SoxD Proteins Influence Multiple Stages of Oligodendrocyte Development and Modulate SoxE Protein Function

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Summary

The myelin-forming oligodendrocytes are an excellent model to study transcriptional regulation of specification events, lineage progression, and terminal differentiation in the central nervous system. Here, we show that the group D Sox transcription factors Sox5 and Sox6 jointly and cell-autonomously regulate several stages of oligodendrocyte development in the mouse spinal cord. They repress specification and terminal differentiation and influence migration patterns. As a consequence, oligodendrocyte precursors and terminally differentiating oligodendrocytes appear precociously in spinal cords deficient for both Sox proteins. Sox5 and Sox6 have opposite functions than the group E Sox proteins Sox9 and Sox10, which promote oligodendrocyte specification and terminal differentiation. Both genetic as well as molecular evidence suggests that Sox5 and Sox6 directly interfere with the function of group E Sox proteins. Our studies reveal a complex regulatory network between different groups of Sox proteins that is essential for proper progression of oligodendrocyte development.

Introduction

Many transcription factors are important regulators of development. Coexpression of related transcription factors is frequently observed and can manifest itself in functional equivalence, complementarity or antagonism (Bouchard et al., 2000; Bylund et al., 2003; Coppola et al., 2005; McEvilly et al., 2002; Sandberg et al., 2005). Within the developing central nervous system (CNS), many transcription factors of the Sox family are expressed (Wegner and Stolt, 2005). All members of group E (i.e., Sox8, Sox9, and Sox10, collectively referred to as SoxE proteins) are found in the oligodendrocyte lineage (Wegner and Stolt, 2005).

Oligodendrocytes as the myelin-forming macroglia of the vertebrate CNS allow saltatory and rapid nerve conduction. They arise from multipotent progenitor cells in defined areas of the ventricular zone (VZ) (Richardson et al., 2006). In the mouse spinal cord, approximately 85%–90% originate from Olig2-positive cells in the pMN domain of the ventral VZ around 11.5 days post coitum (dpc), and the remaining 10%–15% with delay from the dP3–dP5 domains in the dorsal VZ (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). Sox9 occurrence in VZ cells is a prerequisite for oligodendrogenesis (Stolt et al., 2003). Upon specification, oligodendrocyte precursors start to express Sox10 along with Sox9. Both Sox proteins appear to function redundantly in oligodendrocyte precursors so that each can be deleted without adverse effect on the oligodendrocyte precursor. Sox8 is additionally expressed at low levels in the oligodendrocyte lineage but contributes much less to oligodendrocyte development (Stolt et al., 2004, 2005).

From their birthplace, oligodendrocyte precursors spread as still proliferative cells throughout the spinal cord, now additionally marked by expression of the NG2 proteoglycan and the PDGF receptor α (PDGFRα). The majority finally settles in the future white matter where terminal differentiation starts at the end of embryogenesis, characterized by loss of NG2 and PDGFRα, and induction of myelin genes including the genes for myelin basic protein (MBP) and proteolipid protein (PLP). With the onset of terminal differentiation, Sox9 is selectively downregulated in the oligodendrocyte lineage, and Sox10 becomes the major remaining SoxE protein. Sox10 controls the expression of several myelin genes by directly binding to the genes' regulatory region. As a consequence, terminal oligodendrocyte differentiation and myelin production are severely disrupted upon experimental Sox10 removal (Stolt et al., 2002). Even terminally differentiated oligodendrocytes in the adult CNS still express Olig2 and Sox10. Thus, Olig2 and Sox10 mark all stages of oligodendrocyte development.

Here, we show that cells of the oligodendrocyte lineage in addition to SoxE proteins express Sox5 and Sox6, which are closely related to each other. These two SoxD proteins help SoxE proteins to perform different functions at different stages and thereby allow timely progression of oligodendrocyte development.

Results

Sox5 and Sox6 Occur in Several Cell Types of the Spinal Cord

Sox5 and Sox6 expression was studied by immunohistochemistry in the embryonic mouse spinal cord (Figure 1). Weak immunoreactivity was obtained for both SoxD proteins at 10.5 dpc in VZ cells (Figures 1A and 1G). Sox5 was predominantly expressed in the dorsal VZ (Figure 1A), whereas Sox6 was additionally detected in the ventralmost VZ (Figure 1G). By 11.5 dpc, both Sox5 and Sox6 were present throughout the VZ and in

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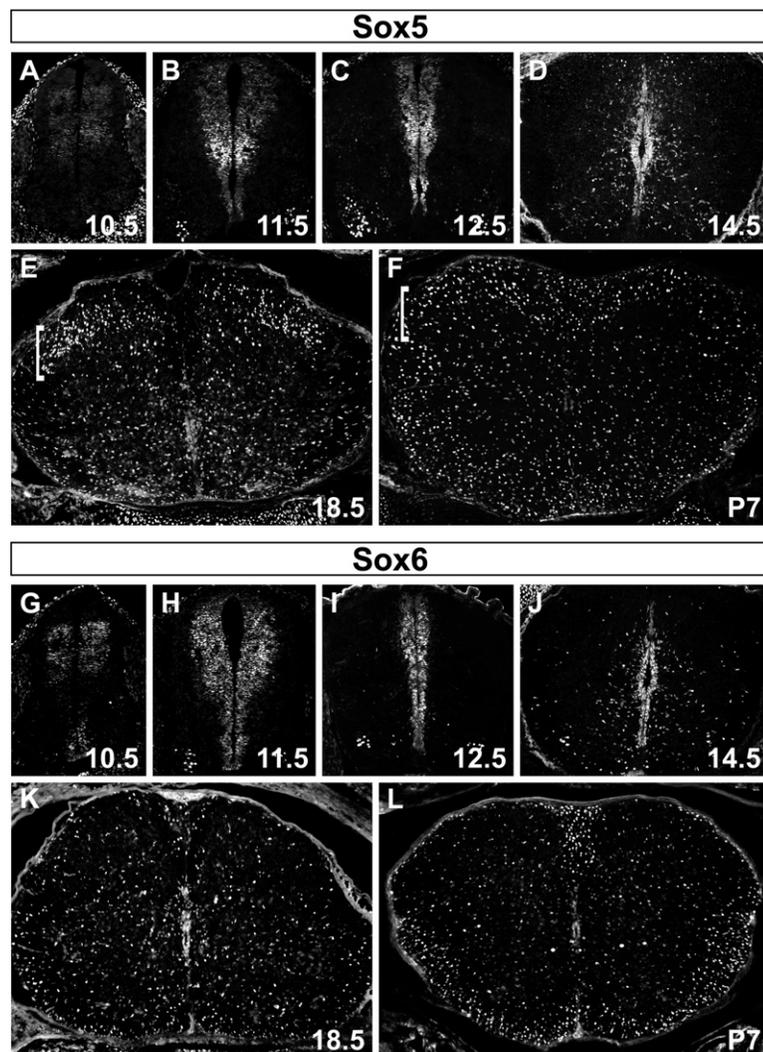
Figure 1. Expression of Sox5 and Sox6 in the Embryonic Spinal Cord

Immunohistochemistry was performed on transverse sections from the forelimb region of wild-type embryos with antibodies directed against Sox5 (A–F) or against Sox6 (G–L) at embryonic stages 10.5 dpc (A and G), 11.5 dpc (B and H), 12.5 dpc (C and I), 14.5 dpc (D and J), and 18.5 (E and K) dpc and at postnatal day 7 (P7) (F and L). The position of dorsal horn neurons in (E) and (F) is indicated by brackets.

cells that by nuclear shape, location in the ventrolateral corner of the mantle zone, and Hb9 expression, correspond to a subset of motoneurons (Figures 1B and 1H and Figures S1A and S1B; see the Supplemental Data available with this article online). From 12.5 dpc onward, both Sox5 and Sox6 were also seen in cells that had emigrated from the ventral VZ (Figures 1C and 1I). Emigration was visible from all VZ regions at 14.5 dpc (Figures 1D and 1J), and emigrated cells had dispersed throughout the mantle zone by 18.5 dpc (Figures 1E and 1K). From time of appearance and pattern of migration, the majority of these cells are glia. Sox6-positive cells had accumulated in the future white-matter region at postnatal day 7, suggesting oligodendrocytic expression (Figure 1L). Sox5-positive cells were more equally distributed between white and gray matter (Figure 1F). In addition to glial expression, there was strong Sox5, but no Sox6 expression, in dorsal horn neurons at 16.5 dpc, 18.5 dpc, and postnatal day 7 (Figures 1E and 1F and Figures S1C–S1J). Sox5-positive dorsal horn neurons also expressed Lmx1b and Tlx3, but very little Lbx1 and Pax2 (Figures S1C, S1E, S1G, and S1I), indicating that Sox5 is present predominantly in glutamatergic dorsal horn neurons of layer 1 and 2 rather than in

GABAergic or layer 3 neurons (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). In the adult spinal cord, the signal for both SoxD proteins was greatly diminished (Figures 2O and 2P). These results argue that Sox5 and Sox6 are both transiently expressed in VZ cells as well as various types of neurons and glia.

