Mutual antagonism between Sox10 and NFIA regulates diversification of glial lineages and glioma subtypes

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Lineage progression and diversification is regulated by the coordinated action of unique sets of transcription factors. Oligodendrocytes (OL) and astrocytes (AS) comprise the glial sub-lineages in the CNS, and the manner in which their associated regulatory factors orchestrate lineage diversification during development and disease remains an open question. Sox10 and NFIA are key transcriptional regulators of gliogenesis associated with OL and AS. We found that NFIA inhibited Sox10 induction of OL differentiation through direct association and antagonism of its function. Conversely, we found that Sox10 antagonized NFIA function and suppressed AS differentiation in mouse and chick systems. Using this developmental paradigm as a model for glioma, we found that this relationship similarly regulated the generation of glioma subtypes. Our results describe the antagonistic relationship between Sox10 and NFIA that regulates the balance of OL and AS fate during development and demonstrate for the first time, to the best of our knowledge, that the transcriptional processes governing glial sub-lineage diversification oversee the generation of glioma subtypes.

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Progression of precursor populations through a developmental lineage is an ordered, stepwise process that culminates in the generation of a differentiated cell with a specific physiological function. A major challenge facing a precursor cell over the course of lineage development is assuring the timely expression of molecular components essential to the physiology and function of the differentiated derivative. Given the importance of transcription factors in the regulation of cell fate decisions, it follows that changes in the transcription factor milieu are an essential component of this tightly regulated process. As a consequence, unique combinations of transcription factors are required to produce appropriate transcriptional outputs at different stages of lineage development.

OL and AS comprise the glial sub-lineages in the CNS, and the manner in which their associated regulatory factors orchestrate lineage diversification during development remains unknown. Previously, we identified nuclear factor I-A (NFIA) as a key transcription factor in the specification of glial identity and differentiation of AS in the CNS[1–3]. NFIA function during these distinct phases of astroglial development is mediated by interactions with different transcription factors; during glial specification, it associates with Sox9, and it collaborates with STAT3 in differentiating AS[1,3]. Recently, we also found that NFIA suppresses OL differentiation by directly repressing myelin gene expression, although how it fits into the existing OL transcriptional network remains undefined[4]. Given the diverse functions of NFIA across glial sub-lineages, delineating its partnerships during OL development will further resolve how transcriptional networks operate during the compartmentalization of glial sub-lineages (that is, AS versus OL).

In addition to central roles in development, transcriptional regulators of gliogenesis have also been implicated in glioma formation[5]. Previous studies have demonstrated roles for STAT3, Olig2 and NFIA in glioma formation, indicating common transcriptional requirements for glial development and glioma tumorigenesis[6–8]. Although these studies established that glial fate determinants have a general role that supports tumoriogenesis, whether their developmental functions in specifying cell identity similarly influence the cellular constituency within glioma remains undefined. This is a key, unresolved question, as glioma is comprised of several subtypes, including astrocytoma and oligodendroglioma, which have vastly different clinical outcomes[9]. Thus, understanding how the generation of glioma tumor subtypes is linked to the developmental processes that regulate glial diversification has important implications for the understanding and treatment of this disease.

Given our previous findings that NFIA and Sox9 form a complex and cooperatively regulate a set of genes, we reasoned that NFIA might also have a functional relationship with Sox10 during oligodendrocyte precursor (OLP) differentiation. Using chick and mouse models, we found that NFIA directly antagonizes Sox10 regulation of myelin gene expression and that the reciprocal relationship exists during AS differentiation. Analysis of Sox10 knockout mice revealed a conversion

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of OLPs to AS, revealing a new role for Sox10 in the suppression of AS fate. Applying this developmental relationship to the generation of glioma subtypes, we used a mouse model of glioma and found that overexpression of NFIA converts an oligodendroglioma to an astrocytoma. In sum, our results reveal that cross-antagonism between Sox10 and NFIA balances OL and AS fate decisions, which in turn regulates the diversification of glial lineages during development and tumorigenesis.

RESULTS

NFIA antagonizes Sox10 induction of myelin genes

NFIA has a dynamic expression pattern during OLP differentiation in the embryonic spinal cord, where it is expressed in the motorneuron domain (pMN) domain and migrating OLPs, but is downregulated before myelin gene expression (**Fig. 1** and **Supplementary Fig. 1**)[4]. Conversely, Sox10 is expressed throughout OLP lineage development, beginning in the pMN domain at embryonic day 12.5 (E12.5) and continuing in mature, myelin gene–expressing OL (**Fig. 1a** and **Supplementary Fig. 1**)[10–12]. These patterns of expression are complemented by functional differences between NFIA and Sox10, where NFIA represses and Sox10 promotes myelin gene expression[4,13].

