endogenous myelin genes *Mbp* and *Cnp* in CG4 cells, but not the control housekeeping gene *Gapdh*. By contrast, the control siRNA against an unrelated gene *GFP* did not reduce *Zfp488* expression and myelin gene expression (Fig. 8C, data not shown). These data therefore suggest that specific knockdown of *Zfp488* in the oligodendroglial cell line leads to the downregulation of myelin gene expression, consistent with a role for *Zfp488* in promoting oligodendrocyte maturation.

DISCUSSION

Oligodendrocyte myelinogenesis is characterized by successive stages of lineage progression from precursors to immature oligodendrocytes and finally to mature myelinating oligodendrocytes (Pfeiffer et al., 1993). Although a series of transcriptional regulators has been found in oligodendrocyte precursors (Rowitch, 2004), few have been identified that are specifically expressed in mature oligodendrocytes in the CNS. In the peripheral nervous system, a zinc-finger transcription factor Krox20, which is restricted to mature myelinating Schwann cells, plays an essential role in Schwann cell myelination (Topilko et al., 1997; Topilko et al., 1994). Thus, identification of transcriptional regulators that are spatially and temporally limited to myelinating oligodendrocytes might contribute to unraveling the molecular mechanisms that control the oligodendrocyte myelination process.

In a screen for mRNAs downregulated in non-myelinating optic nerves of *Olig1*-null mice, we identified an as yet uncharacterized zinc-finger transcription regulator Zfp488. It is specifically expressed in oligodendrocytes at later stages of differentiation but not in myelinating Schwann cells in the peripheral nervous system. Ectopic expression of *Zfp488* together with Notch activation or in collaboration with Olig2 induces precocious oligodendrocyte differentiation in the chick neural tube. In addition, RNAi-mediated *Zfp488* knockdown leads to myelin gene downregulation in an oligodendroglial cell line. Thus, our gain- and loss-of-function data suggest that Zfp488 probably plays an important role in the differentiation and maturation process during oligodendrocyte development.

The oligodendrocyte-specific zinc finger protein Zfp488, a downstream effector of Olig1, cooperates with Olig2 to promote oligodendrocyte differentiation

Expression of *Zfp488* persists in oligodendrocytes but not their precursors throughout the CNS, indicating that *Zfp488* is specific for differentiated oligodendrocytes. The observations that *Zfp488* expression is undetectable in the brain and spinal cord of *Olig1* mutant mice, and that *Olig1* transactivates a candidate *Zfp488* regulatory sequence, suggest that *Zfp488* is a direct or indirect downstream target gene of the transcription regulator Olig1.

