

A Genome-Wide Screen for Spatially Restricted Expression Patterns Identifies Transcription Factors That Regulate Glial Development

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Forward genetic screens in genetically accessible invertebrate organisms such as *Drosophila melanogaster* have shed light on transcription factors that specify formation of neurons in the vertebrate CNS. However, invertebrate models have, to date, been uninformative with respect to genes that specify formation of the vertebrate glial lineages. All recent insights into specification of vertebrate glia have come via monitoring the spatial and temporal expression patterns of individual transcription factors during development. In studies described here, we have taken this approach to the genome scale with an *in silico* screen of the Mahoney pictorial atlas of transcription factor expression in the developing CNS. From the population of 1445 known or probable transcription factors encoded in the mouse genome, we identify 12 novel transcription factors that are expressed in glial lineage progenitor cells. Entry-level screens for biological function establish one of these transcription factors, *Klf15*, as sufficient for genesis of precocious GFAP-positive astrocytes in spinal cord explants. Another transcription factor, *Tcf4*, plays an important role in maturation of oligodendrocyte progenitors.

Introduction

The spatial and temporal expression patterns of transcription factors during development shed light on the specification of vertebrate glia. For example, the basic helix–loop–helix (bHLH) transcription factors *Olig1* and *Olig2*, which promote formation of oligodendrocytes, were identified via their initial expression in the pMN (motor neuron progenitor) domain of the embryonic spinal cord (Lu et al., 2000; Zhou et al., 2000), which gives rise to spinal cord oligodendrocytes (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). *Olig2* knock-out mice fail to develop oligodendrocytes and motor neurons in the spinal cord, whereas *Olig1* knock-out mice show slight delays in oligodendrocyte maturation as well as impaired remyelination (Arnett et al., 2004; Xin et al., 2005). In a similar vein, gliogenic functions of the *Scl* and *Nfia* transcription factors were initially suggested by their develop-

mentally regulated expression in neural progenitors that give rise to astrocytes (Muroyama et al., 2005; Deneen et al., 2006). *Scl* specifies formation of V2b interneurons and astrocytes in a regionally restricted area [p2 (progenitor domain 2)] of the ventral spinal cord. *Nfia* is expressed in *Olig2*-positive (*Olig2*⁺) cells at the onset of gliogenesis in the embryonic spinal cord. Both *Scl* and *Nfia* exhibit antagonistic interactions with *Olig2*.

In studies described here, we have taken the spatial and temporal expression pattern approach to the genome scale with an *in silico* screen of the Mahoney pictorial atlas of transcription factor expression in the developing CNS (Gray et al., 2004). From an entry-level list of >1400 candidates, we have identified 87 transcription factors that are expressed at early times in the germinal zone of developing spinal cord, 12 of which are novel transcription factors that (1) show sustained expression in spinal cord white matter and (2) have not hitherto been linked to glial development. We identify three novel transcription factors related to the astrocyte lineage and nine transcription factors related to the oligodendrocyte lineage. Entry-level screens for biological function establish one of the astrocyte-related transcription factors, *Klf15*, as sufficient for genesis of precocious GFAP-positive astrocytes in spinal cord explants, whereas the oligodendrocyte-related transcription factor *Tcf4* plays an important role in maturation of oligodendrocyte progenitors.

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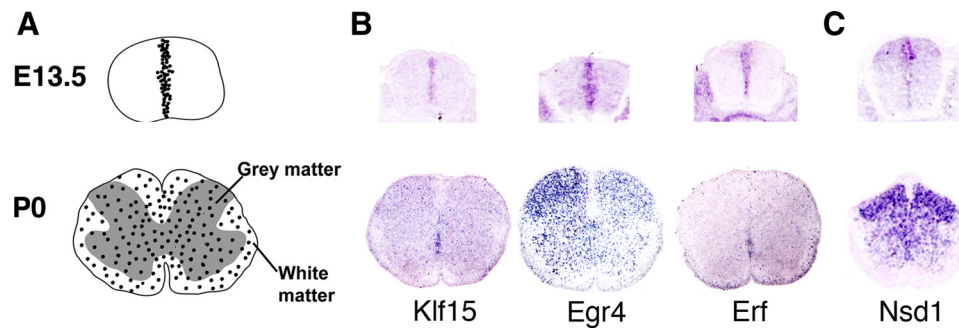


Figure 1. An *in silico* screen for transcription factors that direct formation of glia. **A**, Grayscale drawings are schematic views of glial progenitors in the developing spinal cord. At E13.5, glial progenitors are sequestered within the ventricular zone. At P0, these progenitors have migrated throughout the gray and white matter of the spinal cord. **B**, Color micrographs: three representative examples of transcription factors from the Mahoney database that show spatial/temporal expression patterns consistent with a role in specification/maturation of glia. **C**, Example of a transcription factor with a predominantly gray matter expression pattern at P0. For details, see Results.

Materials and Methods

Animals. Timed-pregnancy CD-1 mice were ordered from Charles River. The genotypes of *Tcf4*^{-/-}, *Nkx6.1*^{-/-}, *Nkx2.2*^{-/-}, *Sox10*^{-/-}, *Klf15*^{-/-}, *Klf15*^{+/-} *LacZ*, *Olig1*^{-/-}, and *Olig2*^{-/-} mice were determined by PCR of the genomic DNA, which has been reported previously (Korinek et al., 1998; Qi et al., 2001; Lu et al., 2002; Stolt et al., 2002; Liu et al., 2003; Fisch et al., 2007; Gray et al., 2007).

In situ hybridization and immunostaining. *In situ* hybridization (ISH) and immunofluorescent (IF) procedures were performed as described previously (Fu et al., 2002; Gray et al., 2004). Double staining was performed by first conducting ISH followed by IF. ISH images were pseudocolored using Adobe Photoshop (Adobe Systems) and then combined with IF images to obtain double-staining images. Antibodies used in this study include the following: anti-Tcf4 (1:200; Upstate), NG2 (1:200; Dr. William Stallcup, Burnham Institute for Medical Research, Cancer Research Center, La Jolla, CA), Sox10 (1:10; Dr. David Anderson, California Institute of Technology, Pasadena, CA), Olig1 [1:500 (Arnett et al., 2004)], Olig2 [1:10,000 (Arnett et al., 2004)], MBP (1:200; Millipore Bioscience Research Reagents), CNPase (1:100; Sigma), GFAP (1:200; Sigma), β -catenin (1:200; BD Biosciences), Ki67 (1:200; Novocastra), Hmgb1 (1:100; Abcam), Hmgb2 (1:500; Abcam), Erf (1:200; Abcam), Rxra (1:100; R & D Systems), and Tcf12 (1:50; Novus Biologicals).

Mixed glia culture. The cortices of neonatal [postnatal day 1 (P1) to P2] rat pups were used for mixed glia culture (McCarthy and de Vellis, 1980). Cortices were physically dissociated and plated on poly-L-ornithine-coated coverslips, grown *in vitro* for 13 d (DMEM/F-12, 10% FBS, and penicillin/streptomycin). Coverslips were fixed in 4% paraformaldehyde for immunostaining.