Sox5 and Sox6 Exhibit an Overlapping Expression Pattern in Cells of the Oligodendrocyte Lineage

Expression of Sox5 and Sox6 was studied in detail during oligodendrocyte development. At 12.5 dpc, specified Sox10-positive oligodendrocyte precursors were discernable at the outer border of the pMN domain (Stolt et al., 2003). Not only VZ cells, but also Sox10-positive oligodendrocyte precursors expressed Sox5 and Sox6 (Figures 2A–2D). Oligodendrocyte precursors were still positive for Sox5 and Sox6 at 16.5 dpc as indicated by colabeling with the oligodendrocyte lineage markers Olig2 and Sox10, and with NG2 or PDGFRα as markers for the precursor stage (Figures 2E–2H and data not shown). Among all Sox6-positive cells in the spinal cord, oligodendrocyte precursors were the ones with the highest expression (Figures 2F and 2H), whereas cells with high Sox5 levels did not belong to the oligodendrocyte lineage
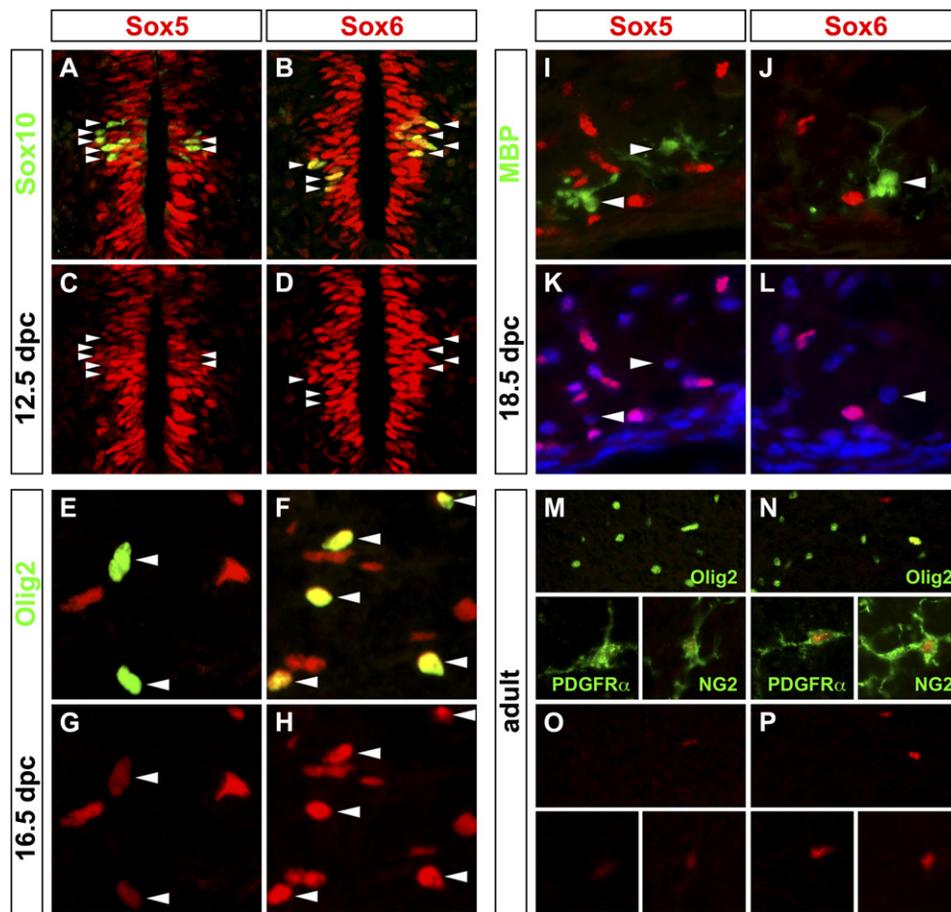
Figure 2. Expression of Sox5 and Sox6 in the Oligodendrocyte Lineage

Immunohistochemistry was performed on transverse sections from the forelimb region of wild-type embryos with Sox5- (A, C, E, G, I, K, M, and O) or Sox6-specific (B, D, F, H, J, L, N, and P) antibodies (red) at several stages of embryonic development. The Sox5- and Sox6-specific staining is shown separately (C, D, G, H, K, L, O, and P) and in combination with stainings for oligodendrocytic marker proteins (green) (A, B, E, F, I, J, M, and N). (A–D) Occurrence of SoxD proteins in newly specified Sox10-positive oligodendrocyte precursors (marked by arrowheads) at the border of the pMN domain at 12.5 dpc. (E–H) Presence of SoxD proteins in Olig2-positive oligodendrocyte precursors (marked by arrowheads) in the spinal cord mantle zone at 16.5 dpc. (I–L) Absence of SoxD proteins in MBP-positive terminally differentiating oligodendrocytes (marked by arrowheads) in the marginal zone of 18.5 dpc old spinal cord. Nuclei were additionally visualized in (K) and (L) by DAPI staining (blue). (M–P) Expression of SoxD proteins in only a small fraction of Olig2-positive cells in the adult spinal cord that can also be stained by PDGFRα and NG2 (inlays).

(Figures 2E and 2G). Colabeling with brain-fatty-acid-binding protein and glutamine synthetase identified the latter as radial glia (Figures S2A, S2C, S2E, S2G, S2I, and S2K) that also expressed low levels of Sox6 (Figures S2B, S2D, S2F, S2H, S2J, and S2L).

When oligodendrocytes start to express myelin proteins such as MBP and undergo terminal differentiation, expression of Sox5 and Sox6 is extinguished (Figures 2I–2L). Very few Olig2-positive cells of the adult spinal cord also express Sox5 or Sox6 (Figures 2M–2P). The remaining Olig2-positive Sox5 or Sox6 expressors label with NG2 and PDGFRα (Figures 2M–2P) and may represent adult oligodendrocyte precursor cells (Noble et al., 1992).

Oligodendrocytic Sox6 Expression Is under Control of SoxE Proteins

Oligodendrocyte precursors fail to be specified in the absence of Sox9 and Sox8 (Stolt et al., 2003, 2005). Following constitutive deletion of the *Sox8* alleles and Nestin Cre-mediated deletion of the *Sox9* alleles, most spinal cords were devoid of Sox9 from 14.5 dpc onward and lacked oligodendrocyte precursors. Some spinal cords, however, still exhibited residual Sox9 expression at 16.5 dpc in the ventral VZ probably due to mosaic expression of the Nestin Cre transgene (Figures 3A and 3B). This correlated with the occurrence of a very limited number of Olig2-positive oligodendrocyte precursors in the mantle zone, which at 16.5 dpc reached 0.5%–5% of wild-type numbers (Figures 3G and 3H and data not shown). The loss of the vast majority of oligodendrocyte precursors was also visible as a 28% and 46% reduction of Sox5- and Sox6-positive cells, respectively (compare Figures 3D and 3F to Figures 3C and 3E). Sox9 immunoreactivity in spinal cords with incomplete Cre-mediated gene deletion was not detected in the residual Olig2-positive oligodendrocyte precursors of the mantle zone (Figures 3A, 3B, 3Q, 3R, 3S, and 3T). The existence of Sox9/Sox8-deficient oligodendrocyte precursors gave us the opportunity to analyze the epistatic relationship between Sox9/Sox8 and SoxD proteins. Sox5
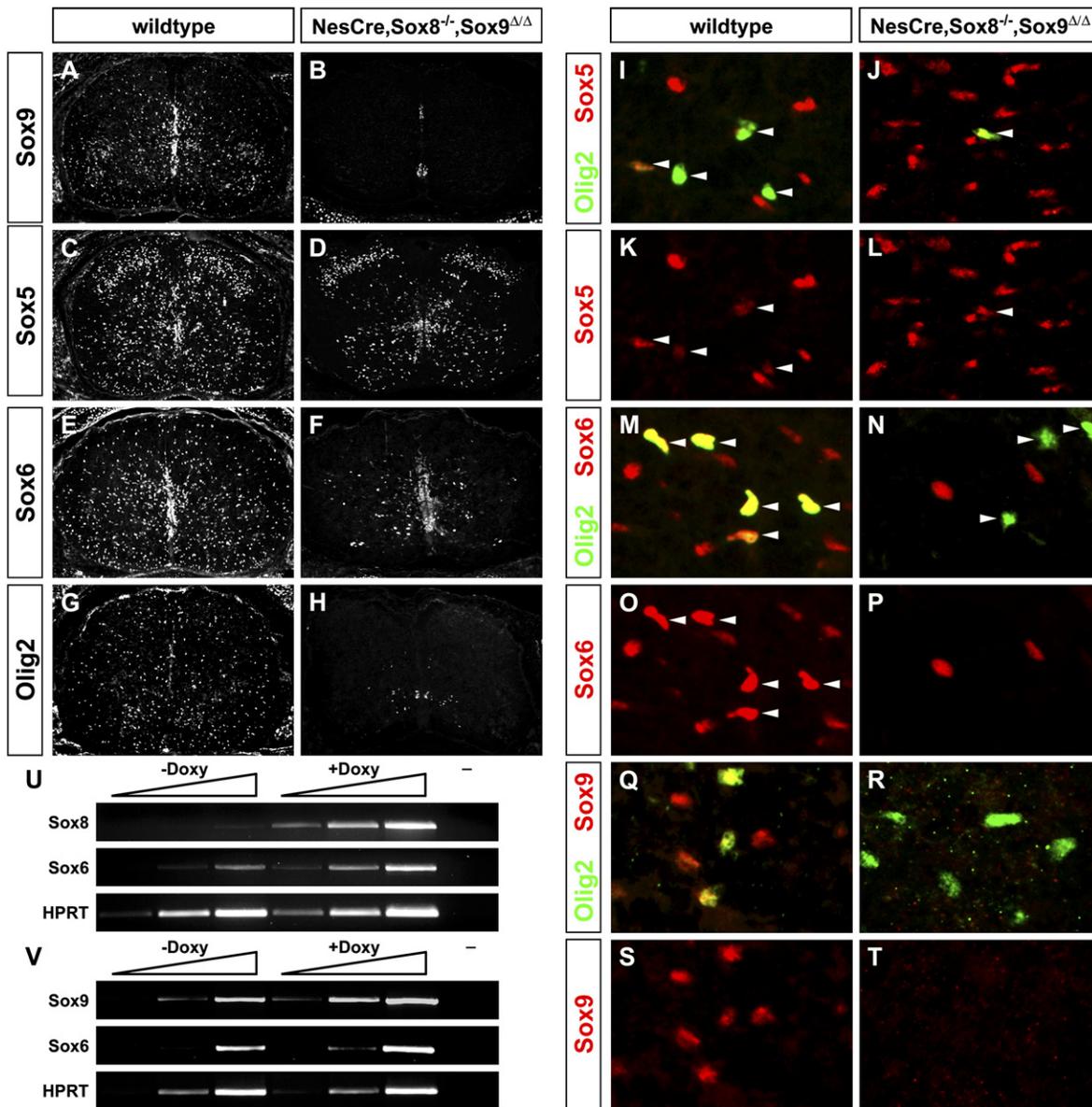
Figure 3. Expression of SoxD Proteins in Spinal Cords of Sox8$^{-/-}$,Sox9$^{\Delta/\Delta}$,Nestin Cre Mice