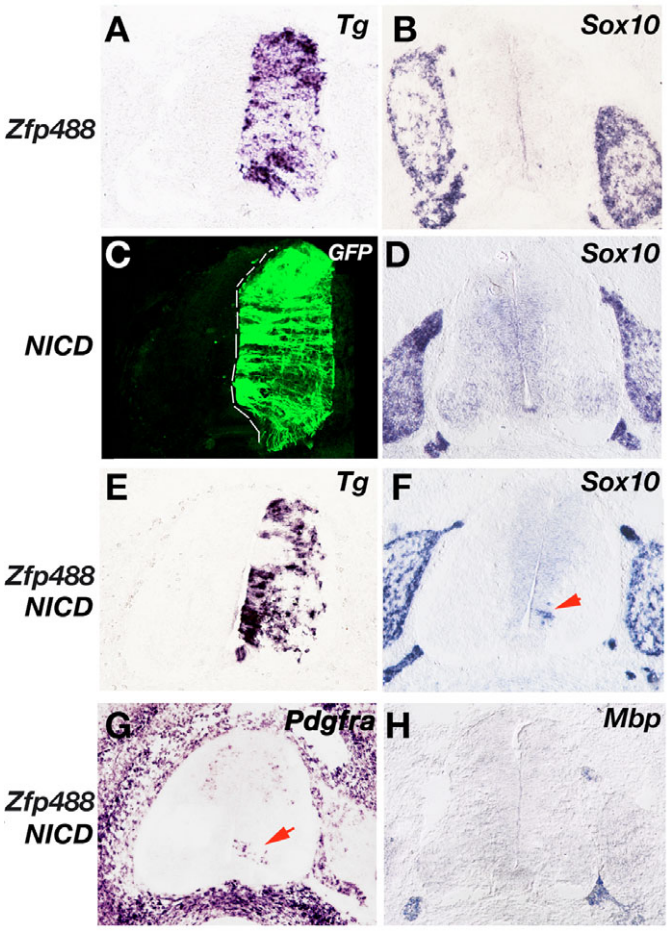

Fig. 6. *Zfp488* induces ectopic oligodendrocyte precursor markers in the presence of Notch signaling activation. Chick neural tubes at the stage HH14 (E2.5) stage were electroporated with expression vectors for *Zfp488* (**A**,**B**), *NICD* (**C**,**D**) or both constructs (**E-H**), and harvested 3 days later at E5.5. Sections of chick neural tube were hybridized with the probes as indicated. Transgene (Tg) was detected by *Zfp488* (A,E) and *NICD* (C). Overexpression of neither *Zfp488* (A,B) nor *NICD* (C,D) alone could induce ectopic oligodendrocyte marker expression. The combination of *NICD* and *Zfp488* induces expression of ectopic oligodendroglial markers *Sox10* (F, arrow) and *Pdgfra* (G, arrow), but not *Mbp* (H).

The cooperation between oligodendrocyte-specific Zfp488 and Olig2 induces ectopic and precocious oligodendrocyte maturation, suggesting that Zfp488 functions as a transcriptional co-regulator for oligodendrocyte differentiation and maturation. Several lines of evidence indicate that interaction of zinc-finger proteins with proneural bHLH proteins is a common theme for inducing neuronal cell fate specification (Acar et al., 2006; Bellefroid et al., 1996; Nakakura et al., 2001). Overexpression of X-Myt1, a *Xenopus* zinc-finger protein, promotes ectopic neuronal differentiation only in combination with proneural bHLH transcription factors (Bellefroid et al., 1996). Recently, Acar et al. showed that a *Drosophila* zinc-finger transcription factor, Senseless, is recruited by bHLH proneural proteins to function as a co-activator in promoting the development of sensory organ precursors (Acar et al., 2006). In keeping with these observations, the physical and functional interaction of the zinc finger protein Zfp488 and the bHLH factor Olig2 in promoting