Spinal cord explant culture and electroporation. The spinal cords of embryonic day 11.5 (E11.5) mouse embryos were dissected out, and only the cervical region was used for tissue culture. The cervical spinal cord was transferred onto 0.8 μ m nitrocellulose membrane (Millipore Corporation) with the ventricular side facing up. After 2 h in culture media (DMEM, 5% FBS, and penicillin/streptomycin) in 37°C incubator, plasmids were electroporated onto the ventricular side of the spinal cord explant culture. After 6 d in culture, the explants were fixed and sectioned for GFAP immunostaining.

Full-length mouse *Olig2*, *Klf15* (Open Biosystems), and *Scl* (from Dr. David Rowitch, Departments of Pediatrics and Neurological Surgery, University of California, San Francisco, CA) cDNA were subcloned into the pIRES2-acGFP1 (BD Biosciences) expression vector for electroporation.

Results

An *in silico* screen for transcription factors that direct formation of glia

The Mahoney atlas was generated by using computer algorithms to search for protein sequences encoded in the existing public and private databases (Sonnhammer et al., 1998; Matys et al., 2003; Thomas et al., 2003; Wheeler et al., 2004). Open reading frames

were classified as transcription factors only if their predicted protein sequence included a Protein Families Database-defined DNA binding domain. By this definition, a total of 1445 known or probable transcription factors were shown to be encoded in the mouse genome. Colorimetric *in situ* hybridization was used to map the expression of >1000 of these transcription factors and transcription factor-coregulator genes in the brains of developing mice (Gray et al., 2004).

We screened this atlas for transcription factors that may play a role in the regulation of glial development. We chose to focus on expression in the spinal cord for our *in silico* screen on the basis of its relatively simple anatomical structure. Our entry-level screen was for transcription factors that are expressed within the germinal zone of the spinal cord at a time (E13.5) that coincides approximately with the neural/glial switch (Fig. 1). A comprehensive list of 87 transcription factors that showed germinal zone-restricted expression at E13.5 is provided in Table 1.

As a secondary screen, we monitored the 87 transcription factors listed in Table 1 for sustained expression at later times in the white matter of the spinal cord. By P0, the majority of the 87 transcription factors detected at E13.5 were no longer expressed or were expressed exclusively in gray matter (Fig. 1C). Transcription factors expressed exclusively in gray matter were discounted. However, 20 members of the initial set of 87 were still expressed at P0 in white matter exclusively or in white matter and gray matter together. Some representative images from the P0 screen are shown in Figure 1, and a comprehensive list of the P0 transcription factors is included in Table 2. It should be noted that all of the transcription factors in this subset are also expressed in neurons. Furthermore, the design of this screen was geared toward identification of transcription factors expressed in glial progenitors. As such, it is possible that some of these transcription factors are involved in maintaining an immature phenotype rather than promoting terminal differentiation. Similarly, the experimental design would naturally exclude transcription factors that are expressed exclusively in gray matter astrocytes.

Note that the E13.5 and P0 inventory of spatially restricted transcription factors in Tables 1 and 2 includes 12 transcription factors that have been linked previously to the formation of glia, including *Olig1*, *Olig2*, *Scl*, *Nfia*, and *Sox9*. The presence of these factors serves in part to validate our screening strategy. In addition, Table 2 lists 12 novel transcription factors that show sustained expression in white matter at P0 and have not been associated previously with glial development. These 12 transcription factors were singled out for additional study.

Table 1. Transcription factors with germinal zone-restricted expression at E13.5

Gene name	Family	LocusID	Gene name	Family	LocusID
Arntl	bHLH	11865	Hmgb1	HMG	15289
Neurod4	bHLH	11923	Sox14	HMG	20669
Hes5 ^a	bHLH	15208	Bbx	HMG	70508
Id2 ^a	bHLH	15902	Irx1	Homeobox	16371
Id1 ^a	bHLH	15901	Irx3	Homeobox	16373
Id3 ^a	bHLH	15903	Lhx2	Homeobox	16870
Srebf1	bHLH	20787	Ihx4	Homeobox	16872
Tcf12	bHLH	21406	LHP/Lhx8	Homeobox	16875
Tcf4	bHLH	21413	Nkx2.2 ^a	Homeobox	18088
E47	bHLH	21423	Nkx6.1 ^a	Homeobox	18096
Tfeb	bHLH	21425	Otx2	Homeobox	18424
Olig2 ^a	bHLH	50913	Pax3	Homeobox	18505
Olig1 ^a	bHLH	50914	Pax7	Homeobox	18509
Mxd3	bHLH	17121	Six5	Homeobox	20475
Smarrcc1	Bromo	20588	Tgif2	Homeobox	228839
Brd7	Bromo	26992	Hoxd3	Homeobox	15434
Crebl1	bZIP	12915	Pknox1	Homeobox	18771
Atf4	bZIP	11911	Pdlim4	Lim	30794
Tsc22d4	bZIP	78829	Csrp2	Lim	13008
Cited4	Cited	56222	Prickle3	Lim	54630
Nfia ^a	CTF/NF-I	18027	Rest	Myb	104383
Nfib ^a	CTF/NF-I	18028	Btf3	Nac	218490
Nfix	CTF/NF-I	18032	Rxra	Nucrec	20181
E2f1	E2f	13555	Rxb	Nucrec	20182
Tfdp1	E2f	21781	Nr1h2	Nucrec	22260
E2f6	E2f	50496	Nr2e1	Nucrec	21907
E2f2	E2f	242705	Trp53	p53	22059
E2f3	E2f	13557	Per2	PAS	18627
E2f5	E2f	13559	Rfx3	RFX	19726
Erf	ETS	13875	Rfx4	RFX	71137
Foxo4	Forkhead	54601	Nfat3	Rhd	18021
FoxM1	Forkhead	14235	Tbpl1	TBP	237336
FoxJ1	Forkhead	15223	Tead2/Klf3	TEA	21677
FoxK1	Forkhead	17425	Ciao1	WD40	26371
Gtf2i	GTF2I	14886	Sall2	Zinc	50524
Hmg20b	HMG	15353	Egr4	ZN C2H2	13656
Sox10 ^a	HMG	20665	Sp1	ZN C2H2	20683
Sox2	HMG	20674	Zfp26	ZN C2H2	22688
Sox9 ^a	HMG	20682	Klf15	ZN C2H2	66277
Tcf3	HMG	21415	Zkscan3	ZN C2H2	72739
Tcf7l2	HMG	21416	Nsd1	Zn PHD	18193
Smarrc1	HMG	57376	Phf21a	ZN PHD	192285
Hmgb2	HMG	97165	Mid1	ZN RING B BOX	17318
Sox21	HMG	223227			

^aGenes known previously to be regulators for development of glia.