(A–T) Immunohistochemistry was performed on transverse sections from the forelimb region of wild-type embryos (A, C, E, G, I, K, M, O, Q, and S) at 16.5 dpc and Sox8$^{-/-}$,Sox9$^{\Delta/\Delta}$,Nestin Cre littermates (B, D, F, H, J, L, N, P, R, and T) with antibodies directed against Sox9 (A, B, Q, R, S, and T), Sox5 (C, D, I, J, K, and L), Sox6 (E, F, M, N, O, and P) (all in red), and Olig2 (G, H, I, J, M, N, Q, and R) (in green) at low (A–H) or high resolution (I–T). Panels (I), (J), (M), (N), (Q), and (R) show the same area as panels (K), (L), (O), (P), (S), and (T) as coimmunohistochemistry with Olig2. Oligodendrocytes are marked by arrowheads in (I)–(N).

(U and V) Induction of Sox6 expression was detected by RT-PCR analysis in Neuro2A Tet-On cells capable of inducibly expressing Sox8 (U) or Sox9 (V). "-Doxy," no doxycycline (low amounts of Sox8 or Sox9); "+Doxy," doxycycline added (high amounts of Sox8 or Sox9). Transcript levels of Sox6, Sox8, and Sox9 were compared semiquantitatively with increasing numbers of amplification cycles. Hypoxanthine phosphoribosyltransferase (HPRT) served as control. "-," water control.

occurred in these oligodendrocyte precursors (compare Figures 3J and 3L to Figures 3I and 3K), whereas Sox6 was not expressed (Figures 3N and 3P) in stark contrast to wild-type oligodendrocyte precursors (Figures 3M and 3O). At least Sox6 expression is therefore under control of Sox9 and Sox8 in the oligodendrocyte lineage. Accordingly, doxycycline-dependent, inducible expression of Sox8 or Sox9 led to induction of Sox6 expression in Neuro2a neuroblastoma (Figures 3U and 3V).

Precocious Oligodendrocyte Specification in the Absence of Sox5 and Sox6

In the forelimb region of the spinal cord, oligodendrocyte precursors start to arise from the pMN domain at 11.5 dpc as documented by the appearance of Sox10-positive cells at the outer edge of the Olig2 stained pMN region (Figures 4A, 4D, 4G, 4J, 4M, and 4P). On average, five oligodendrocyte precursors were present per section at 11.5 dpc, none at 11.0 dpc (Figures 4A, 4J,

Figure 4. Oligodendrocyte Specification in Mice Carrying Sox5 and Sox6 Null Alleles

(A–R) Immunohistochemistry on the pMN domain of 11.0 dpc old (A–I) and 11.5 dpc old (J–R) embryos with antibodies against Sox10 (red) (A, B, C, J, K, and L) and Olig2 (green) (D, E, F, M, N, and O) is presented as single (A–F and J–O) or merged (G–I and P–R) pictures. In addition to the wild-type (A, D, G, J, M, and P), Sox6-deficient (Sox6$^{-/-}$) (B, E, H, K, N, and Q) and double-deficient (Sox5$^{-/-}$,Sox6$^{-/-}$) (C,F,I,L,O,R) spinal cords are shown.

(S and T) The number of Sox10-positive cells (S) and Olig2-positive cells (T) was determined at 11.5 dpc in wild-type, single-deficient (Sox5$^{-/-}$ or Sox6$^{-/-}$) and double-deficient (Sox5$^{-/-}$,Sox6$^{-/-}$) spinal cords, as indicated below the bars. At least 30 separate 10 μm sections from the forelimb region of two independent embryos were counted for each genotype. Data are presented as mean ± standard deviation. Differences to the wild-type were statistically significant for the Sox10-positive cells in Sox6-deficient (Sox6$^{-/-}$) and double-deficient (Sox5$^{-/-}$, Sox6$^{-/-}$) spinal cords as determined by ANOVA and Bonferroni's post-hoc probability test (triple asterisk, $p \leq 0.001$).

(U) The number of Hb9-positive motoneurons at 11.5 dpc in wild-type and double-deficient (Sox5$^{-/-}$,Sox6$^{-/-}$) spinal cords was similarly determined.

and 4S). By contrast, in Sox5- or Sox6-deficient embryos Sox10 staining was already detected at 11.0 dpc in a few cells at the edge of the pMN domain (Figures 4B, 4E, and 4H and data not shown). Their number increased on average to nine to 12 cells per section by 11.5 dpc (Figures 4K, 4N, and 4Q), 2- to 2.5-fold the number found in the wild-type (Figure 4S). Thus, oligodendrocyte precursors appear both earlier and in increased number in mice deficient for either Sox5 or Sox6.

The effect became even more evident in compound mutants that lacked both SoxD proteins. At 11.0 dpc, there were more oligodendrocyte precursors than in the single gene mutants (compare Figure 4B to Figure 4C). At 11.5 dpc, we detected approximately four times as many oligodendrocyte precursors per section than in the wild-type and twice as many as in the single mutant (compare Figure 4L to Figures 4J and 4K). At the same time, no alterations were observed for other Sox proteins with VZ expression such as SoxB1 proteins, Sox21, and Sox9 (Figures S3A–S3F) or for Sox11 (Figures S3G and S3H). Proliferation rate and apoptosis were comparable between spinal cords from wild-type and mutants (Figures S3I and S3J) as was the total number of Olig2-positive cells in the pMN domain (Figure 4T) and the number of Hb9-positive motoneurons (Figure 4U). As Olig2 labels both multipotent progenitors and oligodendrocyte precursors, the additional oligodendrocyte precursors must have been generated at

the expense of multipotent VZ cells. Although the overall Ngn2 expression pattern remained unchanged in the Sox5/Sox6-deficient spinal cord, expression levels appeared reduced at 10.5 and 11.5 dpc (Figures S3K–S3N), possibly favoring oligodendrocyte specification in the pMN domain (Lee et al., 2005).

Distribution of Oligodendrocyte Precursors in the Spinal Cord Is Altered in the Absence of Sox5 and Sox6

After specification, oligodendrocyte development progressed fairly normal in both Sox5-deficient and Sox6-deficient spinal cords so that oligodendrocyte precursor numbers and distribution were comparable to wild-type at 15.5 and 18.5 dpc (data not shown). Oligodendrocyte precursor numbers were also similar in the Sox5/Sox6 double mutant at 15.5 dpc (Figure S4K), as were apoptosis and proliferation rates (Figures S5A and S5B). However, oligodendrocyte precursor distribution was significantly altered in the Sox5/Sox6-double-deficient spinal cord at 15.5 dpc with 72% of oligodendrocyte precursors being in the ventral half of the Sox5/Sox6-double-deficient spinal cord in proximity to the pMN domain compared to only 60% in the ventral half of the wild-type spinal cord (Figures S4C, S4D, and S4K). At the same time, we failed to detect gross abnormalities in developing radial glia (Figures S5C–S5H) and dorsal horn neurons (Figures S5I–S5P). Corroborating a cell-autonomous effect of SoxD proteins, oligodendrocyte precursor migration was also altered at 15.5 dpc in Sox5-deficient spinal cords in which *Sox6* alleles with integrated loxP sites (Dumitriu et al., 2006) had additionally been removed by a Sox10 Cre transgene (data not shown) which ensures oligodendrocyte-selective deletion (Kessaris et al., 2006; Matsuoka et al., 2005).

This difference in oligodendrocyte precursor migration was no longer detected at 18.5 dpc in Sox5-deficient embryos with additional CNS-specific (Figures S4G, S4H, and S4L) or oligodendrocyte-specific (Figures S4I, S4J, and S4L) deletion of *Sox6*. At this stage, conditional deletion of the *Sox6* allele had to be employed because embryos homozygous for both constitutive *Sox5* and *Sox6* null alleles fail to develop beyond 15.5 dpc (Smits et al., 2001).

Precocious Terminal Differentiation of Oligodendrocytes in the Absence of Sox5 and Sox6

Oligodendrocyte precursors start to undergo terminal differentiation at the end of embryogenesis as evidenced by upregulation of *PLP* and *MBP*. Accordingly, very few PLP- or MBP-positive cells were detected at 15.5 dpc in the wild-type spinal cord (Figures 5A and 5B) and many more at 18.5 dpc, predominantly in the prospective white-matter region (Figures 5E and 5F). $Sox5^{-/-},Sox6^{-/-}$ littermates exhibited a strong increase in both PLP- and MBP-positive cells at 15.5 dpc (Figures 5C and 5D). In the $Sox5^{-/-},Sox6^{\Delta/\Delta}$,Nestin Cre genotype, there were 8.5-fold as many PLP-expressing cells and 3.5-fold as many MBP-expressing cells at 18.5 dpc (Figures 5K, 5L, 5O, and 5P). Furthermore, PLP-expressing cells were detected at least a week in advance of their normal appearance in the future gray matter (Figures 5K and 5L). Although terminal oligodendrocyte differentiation could not be followed

postnatally because of the perinatal death of Sox5-deficient embryos (Smits et al., 2001), the precocious and increased myelin gene expression in double-deficient mice before birth indicates that Sox5 and Sox6 help to keep oligodendrocyte precursors in the undifferentiated state and prevent terminal differentiation.