The fact that NFIA and Sox10 are coexpressed in OLP populations before the induction of myelin genes and regulate their expression in an opposing manner led us to hypothesize that NFIA antagonizes Sox10 induction of myelin genes. To test this *in vivo*, we made use of previous observations demonstrating that overexpression of Sox10 in the chick spinal cord promotes early and ectopic expression of the myelin genes *MBP* and *PLP1* (ref. 14). Overexpression of Sox10 resulted in the induction of *MBP*, *PLP1* and *MAG* at E4 in the chick spinal cord (**Fig. 1b–e**). Next, we overexpressed both Sox10 and NFIA and found that inclusion of NFIA inhibited the early and ectopic induction of *MBP*, *PLP1* and *MAG* by Sox10 (**Fig. 1f–i,p**). Moreover, ectopic Olig2 induction by Sox10 was also inhibited by NFIA overexpression (**Supplementary Fig. 2**).

To confirm these findings in a cell system that models OLP differentiation, we used *in vitro* OLP culture and lentiviral-mediated overexpression of Sox10 and NFIA (Online Methods)[4,15]. Consistent with our chicken studies, we found that misexpression of lentiviruses containing Sox10 resulted in increased expression MBP, PLP1 and MAG, and that these effects were inhibited when Sox10 was combined with lentiviruses containing NFIA (**Fig. 1j–o,q**). These *in vitro* and *in vivo* functional studies, in conjunction with the expression dynamics of NFIA and Sox10 during OLP development, indicate that NFIA antagonizes Sox10 induction of MBP, PLP1 and MAG during OLP development.

NFIA directly antagonizes Sox10 function

Having established that NFIA antagonizes Sox10 function during OLP differentiation, we next sought to decipher the biochemical basis of this relationship. Previously, we found that NFIA forms a complex with Sox9 and positively co-regulates a set of genes associated with glial specification in the developing spinal cord[1]. Because *Sox9* and *Sox10* are closely related members of the SoxE subfamily of Sox genes, we reasoned that NFIA antagonism of Sox10 occurs through a direct mechanism. To examine this possibility, we first determined whether NFIA and Sox10 associate using immunoprecipitation (IP) in Oli-Neu cells on ectopically expressed, tagged versions of Sox10 and NFIA, as well as endogenous proteins. Our IP-western analysis of the protein lysates derived from these studies revealed that NFIA and Sox10 co-immunoprecipitate, indicating that they physically associate (**Fig. 2a**).

The fact that NFIA and Sox10 form a complex suggests that NFIA directly antagonizes Sox10 induction of MBP, PLP1 and MAG. To further examine this relationship, we determined whether this antagonism occurs in the promoter regions of *MBP*, *PLP1* and *MAG* regulated by NFIA and Sox10. Previously, we identified key NFIA binding sites in the *MBP*, *PLP1* and *MAG* promoters (**Fig. 2b–d** and **Supplementary Fig. 3**)[4]. Subsequent bioinformatic analysis of these promoter regions identified Sox10 binding sites in relatively close proximity to the NFIA sites (**Fig. 2b–d** and **Supplementary Fig. 3**). Chromatin immunoprecipitation (ChIP) analysis on E12.5 mouse spinal cord revealed that both NFIA and Sox10 associate with the regions containing their binding sites, suggesting that this antagonism occurs on these regulatory regions (**Fig. 2b–d**). To confirm this, we performed reporter assays with the promoter regions of *MBP*, *PLP1* and *MAG* that contain these NFIA and Sox10 binding sites