Segregation into oligodendrocyte- and astrocyte-related subgroups

The CNS white matter contains both oligodendrocytes and GFAP-positive fibrous astrocytes (Privat and Rataboul, 1986). To resolve the cell types marked by novel transcription factors in our P0 “short list,” we performed *in situ* hybridization for the various transcription factors together with immunofluorescence with antibodies to Olig1 or Olig2, a pair of bHLH transcription factors that show >95% overlap in expression (and thus were treated as interchangeable for our purposes here) and are restricted to the nuclei of oligodendrocytes and oligodendrocyte progenitors in the P0 spinal cord (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). Transcription factors that were mainly precluded from Olig2-positive cells were considered to be astrocyte related (Fig. 2*A,B*). Transcription factors that primarily colocalized with Olig2 were considered to be in an oligodendrocyte-related group (Fig. 2*A,B*). To see whether P0 expression patterns reflect adult patterning, we performed expression analysis on Tcf4 and Klf15

Table 2. Transcription factors with glial expression pattern at P0

Gene name	Family	LocusID
Olig2 ^a	bHLH	50913
Olig1 ^a	bHLH	50914
Klf15	ZN C2H2	66277
Rxra	Nucrec	20181
Rxb	Nucrec	20182
Nkx 2.2 ^a	Homeobox	18088
Tcf7l2	HMG	21416
Hmgb2	HMG	97165
Hes5 ^a	bHLH	15208
Id2 ^a	bHLH	15902
Id3 ^a	bHLH	15903
Sox10 ^a	HMG	20665
Sox9 ^a	HMG	20682
E2f2	E2f	242705
Erf	ETS	13875
E2f1	E2f	13555
Six5	Homeobox	20475
Hmgb1	HMG	15289
Egr4	ZN C2H2	13656
Tcf12	bHLH	21406

^aGenes known previously to be regulators for development of glia.

(supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In the postnatal spinal cord, Tcf4 expression is exclusive to oligodendrocyte lineage cells and decreases as myelination proceeds, whereas Klf15 is mainly expressed in the astrocyte lineage. In the brain, Tcf4 is also expressed in neurons, especially in the thalamus. Overall, a group of nine transcription factors are oligodendrocyte related by this criterion and three are astrocyte related.

Our classification of the astrocyte-related transcription factors was further validated by examining the spinal cords of Olig2 knock-out mice and by misexpression studies. Targeted disruption of Olig2 leads to complete ablation of the oligodendrocyte lineage throughout the spinal cord, whereas formation of astrocytes is unperturbed (Lu et al., 2002). All three of the astrocyte-related transcription factors are expressed equally well in wild-type and Olig2 knock-out spinal cords. Representative *in situ* images of Hmgb2 (astrocyte group) and Tcf4 (oligodendrocyte group) in the spinal cords of wild-type and Olig2 knock-out mice are shown in Figure 3*A* (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). With the exception of Tcf4, no striking differences were seen in the oligodendrocyte-related group of transcription factors within the white matter (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Because Tcf4 is the only transcription factor with 100% overlap with Olig2 and the other oligodendrocyte-related transcription factors are also expressed in neurons, this result is not surprising. Tcf12 and Rxra expression is lost in the motor neuron region, consistent with previous reports of motor neuron loss in the Olig2 knock-out mice.

Functional analysis of an astrocyte-related transcription factor

From commercial sources, we were able to obtain full-length cDNA for two of the transcription factors in the astrocyte-related group. For comparative purposes, we also obtained full-length cDNA clones corresponding to two of the nine novel transcription factors in the oligodendrocyte-related group. After validating the nucleic acid sequences, these full-length cDNAs were placed into expression vectors and electroporated into E11.5 “open book” mouse spinal cord explant cultures as described in Materials and

Methods. At day 6 after electroporation, the explant cultures were fixed, sectioned, and immunostained for GFAP. As a positive control for the open book cultures, we used *Scl*, a bHLH transcription factor that has been shown previously to be both necessary and sufficient to promote formation of astrocytes in a regionally restricted domain of the ventral spinal cord (Muroyama et al., 2005). As a negative control, we used the oligodendrocyte-specific bHLH transcription factor *Olig2*, which specifies formation of oligodendrocytes.

As shown in Figure 3*B*, *Scl* and *Klf15* promoted formation of precocious GFAP-positive cells, whereas *Hmgb2* did not (data not shown). In contrast, neither *Olig2* nor the two novel transcription factors from the oligodendrocyte-related group (*Tcf4*, *Tcf12*) displayed this activity (Fig. 3*B*, and data not shown). Physiologic relevance of *Klf15* function in these spinal explant cultures was validated using a *Klf15* β -galactosidase knock-in mouse (Fisch et al., 2007) for fate mapping. As indicated in Figure 3*C*, we observed excellent overlap between β -galactosidase (β -gal) expression and GFAP staining (>60%) and poor overlap with β -gal expression and *Olig1* staining (<15%) in the postnatal spinal cord (quantification not shown). As a technical aside, our spinal cord explants routinely express GFAP-positive astrocytes at day 7 *in vitro*. Because explant cultures are harvested one day earlier (day 6 *in vitro*), we were unable to determine whether overexpression of oligodendrocyte-related transcription factors results in repression of GFAP expression. Overexpression of the oligodendrocyte transcription factors (even control *Olig2*) did not stimulate expression of late-stage oligodendrocyte markers in spinal cord explants, suggesting that additional transcription factors [e.g., *Nkx2.2* (Zhou et al., 2001)] are necessary for oligodendrocyte progenitors to progress to the MBP-expressing developmental stage.

Tcf4 is an oligodendrocyte-related transcription factor

We chose to pursue *Tcf4* for detailed study from our collection of potential pro-oligodendrocyte lineage genes (1) because it was the only transcription factor that showed 100% overlap with *Olig2*, (2) because it was the only oligodendrocyte transcription factor whose expression was disrupted in the *Olig2* knock-out spinal cord, and (3) because of its provocative relationship to the Wnt/ β -catenin signaling pathway. A functional overlap between *Tcf4* and *Olig2* suggested by the phenotype of *Olig2* knock-out spinal cords (Fig. 3*A*) was further resolved with a series of knock-out mouse models that feature a moderate-to-severe impact on myelin development. The list includes *Nkx6.1* and *Sox10* (severe phenotype) and *Olig1* and *Nkx2.2* (moderate phenotype) (Qi et al., 2001; Lu et al., 2002;