Myelin gene expression was also analyzed at 18.5 dpc in embryos with constitutive Sox5 or Sox6 deficiency (Figures 5G, 5H, 5I, and 5J). Whereas myelin gene expression was very similar in wild-type and Sox5-deficient embryos (Figures 5G, 5H, 5O, and 5P), both PLP- and MBP-expressing cells were slightly, but reproducibly, increased by 40%–70% in Sox6-deficient embryos (Figures 5I, 5J, 5O, and 5P). $Sox5^{-/-},Sox6^{\Delta/\Delta}$, Sox10 Cre embryos on the other hand exhibited the same strong increases in PLP- and MBP-expressing cells as the $Sox5^{-/-},Sox6^{\Delta/\Delta}$,Nestin Cre embryos at 18.5 dpc (Figures 5M, 5N, 5O, and 5P), indicating that Sox5 and Sox6 function in a cell-autonomous manner during terminal oligodendrocyte differentiation.

Sox5 and Sox6 Inhibit the SoxE-Dependent Activation of Myelin Genes

Sox5 and Sox6 participate in the activation of Sox9 target genes in chondrocytes as evidenced for *collagen 2a1* where SoxD proteins bind to the same regulatory sequences as Sox9 (Lefebvre et al., 1998). Sox5 and Sox6 also bound to several Sox10 response elements from the promoters of the *Myelin Protein Zero (MPZ)* and *MBP* genes (Figure 6A and data not shown). These included response elements for two cooperatively binding Sox10 molecules (dimeric sites such as C/C′ in Figure 6A), as well as response elements for single Sox10 molecules (monomeric sites such as B in Figure 6A). Whereas the mobility of SoxE-containing complexes was higher on monomeric than on dimeric sites, SoxD-containing complexes exhibited mobilities that were lower than those of SoxE dimers and comparable for both types of response elements, indicating that SoxD proteins bound both sites as dimers. Binding of SoxD dimers to monomeric sites probably requires only one HMG domain to contact DNA (Figure 6B).

When SoxE and SoxD proteins were jointly incubated with a Sox10 response element, only SoxE or SoxD complexes were observed as paradigmatically shown for Sox5 and a shortened Sox10 on the dimeric C/C′ site and confirmed by antibody supershift experiments (Figure 6C). The failure to form heteromeric complexes on Sox10 response elements was also evident in titration experiments in which bound Sox5 protein was challenged with increasing amounts of Sox10 and vice versa (data not shown). Mapped response elements for Sox10 in myelin gene promoters thus did not support joint, but rather mutually exclusive, binding of SoxE and SoxD proteins.

When larger fragments from the MPZ and MBP promoters with multiple recognition sites were used in electrophoretic mobility shift assays (EMSA), a novel complex appeared in the presence of both Sox5 and Sox10 proteins that did not correspond to the SoxD or the SoxE complexes and was supershifted with antibodies directed against either protein (Figures 6D and 6E). The complex migrated with a mobility intermediate between the Sox10-containing and the Sox5-containing
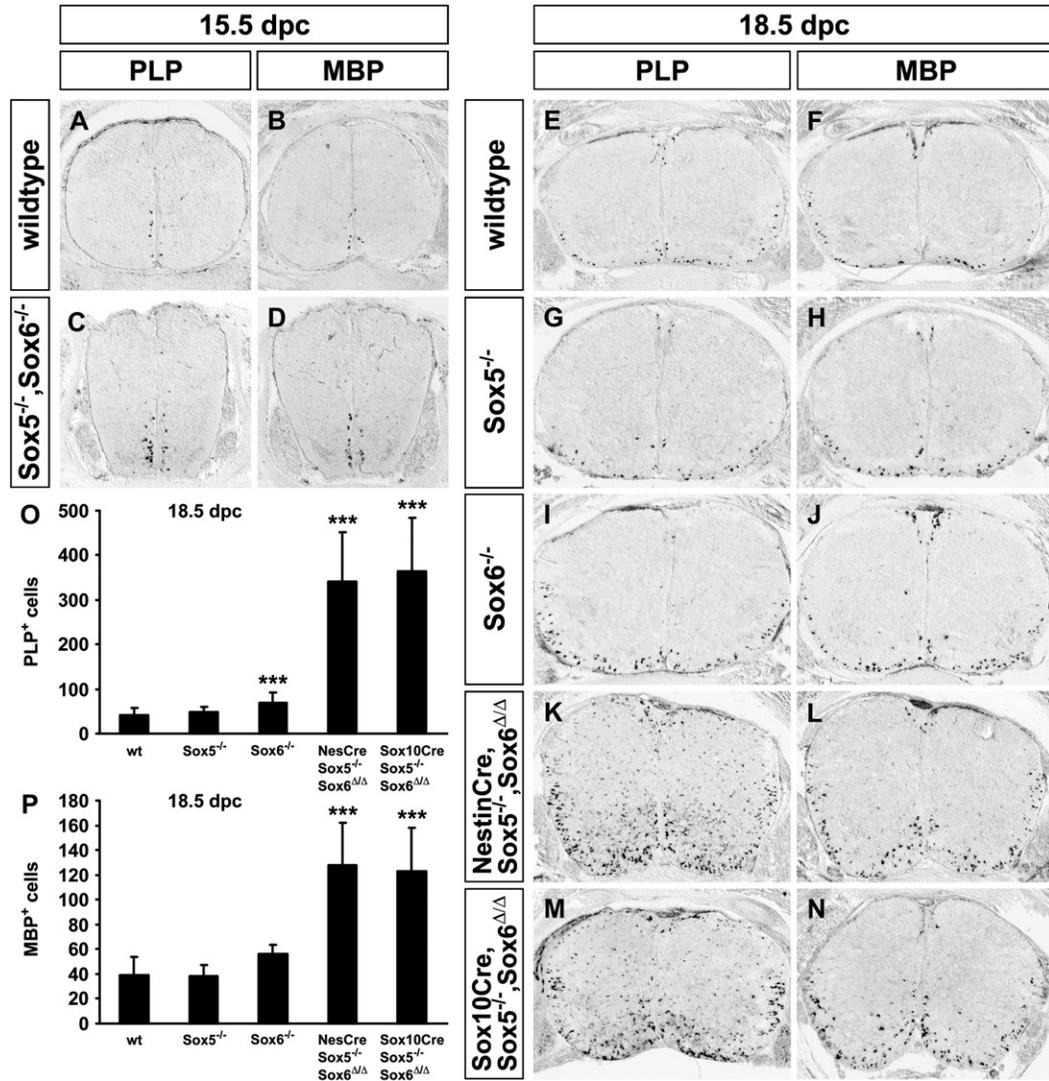
Figure 5. Terminal Differentiation of Oligodendrocytes in Mice Carrying *Sox5* and *Sox6* Mutant Alleles

(A–N) In situ hybridizations were performed on transverse spinal cord sections from the forelimb region of wild-type (A, B, E, and F) and mutant (C, D, and G–N) embryos at 15.5 dpc (A–D) and 18.5 dpc (E–N) with antisense riboprobes specific for PLP (A, C, E, G, I, K, and M) and MBP (B, D, F, H, J, L, and N). Mutant genotypes included: Sox5 deficient (Sox5$^{-/-}$) (G and H), Sox6 deficient (Sox6$^{-/-}$) (I and J), constitutive double deficient (Sox5$^{-/-}$,Sox6$^{-/-}$) (C and D), conditional double deficient (Sox5$^{-/-}$,Sox6$^{\Delta/\Delta}$,Nestin Cre in [K] and [L] and Sox5$^{-/-}$,Sox6$^{\Delta/\Delta}$,Sox10 Cre in [M] and [N]).

(O and P) The number of PLP-positive (O) and MBP-positive (P) cells was determined at 18.5 dpc in wild-type, Sox5-deficient (Sox5$^{-/-}$), Sox6-deficient (Sox6$^{-/-}$), and conditional double-deficient (Sox5$^{-/-}$,Sox6$^{\Delta/\Delta}$,Nestin Cre and Sox5$^{-/-}$,Sox6$^{\Delta/\Delta}$,Sox10 Cre) spinal cords as indicated below the bars. Data acquisition, quantification, and statistical analysis were as described in the legend to Figure 4. Differences to the wild-type were statistically significant in both conditional double-deficient genotypes for PLP and MBP and in the Sox6-deficient genotype for PLP (triple asterisk, p $\leq$ 0.001).

complexes arguing that roughly as many Sox protein molecules were present in this complex as in Sox5- or Sox10-containing complexes and that no additional Sox proteins were recruited into the complex by physical interactions between Sox5 and Sox10 molecules. Coimmunoprecipitation yielded only evidence for protein-protein interactions among SoxD proteins, but not between SoxE and SoxD proteins (Figure 6F). We conclude that SoxE and SoxD proteins also compete for binding sites in the context of myelin gene promoters, which therefore can be occupied by SoxD proteins, SoxE proteins, or both.