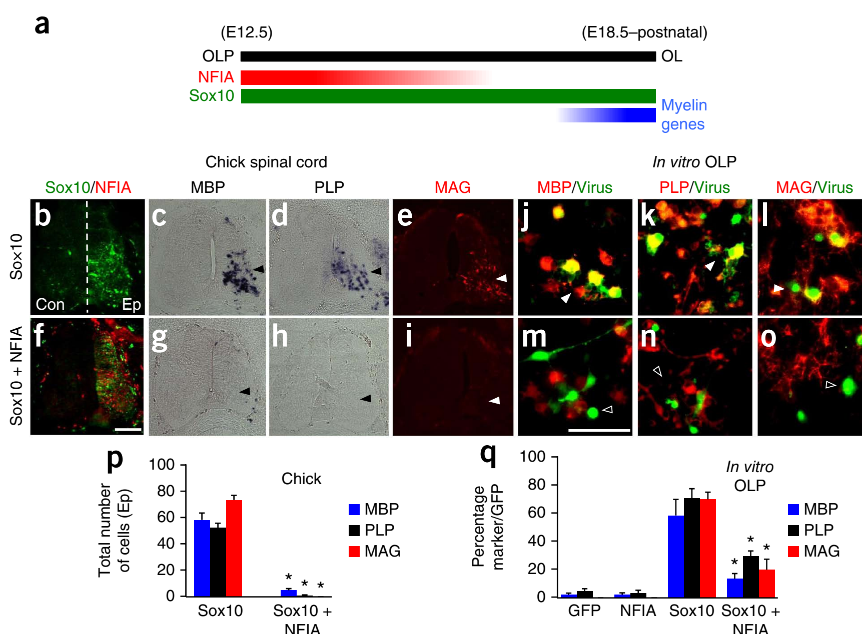

Figure 1 NFIA antagonizes Sox10 induction of myelin genes. (**a**) Schematic summarizing Sox10, NFIA and myelin gene expression during OLP differentiation. (**b**–**i**) Overexpression of Sox10 (**b**–**e**) and Sox10 + NFIA (**f**–**i**) in the chick spinal cord. Ectopic expression in **b** and **e** was detected using antibodies to HA (Sox10) or Flag (NFIA) (**Supplementary Fig. 2**). Arrowheads in **c**–**e** denote ectopic induction of myelin genes. Arrowheads in **g**–**i** denote repression of myelin genes. (**j**–**o**) Overexpression of Sox10 (**j**–**l**) and Sox10 + NFIA (**m**–**o**) in OLP cultures; Arrowheads in **j**–**l** denote Sox10 induction of myelin gene expression, open arrows in **m**–**o** denote non-overlapping expression of transgene and marker. (**p**) Quantification of chick studies is derived from eight independent spinal cords, eight sections per spinal cord (unpaired *t* test values; MBP, $P = 2.4 \times 10^{-12}$; PLP, $P = 2.58 \times 10^{-12}$; MAG, $P = 1.67 \times 10^{-15}$). (**q**) Quantification of *in vitro* studies; performed in triplicate on embryos derived from three independent litters (unpaired *t* test values; MBP, $P = 0.001$; PLP, $P = 0.00009$; MAG, $P = 0.003$). $^{*}P < 0.001$. Error bars represent s.d. Scale bars in **f** and **m** represent 100 μm.

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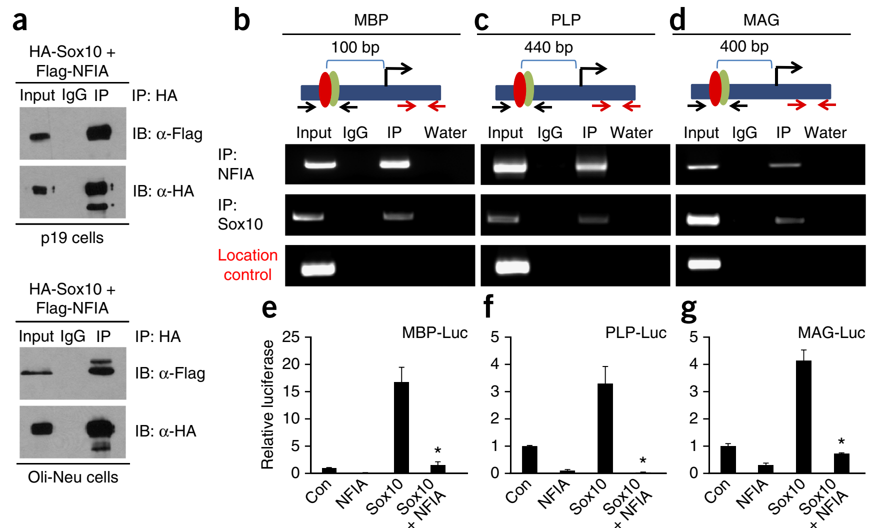