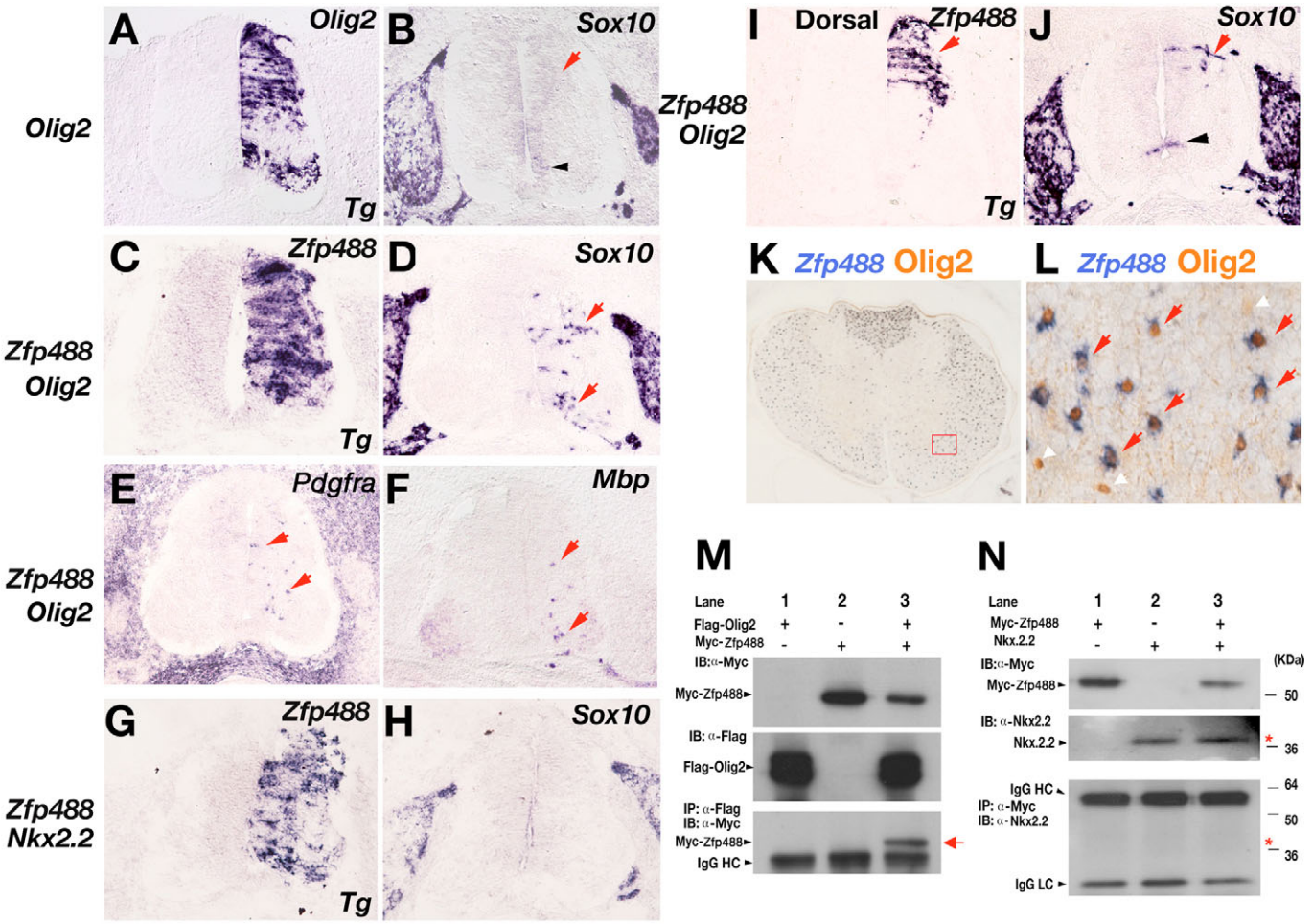

Fig. 7. *Zfp488* cooperates with *Olig2* to promote ectopic and precocious oligodendrocyte differentiation. E2.5 chick embryos were electroporated with expression vectors for *Olig2* (A,B), *Zfp488/Olig2* (C-F,I-J) or *Zfp488/Nkx2.2* (G,H), and harvested 3 days later at E5.5. In situ hybridization of the neural tube was performed with the probes as indicated in panels. (**A**,**B**) Misexpression of the *Olig2* transgene (Tg) alone did not induce oligodendrocyte markers in the dorsal region (red arrow in B), while a small number of *Sox10*+ cells were detected in the ventral domain (black arrowhead). (**C-F**) Co-electroporation of *Zfp488/Olig2* induced robust ectopic expression of *Sox10* (D, arrows), *Pdgfra* (E, arrows) and *Mbp* (F, arrows) in the electroporated side of the neural tube. (**G**,**H**) Co-electroporation of *Zfp488/Nkx2.2* did not induce ectopic *Sox10* expression in the transgenic side of the chick neural tube. (**I**,**J**) Dorsally confined misexpression of *Zfp488* and *Olig2* resulted in ectopic *Sox10* expression in the dorsal region of the spinal cord (J, red arrow) but not in the ventral region (J, black arrowhead). (**K**,**L**) Double in situ labeling for chick ortholog of *Zfp488* (purple) and immunostaining of Olig2 (brown) were performed in the chick spinal cord at E18. Co-expression of chick *Zfp488* and Olig2 was detected in the spinal cord. L is a high magnification of an area outlined in K, showing the co-labeling of *Zfp488* and Olig2 in the same cells of the chick spinal cord (red arrows). White arrowheads in L indicate the cells that express only Olig2. (**M**) Physical interaction between Zfp488 and Olig2. Vectors expressing Myc-tagged Zfp488 and Flag-tagged Olig2 were co-transfected into Cos7 cells. Co-immunoprecipitation (IP) of cell lysates (600 μg total) 48-hours post-transfection was performed