Chromatin immunoprecipitation experiments on 16.5 dpc old spinal cords detected both Sox6 and Sox10 on the MBP promoter, not however on an upstream fragment without known regulatory function (Figure 6G). As 16.5 dpc old spinal cords predominantly contain oligodendrocyte precursors, SoxD and SoxE proteins are both bound to the MBP promoter in oligodendrocyte precursors under in vivo conditions. In contrast, only antibodies against Sox10 retained the ability to precipitate the MBP promoter from chromatin isolated from postnatal spinal cord, where terminally differentiated oligodendrocytes are predominant. Chromatin was equally
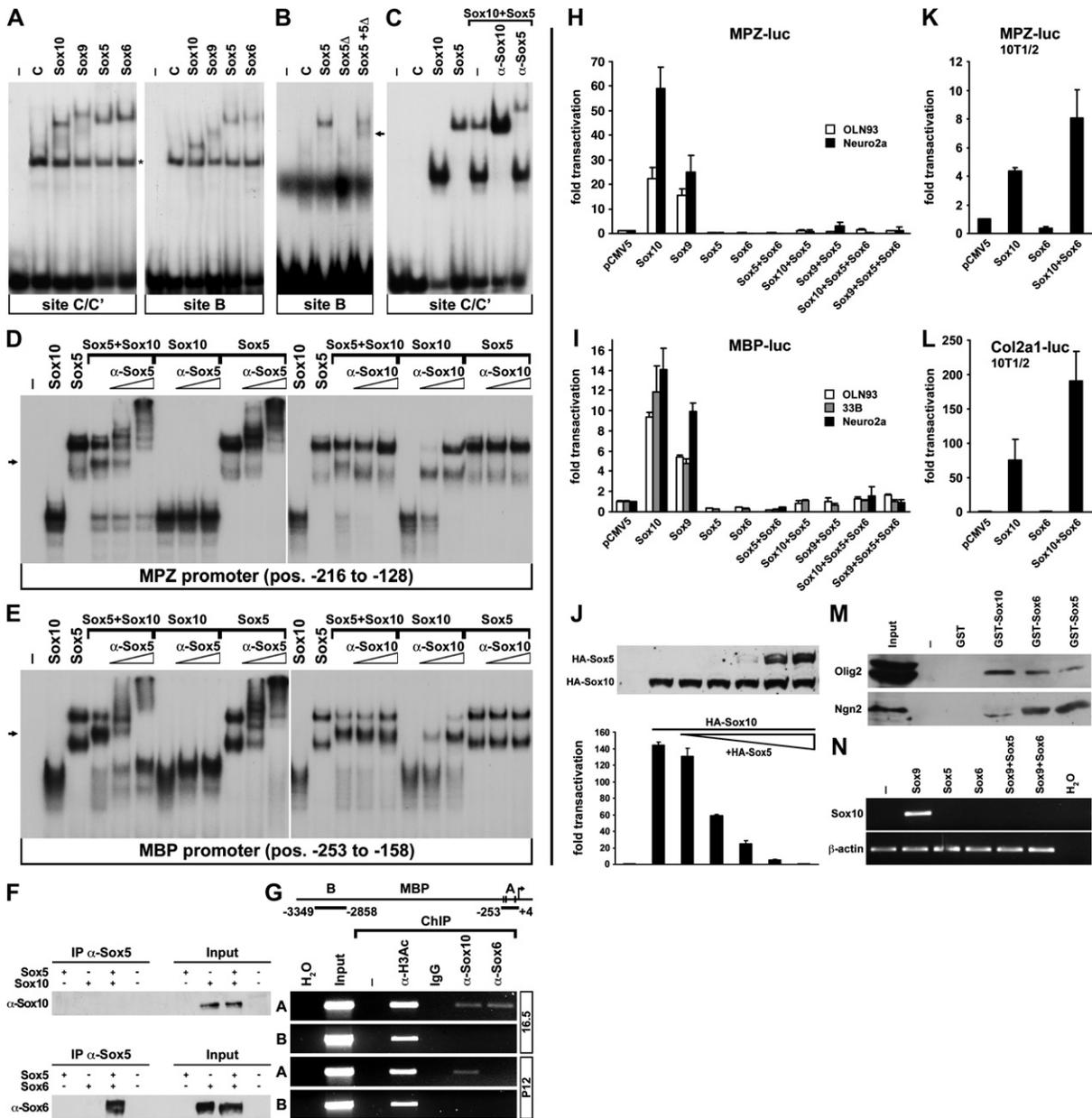
Figure 6. Functional Relationships between SoxD and SoxE Proteins In Vitro

(A) Binding of Sox5 and Sox6 to bona fide SoxE recognition elements was studied in EMSA with the prototypic dimeric Sox10-binding site C/C′ and the prototypic monomeric Sox10-binding site B from the MPZ promoter as probes (Peirano et al., 2000). Extracts from transfected Neuro2a cells served as source for full-length Sox10, Sox9, Sox5, or Sox6. Extract from mock-transfected Neuro2a cells (C) showed the unspecific nature of the complex with the highest mobility (marked by an asterisk). "-," no extract added.

(B) EMSA showed that a carboxyterminally truncated Sox5 without HMG domain (Sox5Δ) failed to bind to DNA when analyzed alone but bound to the site B probe as a heterodimer in the presence of full-length Sox5 (marked by arrow).

(C) EMSA with site C/C′ as probe failed to detect heteromeric complexes between SoxD and SoxE proteins. Extracts from transfected Neuro2a cells served as source for carboxyterminally truncated Sox10 (MIC variant) (Kuhlbrodt et al., 1998b), full-length Sox5, or a mixture thereof. Antibodies directed against Sox10 (α-Sox10) or Sox5 (α-Sox5) were added to some reactions during the incubation period as indicated.

(D and E) SoxD and SoxE proteins can jointly occupy myelin gene promoters as shown by EMSA with MPZ promoter fragments (positions −216 to −128) (D) and MBP promoter fragments (positions −253 to −158) (E). Extracts from transfected Neuro2a cells served as source for Sox5 and shortened Sox10 (MIC variant) or a mixture thereof. Antibodies directed against Sox10 (α-Sox10) or Sox5 (α-Sox5) were added to some reactions during the incubation period as indicated. The complex simultaneously containing Sox5 and Sox10 is marked by an arrow.

(F) Coimmunoprecipitation of Sox10 (upper panel) and Sox6 (lower panel) from extracts of transiently transfected HEK293 cells was carried out with Sox5-specific antibodies in the presence or absence of Sox5. Precipitated proteins were detected by western blot with a Sox10-specific and a Sox6-specific antiserum, respectively. The presence of Sox5, Sox6, and Sox10 in the extract is indicated above each lane.

(G) Chromatin immunoprecipitation was performed on formaldehyde-fixed spinal cords from 16.5 dpc old embryos and 12 day old mice in the absence (−) and presence of antibodies. In addition to guinea pig IgGs (IgG), antisera specifically directed against acetylated histone H3 (α-H3Ac), Sox10 (α-Sox10), and Sox6 (α-Sox6) were employed. PCR was applied on the immunoprecipitate to detect the MBP promoter region

accessible at both times as evident from immunoprecip-itations with anti-acetylated histone H3 antibodies (Figure 6G).

Despite their ability to bind, transiently transfected Sox5 and Sox6 failed to activate transcription from the MPZ or the MBP promoter in the immortalized oligoden-droglial cell line OLN93 (Richter-Landsberg and Hein-rich, 1996), in 33B oligodendroglioma, and in Neuro2a cells when tested alone or in combination (Figures 6H and 6I). This agreed with the reported lack of trans-activation domains in SoxD proteins (Lefebvre et al., 1998). SoxD proteins even lowered basal levels of reporter gene expression, arguing for a repressive function. Importantly, SoxE-dependent transcriptional activation of both MPZ and MBP promoters was effec-tively suppressed by SoxD proteins with equimolar amounts of expression plasmids (Figures 6H and 6I). Even substoichiometric Sox5 amounts were sufficient to suppress Sox10-dependent gene activation (Fig-ure 6J), indicating that repression probably did not require complete Sox10 removal from the target-gene promoter. The inhibition of SoxE-dependent gene acti-vation by SoxD proteins was specific to cell lines of oligodendrocytic or neural origin as the same myelin gene promoters were jointly activated by SoxE and SoxD proteins in mesodermal 10T1/2 cells (as shown for the MPZ promoter) (Figure 6K) similar to the colla-gen2a1 enhancer (Figure 6L) (Lefebvre et al., 1998) and despite the fact that SoxD proteins alone lowered basal expression levels in 10T1/2 cells as much as in neural cell lines (Figure 6K). Whereas SoxD proteins by them-selves thus functioned as repressors, they either stimu-lated or repressed target-gene promoters in a cell-type-specific manner when combined with SoxE proteins.

SoxD proteins may additionally compete with SoxE proteins for interaction partners. In GST pull-down as-says, Sox5 and Sox6 interacted with similar transcrip-tion factors as Sox10 (Figure 6M). Immediately after specification, competitive interaction with Ngn2 may be important, whereas interaction with Olig2 may be rel-evant throughout oligodendrocyte development.

Given the fact that both Sox5 and Sox6 were able to efficiently suppress the Sox9-dependent activation of endogenous *Sox10* gene expression in transfected Neu-ro2a cells (Stolt et al., 2003), it is conceivable that Sox5

and Sox6 also function during oligodendrocyte specifi-cation by interfering with Sox9 activity (Figure 6N). Mechanisms remain to be established.