Figure 2 NFIA associates with Sox10 and inhibits its activity. (**a**) Sox10 and NFIA co-IP from Oli-Neu cell extracts. (**b**–**d**) Schematic of Sox10 and NFIA binding sites in *MBP*, *PLP* and *MAG* promoters (**Supplementary Fig. 3**). Shown is E12.5 mouse spinal cord ChIP demonstrating that Sox10 and NFIA associate with these promoters. Red ovals represent NFIA sites and green ovals represent Sox9 sites. Location controls are intronic regions at least 5 kb from the promoter binding sites. (**e**–**g**) NFIA antagonizes Sox10 activation of myelin gene promoters. Values presented are the average of three independent experiments performed in triplicate (unpaired *t* test values for Sox10 compared with Sox10 + NFIA; **e**, $P = 0.008$; **f**, $P = 0.001$; **g**, $P = 0.005$). ChIP gel images are representative of three independent experiments. $^*P < 0.001$. Error bars represent s.d. Gel and blot images in **a**–**d** are cropped; full-length images are presented in **Supplementary Figure 10**.

and found that NFIA antagonized Sox10 induction of each of these reporter constructs (**Fig. 2e**–**g**). Together, these data indicate that NFIA directly antagonizes Sox10 transcriptional induction of *MBP*, *PLP1* and *MAG*.

Sox10 antagonizes NFIA induction of AS genes

The fact that NFIA antagonizes Sox10 induction of myelin genes during OLP development raises the question of whether Sox10 antagonizes NFIA induction of AS-related genes. Previously, we demonstrated that overexpression of NFIA in the chick spinal cord promotes the precocious migration of AS precursors and induction of GFAP, a marker of mature AS (**Fig. 3**), both of which are key aspects of AS lineage differentiation[2]. To examine whether Sox10 antagonizes NFIA function during AS differentiation, we combined NFIA and Sox10 misexpression in the chick spinal cord and assessed the impact on these NFIA induced, AS-related phenotypes at E7. Combined expression

Figure 3 Sox10 antagonized NFIA induction of AS-related genes. (**a**–**h**) Overexpression of NFIA (**a**–**d**) and Sox10 + NFIA (**e**–**h**) in the chick spinal cord. The regions in the dashed boxes in **a** and **e** are shown at higher magnification in **b** and **g**. Ectopic expression in **a** and **e** was detected using antibodies to HA (Sox10) or Flag (NFIA). AS-related gene expression was detected by *in situ* hybridization (**c**,**d**,**g**,**h**). Arrowheads in **c**, **d**, **g** and **h** denote ectopic induction of AS-related genes by NFIA. (**i**) Quantification is derived from eight independent spinal cords, eight sections per spinal cord (unpaired *t* test values; GFAP, $P = 5.6 \times 10^{-12}$; Apcdd1, $P = 3.4 \times 10^{-16}$; GLAST, $P = 1.89 \times 10^{-36}$). (**j**,**k**) Schematic of Sox10 and NFIA binding sites in *GFAP* and *Apcdd1* promoters; E12.5 mouse spinal cord ChIP. (**l**,**m**) Sox10 antagonized NFIA activation of *GFAP* and *Apcdd1* promoters. For **l** and **m**, experiments were performed on three independent occasions, in triplicate. Comparison of Sox10 and Sox10 + NFIA resulted in unpaired *t* test values of $P = 0.01$ for both *GFAP* and *Apcdd1* promoters. ChIP gel images are representative of three independent experiments. Location controls are intronic regions at least 5 kb from the promoter binding sites. (**n**–**w**) Overexpression of Sox10 and/or NFIA in embryonic rat cortical culture, with FGF (**n**–**q**) and without FGF (**r**–**u**); filled arrowheads denote overlapping expression, open arrowheads denote non-overlapping expression. (**v**,**w**) Quantification of cortical cultures; values presented are the average of five independent experiments performed in triplicate (unpaired *t* test value; **v**, $P = 0.00001$; **w**, GFP compared with Sox10 $P = 0.001$, NFIA compared with Sox10 + NFIA $P = 0.000001$). $^*P < 0.001$, $^{**}P < 0.01$ Error bars represent s.d. Scale bars in **e** and **n** represent 100 µm. Gel images in **l** and **m** are cropped; full-length images are presented in **Supplementary Figure 10**.