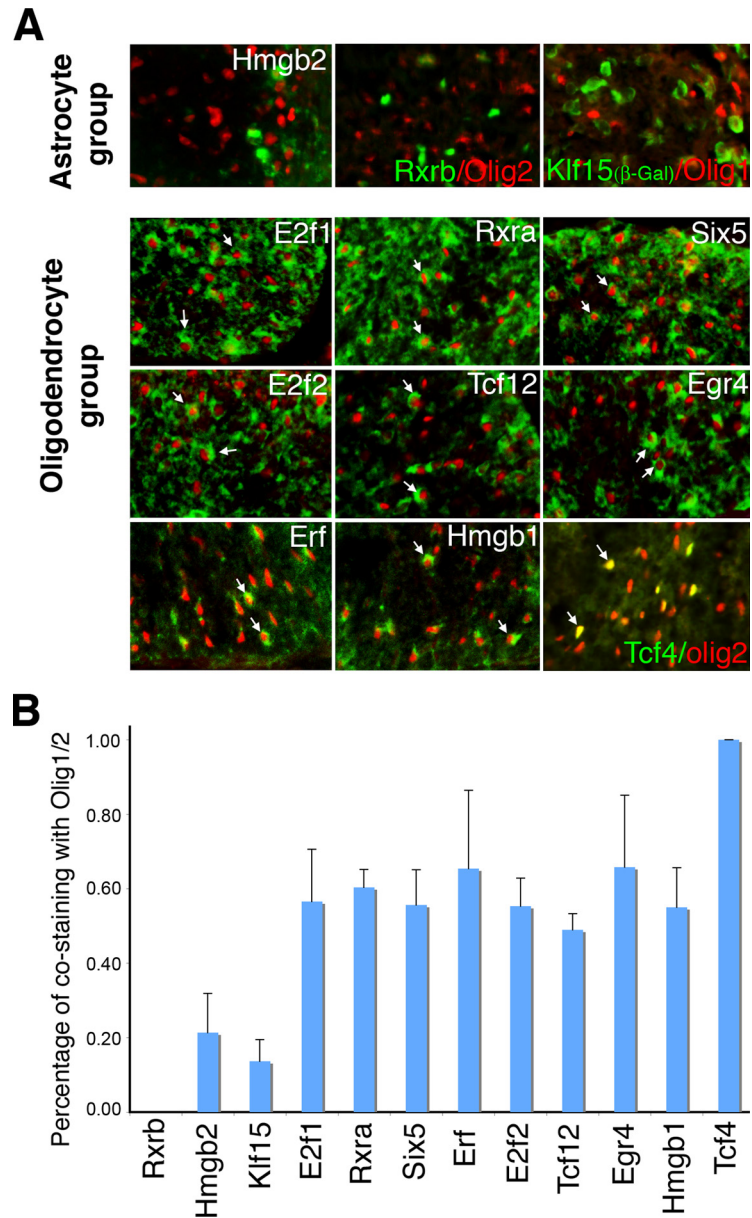


Figure 2. Segregation of glia-related transcription factors into an astrocyte group and oligodendrocyte group. **A**, Expression assays in P0 spinal cords. Oligodendrocytes are imaged by immunofluorescence with antibodies to *Olig2* or (in the case of *Klf15*) *Olig1*. Transcription factors from the Mahoney database are imaged by *in situ* hybridization (green pseudocolor), except for *Klf15* (imaged by β -galactosidase) and *Rxrb* (imaged by immunofluorescence). In the astrocyte group of transcription factors, there is minimal overlap with *Olig2* or *Olig1*. In the oligodendrocyte group, cells double labeled with *Olig2* antibodies (arrows) are far more common. **B**, Quantification of double-stained cells, presented as percentage overlap with *Olig1/2*. *Klf15* was double stained with *Olig1*. All other transcription factors were double stained with *Olig2*. Error bars indicate SD (*n* values: *Rxrb*, 3; *Hmgb2*, 14; *Klf15*, 7; *E2f1*, 7; *Rxra*, 5; *Six5*, 6; *Erf*, 9; *E2f2*, 6; *Tcf12*, 3; *Egr4*, 5; *Hmgb1*, 12; *Tcf4*, 9).

Stolt et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002; Liu et al., 2003). As shown in Figure 4, *Tcf4* expression is ablated or severely delayed in all four of these hypomyelination mouse models. *Olig1* knock-out mice are viable and some *Nkx2.2* animals survive to P7. Low levels of *Tcf4* expression were seen at P5 in *Olig1* and *Nkx2.2* knock-out animals (data not shown).

Physiologic relevance of *Tcf4* ablation in the various hypomyelination models is suggested by *Tcf4* immunostaining experiments at intervals ranging from E18.5 to adult life (Fig. 5). As indicated in Figure 2, all *Tcf4*-positive cells express the oligodendrocyte lineage marker *Olig2*. At E18.5, *Tcf4* protein is detectable but only within a minor subset of cells in the ventral spinal cord

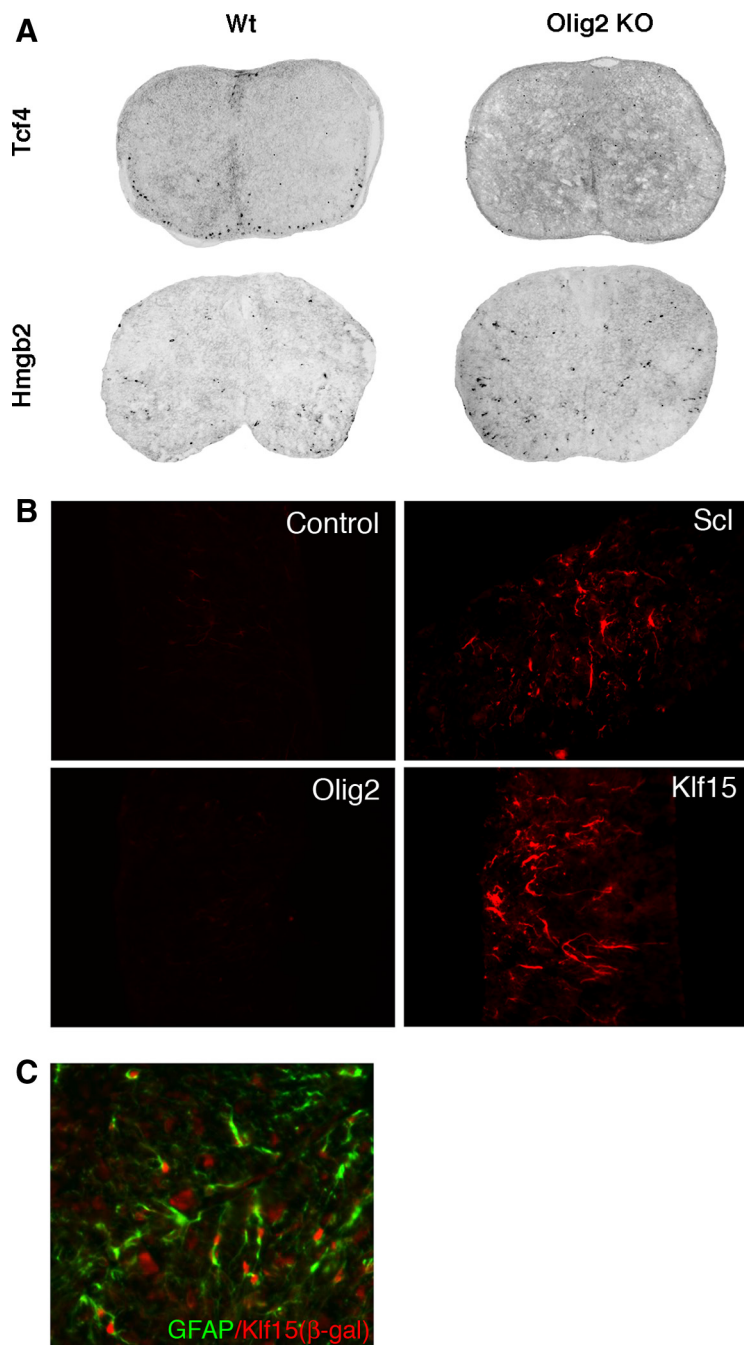


Figure 3. Analysis of astrocyte-related transcription factors. **A**, At E18.5, Hmgb2 has very similar expression in Olig2 knock-out (KO) spinal cords and in wild-type (Wt) littermates, whereas Tcf4 expression is ablated in Olig2 knock-out spinal cords. Tcf4 is used as a negative control. **B**, Klf15, an astrocyte group gene, induces ectopic GFAP expression in mouse spinal cord explant cultures. No GFAP expression is found in Olig2 or empty vector control transfected spinal cord explants. GFAP expression is also observed when the astrocyte-promoting transcription factor *Scl* is electroporated into the spinal cord. **C**, Double immunostaining showed colocalization of GFAP and Klf15 (β -gal) in P14 spinal cord of Klf15 heterozygotes (with β -gal knock-in at the Klf15 locus).