Sox6 Function in Terminally Differentiating Oligodendrocytes Depends on Sox10

The functional relationship between SoxD and SoxE proteins during terminal oligodendrocyte differentiation was also analyzed in Sox6/Sox10-double-deficient mice after ensuring that cell numbers for the oligodendrocyte lineage were still normal in Sox6/Sox10-double-defi-cient spinal cords at 18.5 dpc (Figures 7D and 7F) and that deletion of *Sox10* did not interfere with Sox6 ex-pression (Figures 7A and 7B). If both Sox proteins exert independent effects, defective oligodendrocyte differ-entiation in Sox10-deficient mice may be counteracted by the precocious differentiation in Sox6-deficient mice, leading to at least partial phenotypic rescue in the double-deficient mice. The terminal differentiation defect in the Sox6/Sox10-double-deficient spinal cord was, however, as severe as in the Sox10-deficient spinal cord (compare Figures 7I and 7L to Figures 7H and 7K). There were no PLP- or MBP-expressing cells in either genotype, supporting the assumption that SoxD pro-teins exert their effect on terminal oligodendrocyte dif-ferentiation through interference with Sox10 function.

Discussion

Many Sox proteins are expressed in the developing CNS. However, functions have only been determined for a few (Wegner and Stolt, 2005). SoxB and SoxE pro-teins are, for instance, present in defined cell popula-tions and associated with specific functions including stem cell maintenance, cell-type specification, or termi-nal differentiation events.

In this study, Sox5 and Sox6 were found in many dif-ferent cell types, including VZ progenitors, radial glia, ol-igodendrocyte precursors, and several neuronal sub-populations. Whereas Sox5 and Sox6 were mostly coexpressed in VZ and glial lineages, there were signif-icant differences in their neuronal expression sites as exemplified by the sole presence of Sox5 in developing glutamatergic dorsal-horn neurons.

(A, positions −253 to +4) as well as an upstream region (B, positions −3349 to −2858). These fragments were also amplified from 1/20 of the material used for immunoprecipitation (input). H_2O, water control.

(H and I) SoxD proteins counteract the SoxE-dependent activation of the MPZ (H) and the MBP (I) promoters in transiently transfected OLN93 (white bars), 33B (gray bars), and Neuro2a (black bars) cells as evident from luciferase reporter gene assays. Expression plasmids for Sox10, Sox9, Sox5, Sox6, and various combinations thereof were cotransfected as indicated below the bars. Amounts of expression plasmids were identical for each Sox protein.

(J) To determine the amount of SoxD protein needed to suppress SoxE-dependent MPZ activation in Neuro2a cells, an expression plasmid for HA-tagged Sox5 was transfected in increasing amounts together with an expression plasmid for HA-tagged Sox10 and the MPZ luciferase re-porter. Luciferase assays were performed, and the amounts of HA-tagged Sox5 and HA-tagged Sox10 present in each reaction were simulta-neously detected by western blot as shown above the bars.

(K and L) SoxD proteins increase the SoxE-dependent activation of MPZ promoter (K) and Col2a1 enhancer (L) in transiently transfected 10T1/2 fibroblasts as evident from luciferase reporter gene assays. Expression plasmids for Sox10, Sox6, and their combination were cotransfected as indicated below the bars. Equimolar amounts of expression plasmids were used for each Sox protein. Luciferase activities in extracts from trans-fected cells were determined for (H)–(L) in three independent experiments each performed in duplicates. Data are presented as fold inductions ± standard deviation with the activity of the promoter in the absence of cotransfected Sox protein (−) arbitrarily set to 1.

(M) T7-tagged Ngn2 DNA-binding domain and myc-tagged Olig2 were expressed in transiently transfected HEK293 cells and analyzed for their ability to interact with GST or GST fusions containing HMG domain portions from Sox10, Sox6, and Sox5, incapable of DNA binding. Transcrip-tion factors were detected on western blots via their tags before (input) and after pull-down.

(N) RT-PCR analyses on cDNA obtained from Neuro2A cells transiently transfected with expression plasmids for GFP (−), Sox9, Sox5, Sox6, and various combinations. Transcript levels of Sox10 and β-actin were determined with amplification still being in the linear range. H_2O, water control.
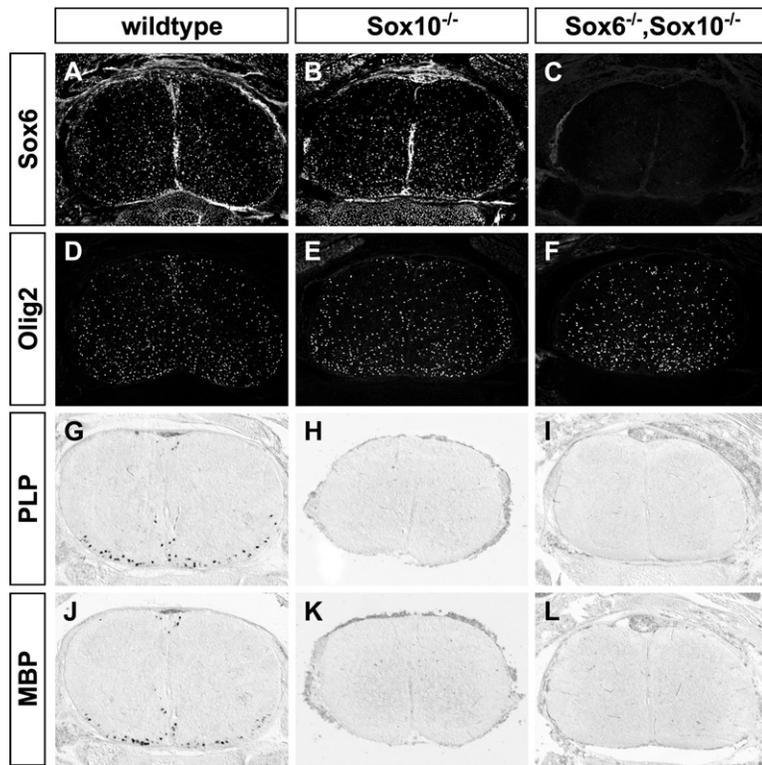
Figure 7. Functional Relationships between SoxD and SoxE Proteins In Vivo

Immunohistochemistry with antibodies against Sox6 (A–C) and Olig2 (D–F) and in situ hybridization with PLP-specific (G–I) and MBP-specific (J–L) riboprobes on transverse sections from the forelimb region of 18.5 dpc old wild-type (A, D, G, and J), $Sox10^{-/-}$ (B, E, H, and K), and $Sox10^{-/-},Sox6^{-/-}$ (C, F, I, and L) embryos.

In VZ and glial lineages, Sox5 and Sox6 overlapped strongly with Sox8 and Sox9 (Stolt et al., 2003, 2005). Despite preceeding Sox9 expression, induction of SoxD gene expression in the spinal cord is not under control of SoxE proteins. This contrasts with the situation in chondrocytes where both *Sox5* and *Sox6* gene induction requires the presence of Sox9 (Akiyama et al., 2002) and in oligodendrocyte precursors where maintenance of *Sox6* expression required the continued presence of Sox8 and Sox9.

In the combined absence of Sox5 and Sox6, specification of oligodendrocyte precursors from pluripotent progenitors of the pMN domain occurred earlier than in the wild-type by at least half a day, arguing that Sox5 and Sox6 act as functionally equivalent repressors. The opposite effect was observed for SoxE proteins, where oligodendrocyte specification is strongly diminished in the absence of Sox9 and lost in the combined absence of Sox9 and Sox8 (Stolt et al., 2003, 2005). It may thus be concluded that SoxD proteins keep the gliogenic activity of SoxE proteins in pluripotent progenitors of the pMN domain in check. This antagonism between SoxE and SoxD proteins is reminiscent of the relationship between SoxB1 and SoxB2 proteins during spinal cord neurogenesis where SoxB1 proteins prevent neurogenesis of VZ cells, while the SoxB2 protein Sox21 promotes neurogenesis by interfering with SoxB1 function (Bylund et al., 2003; Sandberg et al., 2005). SoxD proteins might similarly interfere with SoxE function, for instance by preventing Sox9 from activating Sox10 expression as an important step in oligodendrocyte specification. As both SoxE and SoxD proteins are more widely distributed throughout the VZ than just in the pMN domain, timely specification of cell populations other than oligodendrocytes may also depend on the antagonistic activities of these two groups of Sox proteins.

The sum of pluripotent pMN progenitors and oligodendrocyte precursors remained constant in the Sox5/Sox6-double-deficient spinal cord. Increased oligodendrogenesis therefore is at the expense of pluripotent pMN progenitors. Nevertheless, we do not observe any obvious consequences corresponding to a premature depletion of the pMN progenitor pool. From 11.5 dpc onward, oligodendrocyte precursors are the main cells arising from the pMN domain. As oligodendrocyte precursors are also highly proliferative cells, a shift in the cellular composition of the pMN domain may not affect the rate at which oligodendrocytes are generated. This is supported by the unaltered proliferation rates in the ventral VZ of Sox5/Sox6-double-mutant spinal cords.

Interestingly, Sox5 and Sox6 also influence the pattern in which oligodendrocyte precursors colonize the spinal cord parenchyma after their specification, likely in a cell-autonomous manner. The effect is transient as the Sox5/Sox6-double-deficient spinal cord shows again normal distribution of oligodendrocyte precursors at the time of birth.

Similar to Sox9 (Stolt et al., 2003), both Sox5 and Sox6 are normally extinguished in the oligodendrocyte lineage when cells start to undergo terminal differentiation. Sox9 extinction in differentiating oligodendrocytes may even be directly responsible for Sox6 downregulation. In the absence of Sox5 and Sox6, oligodendrocytes start to differentiate prematurely and at higher numbers.

This effect was even observed when Sox6 was selectively deleted in the oligodendrocyte lineage arguing for a cell-autonomous function of SoxD proteins in oligodendrocyte differentiation.