with anti-Flag antibody. Western blot was carried out to detect the input proteins for Zfp488 (upper panel) and Olig2 (middle panel). The immunoprecipitated Myc-Zfp488 was detected by anti-Flag (lower panel, arrow). (**N**) Absence of Zfp488 and Nkx2.2 interaction. Vectors expressing Myc-tagged Zfp488 and Nkx2.2 were co-transfected into COS7 cells. Co-immunoprecipitation was performed with anti-Myc antibody. Western blot was performed to detect the input proteins for Myc-Zfp488 (upper panel) and Nkx2.2 (middle panel). The co-immunoprecipitated complex was detected by anti-Nkx2.2 (lower panel). Star indicates the prospective Nkx2.2 position.

oligodendroglial maturation may reflect a similar mechanism for zinc finger/bHLH cooperation in regulating glial differentiation during development.

Zfp488 exerts repressive effects on transcription and promotes oligodendrocyte precursor formation upon Notch activation

Zfp488 is a nuclear protein in the different cell lines tested. Like many oligodendrocyte transcriptional regulators, including Olig2, Nkx2.2 and Nkx6.2 (Awatramani et al., 2000; Wei et al., 2005; Zhou et al., 2001), Zfp488 also functions apparently as a transcriptional repressor when assayed by an in vitro transcription reporter system. Its transcriptional repression domain appears localized outside the zinc-finger motifs, suggesting that the repressive regulatory domain in Zfp488 is separate from a potential DNA recognition domain mediated by zinc-finger motifs. It is possible that the repression activity common among oligodendrocyte transcriptional regulators may be required for directing oligodendrocyte differentiation while repressing neurogenic activity during development.

Although overexpression of Zfp488 itself cannot promote oligodendrocyte differentiation, Zfp488 is able to promote precocious oligodendrocyte precursor formation in the chick neural tube under