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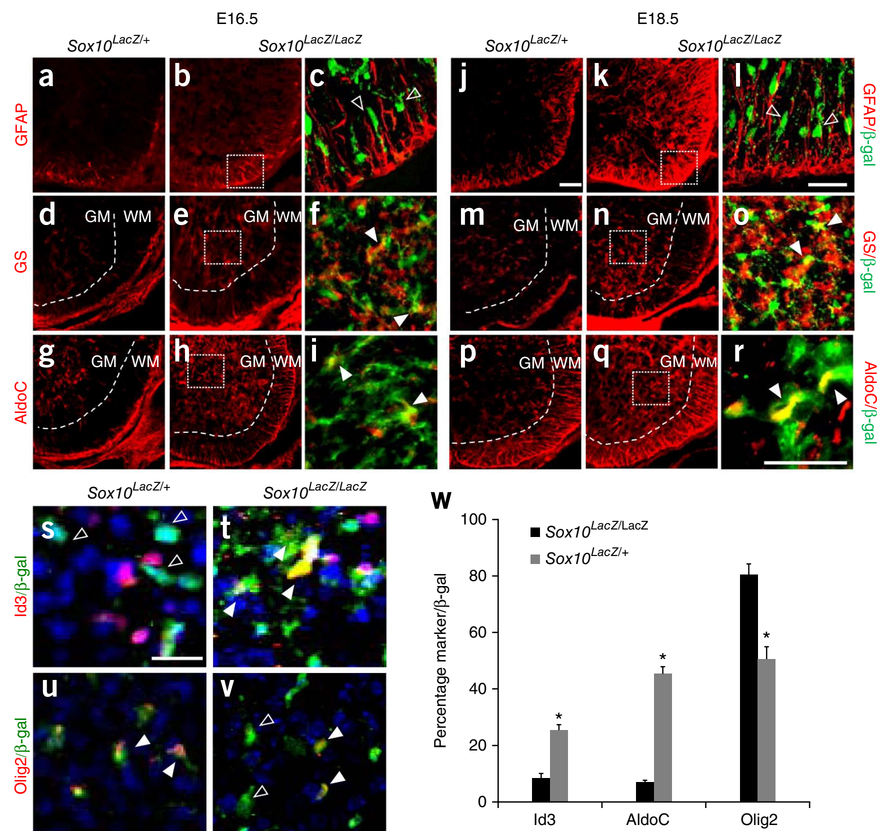

Figure 4 Loss of Sox10 promotes AS development. (**a**–**r**) Analysis of GFAP (**a**–**c**,**j**–**l**), GS (**d**–**f**,**m**–**o**) and AldoC (**g**–**i**,**p**–**r**) expression in spinal cord from *Sox10*$^{LacZ/+}$ and *Sox10*$^{LacZ/LacZ}$ embryos. Dashed line denote white matter (WM) and gray matter (GM) boundary. Double labeling with GFAP/β-gal (**c**,**l**), GS/β-gal (**f**,**o**) and AldoC/β-gal (**i**,**r**). Boxed regions are magnified in adjacent panels. Filled arrowheads denote overlap; open arrowheads denote no overlap. (**s**–**w**) Double labeling with Id3/β-gal (**s**,**t**) and Olig2/β-gal (**u**,**v**); quantification in **w** is derived from three embryos of each genotype from two independent litters (unpaired *t*-test values; Id3, $P = 0.0001$; AldoC, $P = 0.00001$; Olig2, $P = 0.006$). $^*P < 0.006$; Error bars represent s.e.m. **c**, **f**, **l** and **o** are imaged at 20×. **i** and **r** are imaged at 40×. Scale bar represents 50 μm (**j**) and 100 μm (**l**,**r**,**s**).

of Sox10 with NFIA blocked the precocious migration of GLAST-expressing AS precursors and the induction of GFAP (**Fig. 3f–j**). Moreover, Sox10 blocked NFIA induction of Apcdd1, a NFIA target that regulates AS precursor migration (**Fig. 3d,i–k**)[1]. To confirm Sox10 antagonism of NFIA induction of AS-related genes, we employed a cortical progenitor culture system[2,16]. Previously, we demonstrated that overexpression of NFIA in these cortical progenitors promotes the formation of GFAP-expressing AS (**Fig. 3p,r**). Consistent with our chicken studies, we found that combined expression with Sox10 blocked NFIA induced GFAP-expressing AS (**Fig. 3o–r**). Together, these data indicate that Sox10 antagonizes NFIA induction of AS-related genes.