The premature differentiation of Sox5/Sox6-deficient oligodendrocyte precursors is the exact opposite of the failure of Sox10-deficient oligodendrocyte precursors to undergo terminal differentiation (Stolt et al., 2002). Considering that the terminal differentiation defect was equally severe in Sox6/Sox10-double-deficient and Sox10-deficient spinal cords, SoxD proteins are likely to directly counteract Sox10 function. Evidence suggests that SoxD proteins may accomplish this by competing with Sox10 for common interaction partners and for previously identified Sox10 response elements in promoters of myelin genes. Myelin gene promoters may not only be repressed when bound exclusively by SoxD proteins. Rather, promoter co-occupancy by SoxD and SoxE proteins appears sufficient to interfere with SoxE-dependent activation. This may involve cell-type-specific recruitment of corepressors by SoxD proteins as Sox6 has previously been shown to direct the CtBP corepressor to the Fgf3 promoter (Murakami et al., 2001). The antagonistic role of SoxD and SoxE proteins on myelin gene expression also helps to explain the significant gap in the onset of expression of Sox10 and its myelin target genes in oligodendrocytes. It is tempting to assume that SoxD proteins during the precursor stage not only repress the function of Sox10 and other SoxE proteins on myelin genes but additionally support SoxE proteins in the activation of a different set of target genes.

There are obvious similarities in the relationship of SoxD and SoxE proteins in oligodendrocytes and in chondrocytes. Sox9, which is the major SoxE protein expressed in chondrocytes (Lefebvre et al., 1997), is expressed earlier than Sox5 and Sox6 (Lefebvre et al., 1998). Loss of Sox9 furthermore has a more dramatic effect on chondrocyte development than loss of Sox5 and Sox6 (Akiyama et al., 2002; Smits et al., 2001). As in oligodendrocytes, Sox5 and Sox6 appear to function largely redundantly and also influence several consecutive steps of chondrocyte development (Lefebvre, 2002; Smits et al., 2001). Early differentiation of chondrocytes and expression of tissue-specific extracellular matrix genes is similarly influenced by Sox9 and the two SoxD genes so that Sox9 function may be mediated or supported by Sox5 and Sox6 as paradigmatically shown for the regulation of collagen 2a1 gene expression (Lefebvre et al., 2001). However, during terminal hypertrophic maturation, Sox9 and SoxD proteins have opposing roles (Akiyama et al., 2002; Smits et al., 2001).

A third cell type in which SoxE and SoxD proteins are coexpressed and influence the same processes are neural crest cells (Perez-Alcala et al., 2004). Modulation of SoxE protein activity by SoxD proteins may thus be a more general theme. This does in no way exclude that SoxD proteins have additional functions independent of SoxE proteins. Neuronal functions in the CNS should, for instance, be independent of SoxE proteins as SoxE proteins are largely restricted to glia (Wegner and Stolt, 2005).

Crossregulation between SoxD and SoxE proteins might also be relevant under pathological situations.

Sox10 has, for instance, been found in many brain tumors, including oligodendrogliomas (Bannykh et al., 2006), which are believed to be of oligodendrocytic origin but express very low amounts of myelin genes. On the basis of our results, it may be postulated that SoxD proteins are required in these brain tumors to counterbalance Sox10 by keeping the cells in an undifferentiated state and preventing myelin gene expression. Many gliomas indeed contain Sox6 (Ueda et al., 2004a, 2004b).

Experimental Procedures

Animal Husbandry, Genotyping, and BrdU Labeling

Mice were bred with various combinations of the following genetically altered Sox alleles: Sox5⁻ (Smits et al., 2001), Sox6⁻ (Smits et al., 2001), Sox6^{loxP} (Dumitriu et al., 2006), Sox8^{lacZ} (Sock et al., 2001), Sox9^{loxP} (Akiyama et al., 2002), and Sox10^{lacZ} (Britsch et al., 2001). For tissue-specific deletion of Sox genes with loxP recognition sites, Cre transgenes under control of nestin regulatory sequences (Nestin Cre) (Tronche et al., 1999) or Sox10 regulatory sequences (Sox10 Cre) (Kessaris et al., 2006; Matsuoka et al., 2005) were additionally crossed into male mice. Genotyping was performed by PCR (Britsch et al., 2001; Smits et al., 2001; Sock et al., 2001; Stolt et al., 2003). For BrdU labeling, pregnant mice were injected intraperitoneally with 100 μg BrdU (Sigma) per gram body weight 1 hr before embryo preparation (Stolt et al., 2003).

Tissue Preparation, Immunohistochemistry, and In Situ Hybridization

Embryos were isolated at 10.5 dpc to 18.5 dpc from staged pregnancies. After fixation in 4% paraformaldehyde, specimens were cryoprotected by overnight incubation at 4°C in 30% sucrose, embedded in OCT compound at −80°C, and sectioned on a Leica cryotome (Bensheim, Germany) at 10 μm or at 20 μm thickness (Stolt et al., 2003, 2004).

Immunohistochemistry was performed on 10 μm thick sections. Sources and working dilutions of primary antibodies are listed in the Supplemental Data. Guinea pig antisera against Sox5 and Sox6 were generated against purified bacterially expressed proteins consisting of amino acids 329–373 of mouse Sox5 (Accession number: AJ010604) fused to amino acids 416–462 or consisting of amino acids 445–497 of mouse Sox6 (Accession number: NM_011445) fused to amino acids 544–610. The anti-Sox5 antiserum failed to yield immunohistochemical staining of sections from Sox5-deficient embryos and recognized Sox5, but not Sox6, in western blots. The anti-Sox6 antiserum behaved analogously indicating that these antibodies were highly specific (Figure S6). Secondary antibodies conjugated to Cy2 and Cy3 immunofluorescent dyes (Dianova) were used for detection. TUNEL assays were performed according to the manufacturer's protocol (Chemicon). Immunofluorescence was detected and documented with a Leica inverted microscope (DMIRB) equipped with a cooled SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI).

In situ hybridizations were performed on 20 μm thick sections with DIG-labeled antisense riboprobes for Ngn2 (gift of Lukas Sommer, ETH, Zurich), MBP, and PLP (Stolt et al., 2002). All steps except probe hybridization and final colorimetric detection were performed automatically on a Biolane HTI (Hölle & Hüttner AG, Tübingen, Germany). In situ hybridizations were analyzed and documented with a Leica MZFLIII stereomicroscope equipped with an Axiocam (Zeiss, Oberkochem, Germany).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed as described (Schlierf et al., 2006). Briefly, cellular protein and genomic DNA from freshly prepared spinal cords of 16.5 dpc old embryos and 12 day old pups were cross-linked in 1% formaldehyde before chromatin extraction and sonication to an average fragment length of 300 to 600 bp. Immunoprecipitations were performed overnight at 4°C with polyclonal IgG against acetylated histone H3 from rabbit (Upstate #06-599) and against Sox10 or Sox6 from guinea pig (see

Supplemental Data). DNA was purified from precipitates after cross-link reversal and subjected to PCR. For detection of the MBP promoter (positions −253 to +4), 5′-CCTCGTACAGGCCCACATTC-3′ and 5′-TGAAGCTCGTCGGACTCTGA-3′ were used as primers in 33 cycles of standard PCR with an annealing temperature of 60°C. Primers 5′-GCCACACTTGGAAGGTACAG-3′ and 5′-CAAGGTGCAG TCACCTAGGA-3′ were employed to detect a distal fragment from the *MBP* upstream region (positions −3349 to −2858).

Cell Culture, RT-PCR, and Luciferase Assays
HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and transfected by the calcium-phosphate technique. Neuro2A, OLN93, and 33B cells were maintained in DMEM containing 5% FCS and transfected with Superfect reagent (Qiagen). Stable Neuro2A cell clones capable of doxycycline-dependent, inducible Sox8 or Sox9 expression were generated as described for Sox10 (Peirano et al., 2000). RNA was prepared from transiently and stably transfected Neuro2A cells, from the latter in both the induced and uninduced state. After reverse transcription to cDNA, semiquantitative PCR was performed to detect products specific for Sox6, Sox8, Sox9, Sox10, β-actin, or hypoxanthine phosphoribosyl-transferase (HPRT).

For luciferase assays, Neuro2A cells were transfected transiently in duplicates in 24-well plates with 200 ng of luciferase reporter plasmid and 200 ng of effector plasmids per well if not stated otherwise. Luciferase assays in 10T1/2 cells were carried out similarly in the presence of 10% FCS. OLN93 and 33B cells were transiently transfected in 35 mm plates with 0.5 μg of luciferase reporter and 2 μg of effector plasmid. Luciferase reporters containing the myelin protein zero (MPZ) promoter (positions −435 to +48), the MBP promoter (positions −656 to +31), or four copies of the 48 bp collagen2a1 enhancer in front of the gene's minimal promoter were used (Lefebvre et al., 1997; Peirano et al., 2000; Stolt et al., 2002). Effector plasmids corresponded to pCMV5-based expression plasmids for Sox5, Sox6, Sox9, Sox10, and HA-tagged versions of Sox5 and Sox10 (Kuhlbrodt et al., 1998a; Peirano et al., 2000; Sock et al., 2003). Cells were harvested 48 hr posttransfection, and extracts assayed for luciferase activity (Stolt et al., 2002).