(Fig. 5, compare E18.5 Tcf4 image with E18.5 Olig2). Between P0 and P14, Tcf4-positive cells become more abundant, and they are dispersed throughout the gray and white matter of the spinal cord. However, in adult mice, Tcf4-positive cells can no longer be detected in white matter of the spinal cord. Likewise, Tcf4 protein is not expressed in adult corpus callosum (data not shown). Note that Tcf4 protein expression does not strictly overlap the expression of Tcf4 mRNA (Fig. 5, compare weak Tcf4 protein expression at E18.5 with robust mRNA expression in Mahoney atlas).

The disconnect between mRNA and protein expression may reflect some level of translational or posttranslational regulation of Tcf4.

Tcf4 marks postmitotic/premyelinating oligodendrocytes

The relationship between expression of the Tcf4 and Olig2 proteins is not symmetric. Although all Tcf4-positive cells express Olig2, many Olig2-positive cells do not express Tcf4. To identify the subset of oligodendrocyte progenitors marked by Olig2 and Tcf4, we turned to mixed glial cultures from neonatal rat cortex. Oligodendrocyte progenitors in mixed glial cultures differentiate into myelin-positive oligodendrocytes over a 2 week period of time. Differentiation is asynchronous so that, at any given time, a mixed population of immature, partially mature, and mature oligodendrocytes can be observed (McCarthy and de Vellis, 1980). A cellular “deletion map” of maturational stages in these cultures can be obtained with a set of marker proteins that are expressed at timed intervals as shown (Fig. 6A).

As shown in Figure 6B, Tcf4 protein is expressed in a subset of the cells with the sustained early markers (Olig1, Olig2 or Sox10). Tcf4 is also expressed in a subset of cells marked by proteins that are expressed at early (NG2) or late (MBP) stages of the maturational process. However, the marker most closely linked with Tcf4 (~90% coexpression) is CNPase, a protein expressed as oligodendrocytes exit the cell cycle for terminal differentiation (Barradas et al., 1995). The linkage between Tcf4 expression and cell cycle exit is further confirmed by double immunostaining with antibodies to Ki67, a marker for dividing cells. As indicated (Fig. 6C), there is no overlap between Tcf4 and Ki67 at any stage of development, showing that Tcf4 is expressed only in postmitotic cells.

Tcf4 expression is required for maturation of oligodendrocyte progenitors but is dispensable for their initial specification

Ablation of white matter by targeted disruption of pro-myelin transcription factors leads to loss of Tcf4-positive cells

(Figs. 3A, 4). However, these data do not address the role of Tcf4 itself in formation of myelin. As shown in Figure 7B, the spinal cords of Tcf4 knock-out mice show little difference in the number of cells that express early markers of the oligodendrocyte lineage such as Olig2 or platelet-derived growth factor receptor α (PDGFR α) when examined at P0. Thus, Tcf4 is dispensable for the initial specification of oligodendrocyte progenitors (see schematic in Fig. 6A). However, as shown in Figure 7A, there are significant differences in expression of later markers for oligoden-

drocytes. For example, CNPase is almost nonexistent in *Tcf4* knock-out mice at P0. Moreover, the mRNA levels of the late marker proteins MBP and proteolipid protein (PLP) are decreased to approximately one-third the levels in *Tcf4* knock-out mice relative to their wild-type littermates at P0. Thus, *Tcf4* promotes the maturation of oligodendrocyte progenitors, a function also ascribed to the bHLH transcription factor *Olig1* (Arnett et al., 2004; Xin et al., 2005).

One important remaining question is whether *Tcf4* expression must be extinguished before full maturation of oligodendrocytes. Unfortunately, *Tcf4* knock-out mice die immediately after birth. Because spinal cord explant cultures are not technically feasible for postnatal stages and oligodendrocyte cultures are difficult to transfect and mature *in vitro*, additional biological tools, including the development of inducible *Tcf4* overexpression mouse models, will be required to fully define the role of *Tcf4* in development and repair of CNS white matter.

***Tcf4* probably functions as a transcription activator in oligodendrocyte progenitors**

As a downstream effector of the Wnt signaling axis, *Tcf4* can function as either a repressor or an activator of gene expression depending on the activation state of β -catenin (Clevers, 2006). In the absence of Wnt signaling, β -catenin is phosphorylated by the serine/threonine kinase GSK3 and rapidly proteolyzed in the cell cytosol. Under these conditions, *Tcf4* is a transcription repressor. In the presence of Wnt signaling, GSK3 function is silenced and the stabilized β -catenin moves into the cell nucleus. Under these conditions, *Tcf4* is a transcription activator.

To obtain insight into the molecular mechanism whereby *Tcf4* promotes the timely maturation of oligodendrocyte progenitors (Fig. 7A), we conducted double immunostaining with antibodies to *Tcf4* and β -catenin. As shown in Figure 8, all of the *Tcf4*-positive cells in the P5 spinal cord and also in our mixed glial cultures show β -catenin within their nuclei. Accordingly, *Tcf4* probably functions as a transcription activator during the process of oligodendrocyte maturation.

Discussion

Transcription factors that specify formation of glia in genetically accessible organisms such as *Drosophila* and nematode worms have been essentially displaced and re-assigned to different functions in vertebrate animals (Wegner and Riethmacher, 2001). For vertebrate CNS development, a short list of glial transcription factors have been identified via spatially

restricted expression patterns in developing spinal cord, including *Olig1*, *Olig2*, *Scl*, *Sox 9*, *Sox10*, *Nfia*, and *Nfib* (Lu et al., 2000; Zhou et al., 2000; Stolt et al., 2002, 2003; Muroyama et al., 2005; Deneen et al., 2006). We show here how a visual database can be used to expand on this gene discovery strategy.