Next, we examined whether Sox10 antagonism of NFIA occurs on the regulatory regions of these AS-related genes by first determining whether the *GFAP* and *Apcdd1* promoter regions also contain NFIA and Sox10 binding sites in close proximity. Using bioinformatics, we found that this was indeed the case and confirmed that both NFIA and Sox10 associate with these regulatory regions by performing ChIP assays on E12.5 spinal cord (**Fig. 3j,k** and **Supplementary Fig. 3**). To determine whether this antagonism occurs at the *Apcdd1* and *GFAP* regulatory regions, we performed reporter assays with these regulatory elements in the presence of various combinations of Sox10 and NFIA. Overexpression of NFIA induced activation of these reporter constructs, whereas combined expression with Sox10 resulted in either a loss of activity (*Apcdd1*; **Fig. 3m**) or attenuated activation (*GFAP*; **Fig. 3l**). Together, our functional and biochemical results suggest that Sox10 antagonizes NFIA induction of AS-related genes.

That Sox10 antagonizes the ability of NFIA to promote AS differentiation raises the possibility that Sox10 itself suppresses AS differentiation. To examine this possibility, we made use of the cortical progenitor culture system, where AS differentiation can be induced via removal of FGF[2]. As expected, overexpression of NFIA promoted AS differentiation, which was reduced when combined with Sox10, indicating that Sox10 is capable of antagonizing NFIA function under these conditions as well (**Fig. 3o,p,r**). Next, we overexpressed Sox10 on its own and found that it reduced the generation of GFAP-expressing AS by fivefold compared with control populations (**Fig. 3s,t,w** and **Supplementary Fig. 4**), suggesting that it functions to suppress AS differentiation *in vitro*.

Sox10 suppresses AS development

In the developing spinal cord, Sox10 expression is restricted to the pMN domain and migrating OLP populations. Although Sox10 is not expressed in migrating or mature AS populations, multiple lineage tracing studies have found that a subset of pMN-derived cells generate AS in the ventral spinal cord, likely through radial glial transformation[17,18] (D. Rowitch, personal communication). These observations suggest that Sox10-expressing cells have the potential to give rise to AS; indeed, Sox10 was coexpressed with GLAST in the pMN domain, further supporting this possibility (**Supplementary Fig. 1**). To confirm this, we used a *Sox10-Cre; Rosa26-LacZ* mouse line to trace the fate of Sox10-expressing cells in the spinal cord and found that they similarly gave rise to a subset of both gray- and white-matter AS (GMAs and WMAs, respectively) in ventral and ventral-lateral regions of the spinal cord (**Supplementary Fig. 5**)[19].

The fact that Sox10-expressing cells have the potential to give rise to AS populations, coupled with our functional results indicating that Sox10 antagonizes NFIA regulation of AS-related genes, suggest that Sox10 functions early in OLP populations to ensure lineage integrity by suppressing AS development programs. Thus, we postulated that the loss of Sox10 would disrupt repression of these AS programs, resulting in enhanced astrocytogenesis or differentiation. To investigate this possibility, we made use of the *Sox10-LacZ* mouse line[20], where *LacZ* is inserted into the *Sox10* locus and generated E16.5 and E18.5 embryos from the *Sox10*$^{LacZ/+}$ and *Sox10*$^{LacZ/LacZ}$ lines. To assess AS differentiation, we analyzed expression of GFAP, glutamine synthase (GS) and AldoC, finding that expression of these markers was substantially enhanced in *Sox10*$^{LacZ/LacZ}$ embryos at both time points in gray and white matter regions of the spinal cord (**Fig. 4**). This *in vivo* analysis was supplemented with *in vitro* studies, where we found increased astrocytogenesis from neural stem cells derived

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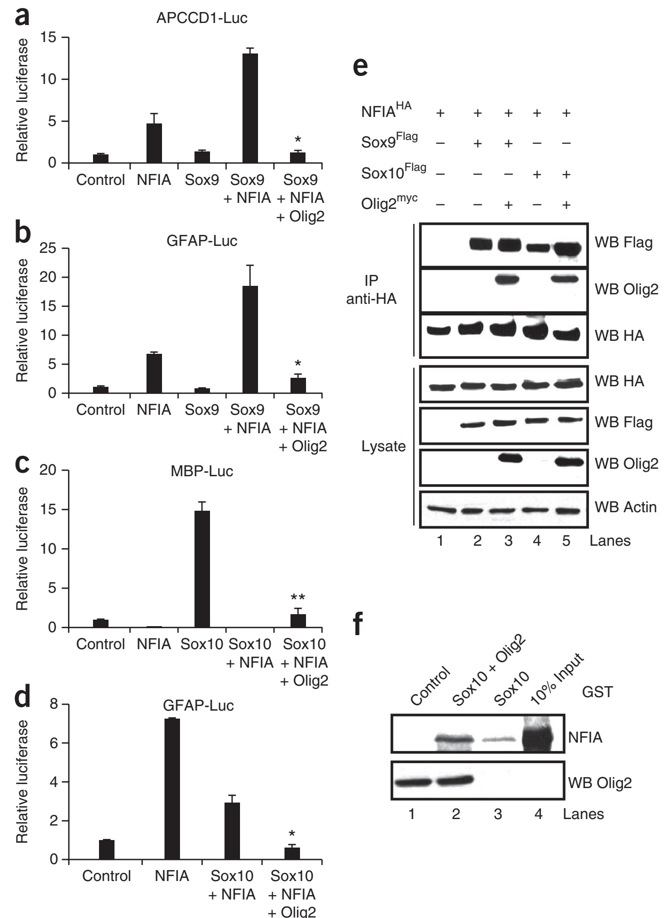