EMSA
Extracts from transfected Neuro2A cells (10 cm dishes) ectopically expressing full-length Sox5, Sox6, Sox9, Sox10, a truncated Sox5 without HMG domain, or the carboxyterminally shortened MIC version of Sox10 were prepared as described (Kuhlbrodt et al., 1998b). With these extracts, EMSA were performed in the presence of poly(dGdC) as unspecific competitor with ^{32}P-labeled fragments from MBP (positions −253 to −158) and MPZ (positions −216 to −128) promoter and oligonucleotides containing the prototypic dimeric binding site C/C′ (5′-TACACAAAGCCCTCTGTGTAAG-3′, position −206 to −185) or the monomeric binding site B (5′-CCAG CGTATACAATGCCCCTT-3′, position −155 to −134) from the MPZ promoter (Peirano et al., 2000). For super-shift experiments, Sox10- or Sox5-specific antisera were additionally added.

GST Pull-Downs, Coimmunoprecipitations, and Western Blots
For GST pull-downs, GST, or GST fusion proteins with amino acids 133–203 of Sox10 (Wißmüller et al., 2006), amino acids 648–717 of Sox6 (accession number NM_011445), and amino acids 499–568 of Sox5 (accession number AJ010604) were produced in *E. coli* strain BL21 DE3 pLysS and bound in the presence of DNaseI to glutathione Sepharose 4B beads. An aliquot of the washed and equilibrated beads, now carrying GST or the GST fusion protein, was incubated with extract from HEK293 cells transiently transfected with myc-tagged Olig2 or the T7-epitope tagged DNA-binding domain of Ngn2 in the presence of DNaseI for 2 hr at 4°C, spun down, and extensively washed (Wißmüller et al., 2006).

For coimmunoprecipitation, extracts of HEK293 cells transfected with Sox5, Sox6, and Sox10 in various combinations were incubated in the presence of DNaseI at 4°C with Sox5-specific antibodies for 1 hr, before addition of protein A-Sepharose beads (Amersham Biosciences) and 2 hr further incubation. Beads were separated from the extract and washed extensively.

Bead-bound proteins from GST pull-downs and coimmunoprecipitations were subjected to SDS-PAGE. Western blotting was performed with specific antisera against Sox6, Sox10 (1:3,000 dilution), the myc tag (1:200 dilution, 9E10 hybridoma supernatant), the HA tag (1:200 dilution, 12CA5 hybridoma supernatant), or the T7 epitope tag (1:10,000 dilution, Novagen) as primary antibodies and horseradish-peroxidase-coupled secondary antibodies with enhanced chemiluminescence.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures and six figures and are available at http://www.developmentalcell. com/cgi/content/full/11/5/697/DC1/.

References

Akiyama, H., Chaboissier, M.-C., Martin, J.F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. *16*, 2813–2828.

Bannykh, S.I., Stolt, C.C., Kim, J., Perry, A., and Wegner, M. (2006). Oligodendroglial-specific transcriptional factor SOX10 is ubiquitously expressed in human gliomas. J. Neurooncol. *76*, 115–127.

Bouchard, M., Pfeffer, P., and Busslinger, M. (2000). Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. Development *127*, 3703–3713.

Britsch, S., Goerich, D.E., Riethmacher, D., Peirano, R.I., Rossner, M., Nave, K.A., Birchmeier, C., and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. *15*, 66–78.

Bylund, M., Andersson, E., Novitch, B.G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci. *6*, 1162–1168.

Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., Li, Q., Sander, M., and Qiu, M. (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron *45*, 41–53.

Cheng, L., Samad, O.A., Xu, Y., Mizuguchi, R., Luo, P., Shirasawa, S., Goulding, M., and Ma, Q. (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat. Neurosci. *8*, 1510–1515.

Coppola, E., Pattyn, A., Guthrie, S.C., Goridis, C., and Studer, M. (2005). Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. EMBO J. *24*, 4392–4403.

Dumitriu, B., Dy, P., Smits, P., and Lefebvre, V. (2006). Generation of mice harboring a Sox6 conditional null allele. Genesis *44*, 219–224.

Fogarty, M., Richardson, W.D., and Kessaris, N. (2005). A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. Development *132*, 1951–1959.

Gross, M.K., Dottori, M., and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron *34*, 535–549.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., and Richardson, W.D. (2006). Competition waves of oligodendrocytes in the forebrain and postnatal elimination of an early embryonic lineage. Nat. Neurosci. *9*, 173–179.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I., and Wegner, M. (1998a). Sox10, a novel transcriptional modulator in glial cells. J. Neurosci. *18*, 237–250.

Kuhlbrodt, K., Schmidt, C., Sock, E., Pingault, V., Bondurand, N., Goossens, M., and Wegner, M. (1998b). Functional analysis of Sox10 mutations found in human Waardenburg-Hirschsprung patients. J. Biol. Chem. *273*, 23033–23038.

Lee, S.-K., Lee, B., Ruiz, E.C., and Pfaff, S.L. (2005). Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev. *19*, 282–294.

Lefebvre, V. (2002). Toward understanding the functions of the two highly related Sox5 and Sox6 genes. J. Bone Miner. Metab. *20*, 121–130.

Lefebvre, V., Huang, W., Harley, V.R., Goodfellow, P.N., and DeCrombrugghe, B. (1997). Sox9 is a potent activator of the chondrocyte-specific enhancer of the proα1(II) collagen gene. Mol. Cell. Biol. *17*, 2336–2346.

Lefebvre, V., Li, P., and de Crombrugghe, B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J. *17*, 5718–5733.

Lefebvre, V., Behringer, R.R., and de Crombrugghe, B. (2001). L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage *9*, S69–S75.

Matsuoka, T., Ahlberg, P.E., Kessaris, N., Iannarelli, P., Dennehy, U., Richardson, W.D., McMahon, A.P., and Koentges, G. (2005). Neural crest origins of the neck and shoulder. Nature *436*, 347–355.

McEvilly, R.J., de Diaz, M.O., Schonemann, M.D., Hooshmand, F., and Rosenfeld, M.G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. Science *295*, 1528–1532.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron *34*, 551–562.

Murakami, A., Ishida, S., Thurlow, J., Revest, J.M., and Dickson, C. (2001). SOX6 binds CtBP2 to repress transcription from the Fgf-3 promoter. Nucleic Acids Res. *29*, 3347–3355.

Noble, M., Wren, D., and Wolswijk, G. (1992). The O-2A(adult) progenitor cell: a glial stem cell of the adult central nervous system. Semin. Cell Biol. *3*, 413–422.

Peirano, R.I., Goerich, D.E., Riethmacher, D., and Wegner, M. (2000). Protein zero expression is regulated by the glial transcription factor Sox10. Mol. Cell. Biol. *20*, 3198–3209.

Perez-Alcala, S., Nieto, M.A., and Barbas, J.A. (2004). LSox5 regulates RhoB expression in the neural tube and promotes generation of the neural crest. Development *131*, 4455–4465.

Richardson, W.D., Kessaris, N., and Pringle, N. (2006). Oligodendrocyte wars. Nat. Rev. Neurosci. *7*, 11–18.

Richter-Landsberg, C., and Heinrich, M. (1996). OLN-93: a new permanent oligodendroglia cell line derived from primary rat brain glial cultures. J. Neurosci. Res. *45*, 161–173.

Sandberg, M., Källstrom, M., and Muhr, J. (2005). Sox21 promotes the progression of vertebrate neurogenesis. Nat. Neurosci. *8*, 995–1001.

Schlierf, B., Werner, T., Glaser, G., and Wegner, M. (2006). Expression of Connexin47 in oligodendrocytes is regulated by the Sox10 transcription factor. J. Mol. Biol. *361*, 11–21.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J.M., Behringer, R.R., de Crombrugghe, B., and Lefebvre, V. (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev. Cell *1*, 277–290.

Sock, E., Schmidt, K., Hermanns-Borgmeyer, I., Bösl, M.R., and Wegner, M. (2001). Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. Mol. Cell. Biol. *21*, 6951–6959.

Sock, E., Pagon, R.A., Keymolen, K., Lissens, W., Wegner, M., and Scherer, G. (2003). Loss of DNA-dependent dimerization of the transcription factor SOX9 as a cause for campomelic dysplasia. Hum. Mol. Genet. *12*, 1439–1447.

Stolt, C.C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., Bartsch, U., and Wegner, M. (2002). Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. Genes Dev. *16*, 165–170.

Stolt, C.C., Lommes, P., Sock, E., Chaboissier, M.-C., Schedl, A., and Wegner, M. (2003). The Sox9 transcription factor determines glial fate choice in the developing spinal cord. Genes Dev. *17*, 1677–1689.

Stolt, C.C., Lommes, P., Friedrich, R.P., and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development *131*, 2349–2358.

Stolt, C.C., Schmitt, S., Lommes, P., Sock, E., and Wegner, M. (2005). Impact of transcription factor Sox8 on oligodendrocyte specification in the mouse embryonic spinal cord. Dev. Biol. *281*, 323–331.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. *23*, 99–103.

Ueda, R., Iizuka, Y., Yoshida, K., Kawase, T., Kawakami, Y., and Toda, M. (2004a). Identification of a human glioma antigen, SOX6, recognized by patients' sera. Oncogene *19*, 1420–1427.

Ueda, R., Yoshida, K., Kawakami, Y., Kawase, T., and Toda, M. (2004b). Immunohistochemical analysis of SOX6 expression in human brain tumors. Brain Tumor Pathol. *21*, 117–120.

Vallstedt, A., Klos, J.M., and Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron *45*, 55–67.

Wegner, M., and Stolt, C.C. (2005). From stem cells to neurons and glia: a soxist's view of neural development. Trends Neurosci. *28*, 583–588.

Wißmüller, S., Kosian, T., Wolf, M., Finzsch, M., and Wegner, M. (2006). The high-mobility-group domain of Sox proteins interacts with the DNA-binding domains of many transcription factors. Nucleic Acids Res. *34*, 1735–1744.