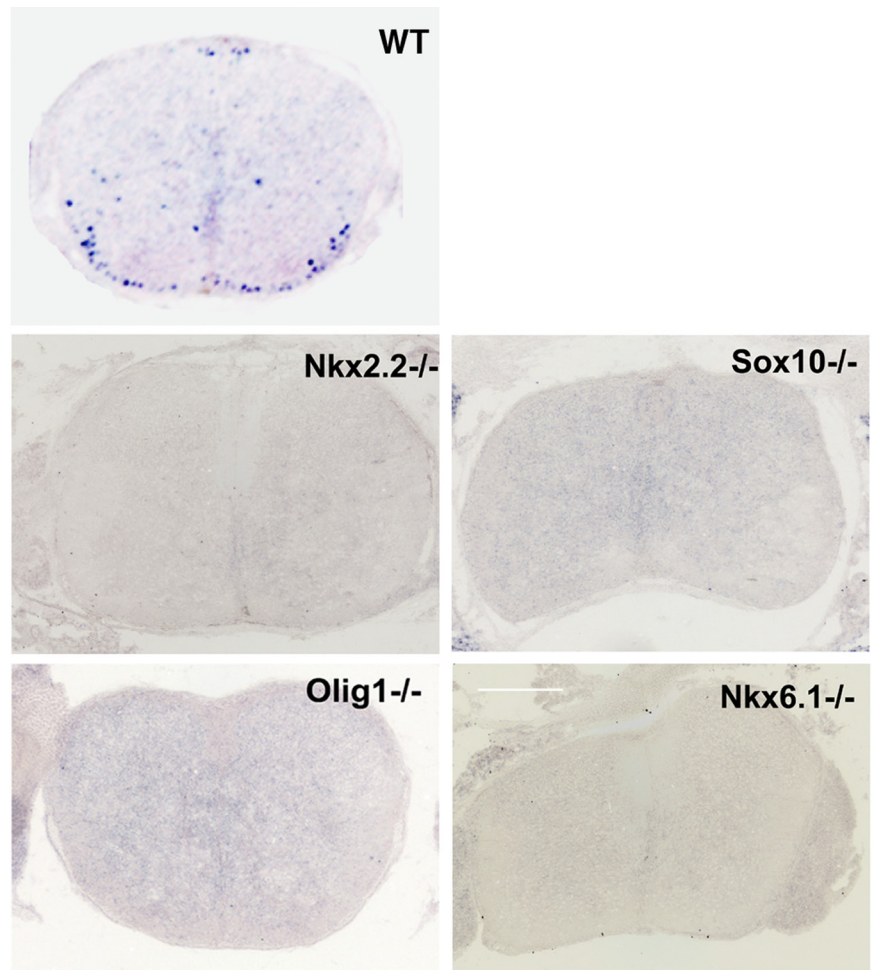


Figure 4. *Tcf4* expression is ablated or attenuated in multiple hypomyelination mouse models. All of the *in situ* images shown are from E18.5 spinal cords. WT, Wild type. Scale bar, 100 μ m.

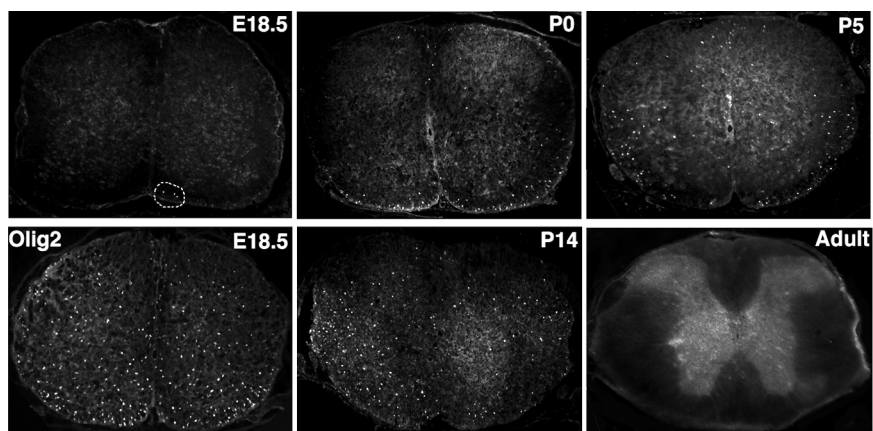


Figure 5. Temporal expression of *Tcf4* in developing spinal cord. In timed mating experiments, *Tcf4* protein is barely detectable before E18.5 in the spinal cord (3 positive cells are circled). For comparative purpose, the expression pattern of *Olig2* in E18.5 mouse spinal cord is shown below the *Tcf4* image. Between P0 and P14, *Tcf4* protein is readily detectable. In mature white matter of the adult spinal cord, however, *Tcf4* is no longer expressed.