Figure 5 Olig2 facilitates Sox10/NFIA interactions NFIA and Sox activity on AS and myelin gene promoters. (**a**,**b**) Olig2 antagonized NFIA/Sox9 activation of *APCCD1* and *GFAP1* promoters (unpaired *t* test values comparing Sox9 + NFIA and Sox9 + NFIA + Olig2; **a**, $P = 0.0007$; **b**, $P = 0.01$). (**c**) NFIA suppressed the ability of Sox10 to activate the *MBP* promoter in luciferase assays. The addition of Olig2 mildly alleviated this repression. (**d**) Olig2 potentiated Sox10 suppression of NFIA-induced GFAP induction (unpaired *t* test values comparing Sox10 + NFIA and Sox10 + NFIA + Olig2; **c**, $P = 0.0007$; **d**, $P = 0.001$). Immunoblot (IB) of IP extracts from HEK293 cells expressing HA-NFIA and either Flag-Sox9 or Flag-Sox10 in the presence or absence of Myc-Olig2 (**e**). (**e**) Sox10/NFIA co-IP was enhanced in the presence of Olig2 (lanes 4,5). Olig2 did not affect Sox9/NFIA interaction (lanes 2,3). (**f**) Interaction of NFIA, Olig2 and Sox10 in GST pulldown experiments. The amount of radiolabeled-NFIA present in one-tenth of the extract before pulldown (1/10 input) is shown in lane 4. NFIA directly bound the Sox10 HMG domain (lane 3). Binding of Sox10 was enhanced in the presence of non–radio-labeled Olig2 (lane 2). The presence of *in vitro* translated Olig2 was verified by IB. Values in **a**–**d** presented are the average of three independent experiments performed in triplicate in HEK293 cells. Images in **e** and **f** are representative of three independent experiments. $*P < 0.001$, $**P < 0.01$. Error bars represent s.d. Blot images in **e** and **f** are cropped; full-length images are presented in **Supplementary Figure 10**.

from $Sox10^{LacZ/LacZ}$ embryos and rescue of these effects with ectopic expression of Sox10 (**Supplementary Fig. 6**). Together, these observations indicate that a loss of Sox10 enhances AS differentiation.

The enhanced AS differentiation witnessed in the absence of Sox10 could be a result of either accelerated differentiation of AS precursor populations or a conversion of Sox10-expressing cells to the AS fate. To distinguish between these possibilities, we used the LacZ inserted into the Sox10 locus to assess the fate of the Sox10-LacZ–expressing populations in $Sox10^{LacZ/LacZ}$ embryos. Double-labeling experiments revealed minimal overlap between GFAP and β-galactosidase (β-gal) in white matter regions in both $Sox10^{LacZ/+}$ and $Sox10^{LacZ/LacZ}$ animals (**Fig. 4c**,**l**). Analysis of Pax6 and Nkx6.1, markers of ventral WMAs, revealed no change in the number of Pax6/Nkx6.1-expressing WMAs, indicating that the observed increase in GFAP expression in white matter regions was not a result of an increase in the number of WMAs, but rather an increase in GFAP expression in individual AS (**Supplementary Fig. 7**)[21]. Analysis of GS, AldoC and Id3 in GMAs revealed an increase in the extent GS^+/β-gal^+ and a six- and three-fold increase in the number of $AldoC^+$/β-gal^+ and $Id3^+$/β-gal^+ cells in $Sox10^{LacZ/LacZ}$ spinal cord, respectively (**Fig. 4d–t**,**w**). Together, these data suggest that increased expression of these markers in the gray matter is a result of a conversion of OLP populations to the AS fate in $Sox10^{LacZ/LacZ}$ embryos. Consistent with a conversion to AS, we found a 30% decrease in the number of OLPs ($Olig2^+$/β-gal^+) in the gray matter of $Sox10^{LacZ/LacZ}$ embryos (**Fig. 4u–w**). Finally, these changes in cellular constituency were not accompanied by increases in cell death or proliferation (**Supplementary Fig. 7**). Taken together, our analysis indicates that a loss of Sox10 has differential effects on the differentiation of AS populations in the spinal cord: in the white matter, it accelerates the differentiation of individual GFAP-expressing AS, whereas, in the gray matter, it results in a conversion of OLPs to AldoC-, GS- and Id3-expressing AS.