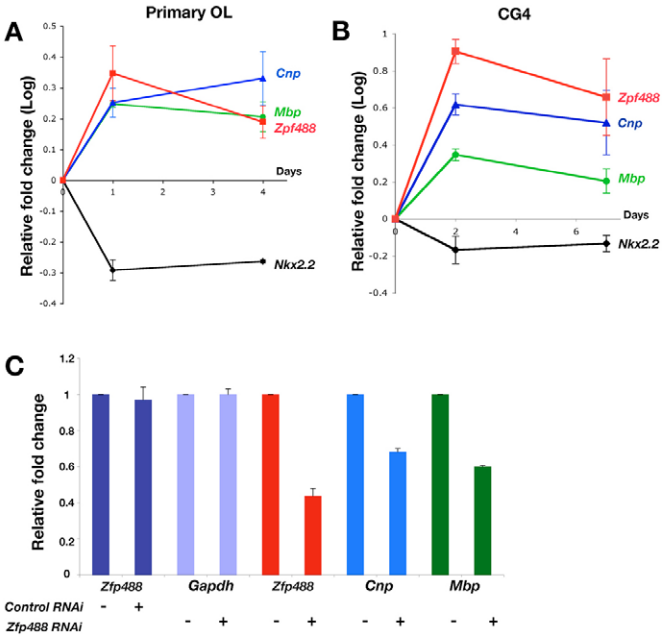

Fig. 8. Expression of *Zfp488* is correlated with oligodendrocyte differentiation. (**A**) Expression of *Zfp488* increased as oligodendroglial cells underwent differentiation. Primary rat OPCs were isolated from neonate rat at P2 and cultured in growth medium. Total RNA was harvested from cell lysates before and after switching to differentiation medium at the time indicated. Real-time RT-PCR was performed to determine the relative amounts of *Zfp488*, *Cnp, Mbp* and *Nkx2.2* expression. (**B**) CG4 cells were analogously treated and analyzed by real-time RT-PCR as described above in A. (**C**) Cultured CG4 cells in growth medium were transfected with control *GFP* siRNA and *Zfp488* siRNA (100 nM) for 48 hours. Total RNAs were isolated before and after siRNA transfection. Real-time PCR was performed to determine the expression level of *Zfp488*, *Cnp, Mbp* and *Gapdh*. All data were derived from three independent experiments and shown as mean±s.d. *Gapdh* gene expression was used as the internal control.

the condition of constitutive Notch activation. One potential mechanism is that activation of Notch signaling may provide an environment to facilitate Zfp488 in promoting oligodendroglial specification either by repressing neurogenic activity of proneural proteins, as occurs when Notch activation facilitates promotion of OPC formation by Olig2 (Zhou et al., 2001) or by destabilizing proneural bHLH proteins (Sriuranpong et al., 2002). These data suggest a more general underlying mechanism where Notch activation provides a permissive environment for transcription regulators to induce oligodendrocyte precursor specification.

Dynamic coupling of stage-specific transcription regulators may control successive waves of oligodendrocyte maturation during CNS development

Olig2 is essential for oligodendrocyte lineage development. Its expression is detected in OPCs during early CNS development and persists in mature myelinating oligodendrocytes in adulthood. At present it is not fully understood how the transition from oligodendrocyte precursors to mature myelinating oligodendrocytes is regulated by Olig2 at different developmental stages (Pfeiffer et al., 1993; Rowitch, 2004). A recent study suggests that an interplay between transcriptional activators and inhibitory factors may orchestrate myelin gene expression (Gokhan et al., 2005; Kagawa et al., 2001; Wegner, 2001). In the developing neural tube, the transient overlap of Olig2 and Nkx2.2 expression domains in the ventricular zone specifies oligodendrocyte precursors, suggesting that Olig2 cooperates with Nkx2.2 for the early specification of OPCs (Zhou et al., 2001). However, the expression of Nkx2.2 is downregulated in oligodendroglial cells undergoing terminal differentiation into myelinating oligodendrocytes (Watanabe et al., 2004; Wei et al., 2005) (Fig. 8). There is also evidence that Nkx2.2 represses *Mbp* expression in oligodendrocytes in vitro (Wei et al., 2005). Thus, the effects of Nkx2.2 may depend on its temporal context, i.e. Nkx2.2 may cooperate with Olig2 for oligodendrocyte differentiation at an early developmental stage while repressing oligodendrocyte maturation at later stages.

Zfp488 is upregulated as immature oligodendroglial cells become differentiated. Increased Zfp488 may subsequently interact with Olig2 to promote oligodendrocyte terminal differentiation. The functional interaction between Zfp488 and Olig2 correlates well with their co-expression in oligodendrocytes. Expression of Zfp488 specifically in differentiated and mature oligodendrocytes, coupled with downregulation of Nkx2.2 during oligodendrocyte maturation, suggests that Zfp488 may potentially cooperate with Olig2 at a later stage of differentiation to promote oligodendrocyte maturation and myelination. Thus, our study supports the notion that dynamic coupling of stage-specific oligodendroglial regulators may control successive waves of oligodendrocyte maturation during CNS development.

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