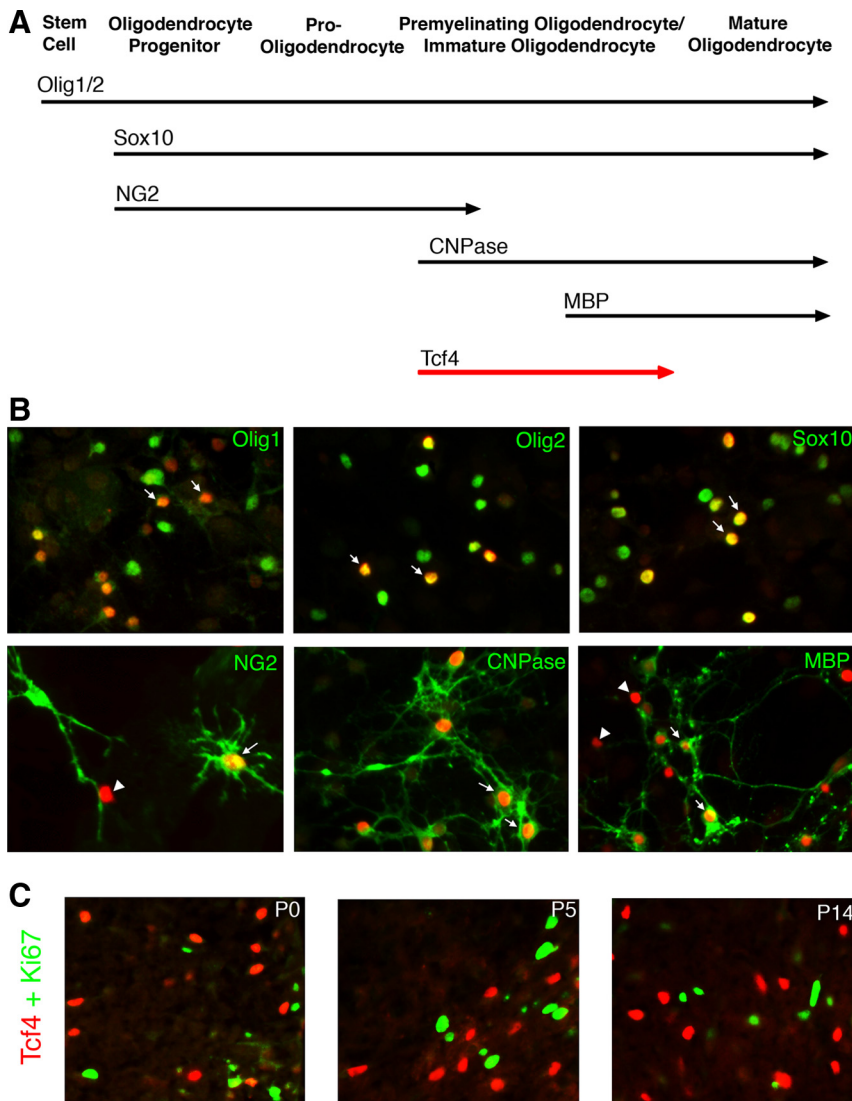


Figure 6. Tcf4 marks postmitotic/premyelinating oligodendrocytes. **A**, Schematic drawing of stage-specific markers for oligodendrocyte development. **B**, Double immunofluorescence of Tcf4 (red) with stage-specific markers (green) in mixed glia cultures. Arrows point to the double-labeled cells, and arrowheads point to cells that are only Tcf4⁺. As expected, Tcf4 is expressed in a subpopulation of Olig1⁺, Olig2⁺, and Sox10⁺ cells because these markers are detectable at all stages of oligodendrocyte formation [$36 \pm 11\%$ ($n = 9$), $40 \pm 12\%$ ($n = 3$), and $35 \pm 8\%$ ($n = 7$), respectively]. However, Tcf4 shows a near 90% overlap with CNPase [$88 \pm 2\%$ ($n = 9$)] and only partial overlap with NG2 [$47 \pm 20\%$ ($n = 6$)] or MBP [$36 \pm 12\%$ ($n = 5$)]. **C**, Double immunofluorescence with antibodies to Tcf4 (red) and Ki67 (green), a marker for dividing cells. As indicated, there is no overlap between Tcf4 and Ki67.

From an entry-level list of >1400 candidates, we identified 87 transcription factors that are expressed at early times in the germinal zone of the developing spinal cord. From this spatially restricted cohort of 87 candidates, we identified a subset of 12 novel transcription factors that (1) show sustained expression in spinal cord white matter and (2) have not hitherto been linked to glial development. Both of the gene sets contain chromatin remodeling proteins and regulators of cell division. More detailed studies of those genes could lead to a greater understanding of chromatin modification during glial development and possibly the molecular mechanisms coupling cell division with cell differentiation. Knock-out mice for many of the 12 novel genes exist. Most of them can be categorized into two groups, embryonic lethal and those with little or no phenotype in the CNS. The former genes will require conditional knock-out strategies, whereas the latter may benefit from combinatorial knock-outs.

One caveat to an entry-level screen based on *in situ* expression data is that the presence of mRNA does not always equate to the expression of protein. Tcf4 mRNA, for example, is seen in oligodendrocyte progenitors as early as E13.5 by *in situ* hybridization. However, Tcf4 protein, as detected by immunostaining, is not seen until E18.5. In contradistinction, Tcf4 mRNA expression in neurons correlates well with expression of the protein (data not shown). We and others have noted a similar disconnect in Olig1, which has been linked to maturation of oligodendrocyte progenitors. Expression of Olig1 mRNA can be seen at times as early as E9 in developing spinal cord (Lu et al., 2000). However, multiple different antibody preparations fail to detect Olig1 protein until E18.5 when oligodendrocyte progenitors have begun to mature (J. A. Alberta and C. D. Stiles, unpublished observations). It seems likely that the function of these transcription factors is regulated in part by posttranscriptional mechanisms. Sequence analysis of Olig1 and Tcf4 mRNA transcripts reveals multiple potential binding sites for microRNAs (MiRBase, <http://microrna.sanger.ac.uk>).

From a cluster of novel transcription factors with an oligodendrocyte-like expression pattern (Fig. 2), Tcf4 was chosen for functional analysis in part because of its known involvement in the Wnt signaling pathway (Kühl et al., 2000). A broad body of literature documents Tcf4 functions in the development of intestinal stem cells as well as in colorectal tumor formation (Korinek et al., 1997, 1998; Morin et al., 1997). More recently, a strong link between Tcf4 and type 2 diabetes has been described (Grant et al., 2006). Tcf4 expression in the developing CNS, however, correlates strongly with oligodendrocyte maturation. Relative to their wild-type littermates, Tcf4 knock-out mice express much less CNPase protein and MBP and

PLP mRNA at P0 (Fig. 7). Moreover, Tcf4 expression is attenuated in several different hypomyelination models (Figs. 3, 4). Preliminary results culturing Tcf4 knock-out cells suggest that oligodendrocyte progenitors are present in similar numbers but show a developmental delay in maturation that mimics the *in vivo* situation. It is unclear whether these cells would eventually catch up to their wild-type counterparts. In other studies, we have seen that Tcf4 is upregulated during the remyelination stage in the cuprizone model of relapsing/remitting multiple sclerosis (data not shown), further suggesting that Tcf4 plays a role in oligodendrocyte maturation. However, *in silico* analysis of the 10 kb upstream promoter regions of CNPase, MBP, and PLP genes failed to discover any Tcf4 binding sites.

He et al. (2007) have shown that targeted disruption of the Ying Yang 1 (YY1) transcription factor leads to overexpression of Tcf4 and an accumulation of immature oligodendrocytes.

This YY1 knock-out phenotype would be predicted from our own observations that Tcf4 is expressed in premyelinating oligodendrocyte progenitors but not in mature oligodendrocytes. He et al. also showed that ectopic Tcf4 suppresses the expression of myelin reporter constructs. However, it is well known that Tcf4 by itself is a transcription inhibitor. Coexpression with activated β -catenin is required to convert Tcf4 into a transcription activator (Clevers and van de Wetering, 1997). We find that all Tcf4-positive spinal cord cells express β -catenin in their nuclei, suggesting that Wnt signaling is important for this stage of oligodendrocyte differentiation (Fig. 8). Our results appear to contradict previous reports suggesting that Wnt signaling prevents oligodendrocyte differentiation (Shimizu et al., 2005; Kim et al., 2008). However, both studies address the role of Wnt signaling during the window of oligodendrocyte specification and/or proliferation, a time before Tcf4 protein expression. Furthermore, the pharmacological activation of Wnt signaling resulted in increased apoptosis and few surviving oligodendrocytes, making it difficult to interpret the role of Wnt signaling on differentiation. While this manuscript was under review, Fancy et al. (2009) published a survey of transcription factors involved in the repair of demyelinated lesions in the postnatal CNS, and Ye et al. (2009) characterized the role of Wnt pathway signaling in oligodendrocyte development. Both of these studies converged on Tcf4 as a central component of oligodendrocyte progenitor maturation, thus providing independent support of the conclusions drawn here.

To this point, there are only a few examples of transcription factors that have been shown to be both necessary and sufficient for the genesis of vertebrate astrocytes. The Sox9 and Nfia/b gene products appear to play broad roles in astrocyte development. However, Sox9 and Nfia/b are both expressed in oligodendrocytes as well as astrocytes, and the formation of these two cell types is mutually exclusive. For example, bone morphogenic proteins promote astrocytes and inhibit oligodendrocytes (Gross et al., 1996; Mekki-Dauriac et al., 2002; Gomes et al., 2003), whereas Olig2 promotes formation of oligodendrocytes and inhibits formation of astrocytes (Zhou and Anderson, 2002; Fukuda et al., 2004; Setoguchi and Kondo, 2004). Accordingly, the pivotal functions of Sox9 and Nfia/b in formation of astrocytes may be to inhibit neuronal fate choice, making the resulting cells permissive for glial development.

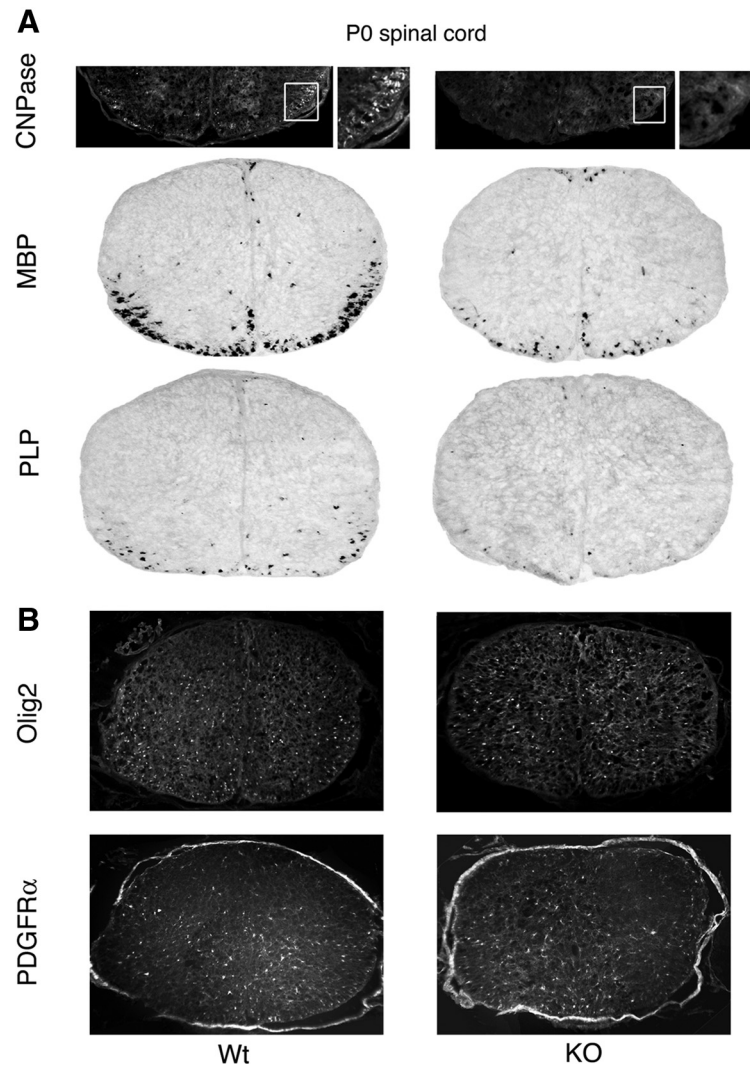


Figure 7. Tcf4 expression is required for maturation of oligodendrocyte progenitors but is dispensable for their initial specification. **A**, At P0, expression of late markers of the oligodendrocyte lineage (CNPase, MBP, and PLP) is reduced or delayed in the Tcf4 knock-out mice relative to wild-type littermates [MBP: knock-out, $29 \pm 3\%$ ($n = 3$)]. CNPase is detected by immunofluorescence with insets (white boxes) shown at higher magnification. MBP and PLP are detected by *in situ* hybridization. **B**, In contrast to late markers, early markers of the oligodendrocyte lineage (Olig2 and PDGFR α) are unaffected by Tcf4 knock-out at P0. Wt, Wild type; KO, knock-out.

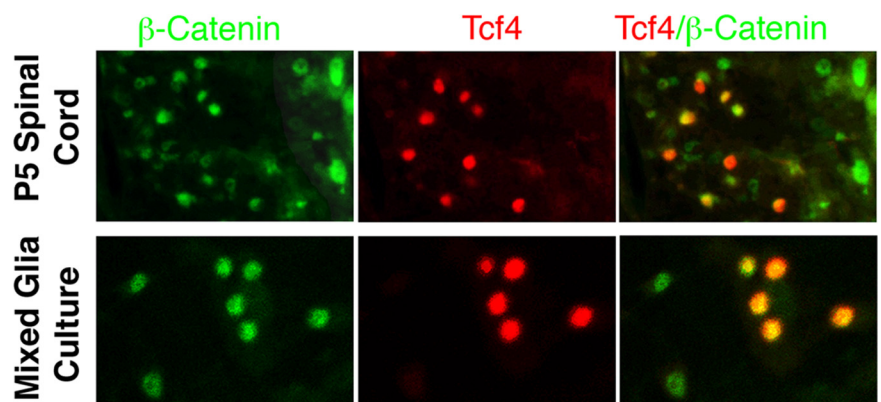


Figure 8. Tcf4 probably functions as a transcription activator in oligodendrocyte progenitors. Double immunofluorescence with antibodies to Tcf4 (red) and β -catenin (green) shows that Tcf4 is coexpressed with nuclear β -catenin in both P5 spinal cord white matter (top row) and mixed glia cell cultures (bottom row). Thus, Tcf4 is most likely functioning as a transcription activator in these cells. For details, see Results.

We identified three novel transcription factors that meet our criteria for being astrocyte specific. *Klf15* is biologically active in a spinal cord explant assay for the formation of ectopic astrocytes and is expressed in astrocytes of the postnatal spinal cord (Fig. 3). We note, in addition, that recent work in the Barres laboratory has identified *Klf15* as one of the best candidates for an astrocyte-specific transcription factor using expression array data obtained from highly purified astrocytes (Cahoy et al., 2008).

Overexpression of *Klf15* in spinal cord explants leads to overexpression of GFAP. Loss-of-function studies with *Klf15* (data not shown) were uninformative. The *Klf15* knock-out mice are viable and fertile with no outward signs of a CNS defect such as tremor, seizure, or ataxia (Fisch et al., 2007; Gray et al., 2007). *Klf15* knock-out mice show no differences in glial marker expression (GFAP, glutamate-aspartate transporter, glutamate synthase, and S-100 β) from wild-type littermates between E13.5 and P14, the critical stages in the development of astrocytes (data not shown). *Klf15* is a member of the Kruppel-like family (Klf) of transcription factors, which play functional roles in cell growth and differentiation in a wide range of mammalian cell types (Black et al., 2001; Pearson et al., 2008). It is thus possible that other members of the Klf family are functionally redundant with *Klf15* in the specification, maturation, or survival of astrocytes in the vertebrate CNS.

Several pictorial atlases of gene expression in the vertebrate CNS have appeared in recent years (Gong et al., 2003; Gray et al., 2004; Visel et al., 2004; Christiansen et al., 2006; Magdaleno et al., 2006; Lein et al., 2007). The content of these newer databases transcends transcription factors to include a wide range regulatory gene classes. Expression screens of these datasets along lines described here might identify novel growth factors, morphogenic proteins, cell surface receptors, and cytoplasmic signal generating proteins involved in the genesis of glial cells. In the fullness of time, identification of the agents that regulate glial development could have practical overtones for the therapy of a wide range of neurological disease states, including multiple sclerosis, hypoxic injury, and seizure disorders.

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