Olig2 facilitates selectivity of NFIA and Sox family interactions

Our finding that Sox10 and NFIA have an antagonistic relationship is in contrast with the cooperativity that exists between Sox9 and NFIA. That these seemingly similar complexes have opposing functional outcomes is paradoxical and led us to further investigate the nature of these relationships. Given that Sox9 and NFIA are also expressed in pMN domain, yet this domain does not robustly generate AS precursors, we reasoned that factors specifically expressed in pMN domain interfere with the Sox9/NFIA relationship. Among transcription factors specifically expressed in the pMN domain, Olig2 is important for the specification of the OL lineage during early embryogenesis. To test whether Olig2 can antagonize the cooperativity between Sox9/NFIA, we performed reporter assays on two AS-related genes, *APCDD1* and *GFAP*, in HEK293 cells. Sox9/NFIA synergistically activated both reporters, whereas inclusion of Olig2 suppressed this activation (**Fig. 5**). Next, we performed co-IP studies, finding that Sox9 and NFIA continued to associate in the presence of Olig2 and immunoprecipitated with Olig2 (**Fig. 5e**). These observations suggest that Olig2 associates with Sox9/NFIA complexes and neutralizes their capacity to cooperatively drive AS gene expression. That Olig2 antagonizes Sox9/NFIA cooperativity raises the complementary question of whether it facilitates Sox10/NFIA antagonism. To address this question, we performed reporter assays and found that Olig2 weakly attenuated NFIA suppression of Sox10-dependent induction of MBP-reporter activity and potentiated Sox10-dependent repression of NFIA-induced GFAP reporter activity (**Fig. 5c**,**d**). Together, these findings suggest that Olig2 reinforces the antagonistic relationship that exists between Sox10 and NFIA. To dissect the biochemical basis for this, we performed co-IP studies and found that the extent of Sox10/NFIA association was substantially enhanced in the presence of Olig2 (**Fig. 5e**). These data suggest that Olig2 facilitates the interaction between Sox10 and NFIA, reinforcing the antagonism that exists between these factors. To directly test this possibility, we performed *in vitro* GST-pulldown studies and found that NFIA binding to Sox10 was increased in the presence of Olig2 (**Fig. 5f**), indicating that Olig2 mediates the biochemical relationship between Sox10 and NFIA.

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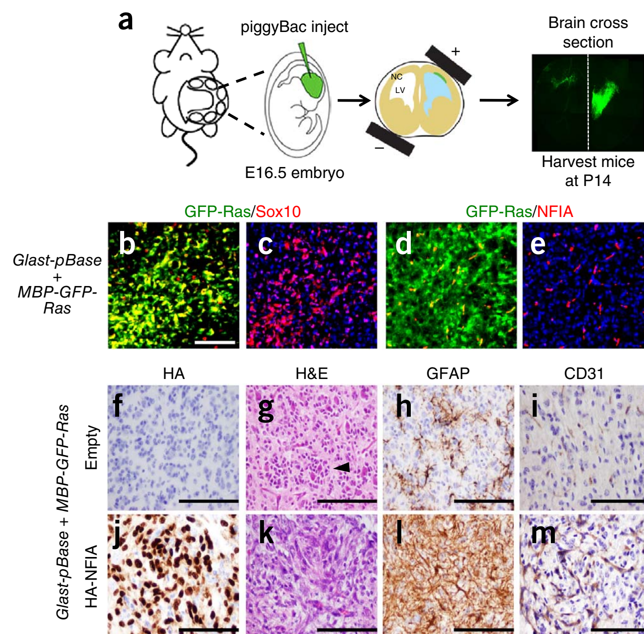

Figure 6 NFIA regulates generation of glioma subtypes. (**a**) Schematic of IUE mouse glioma model; dashed line in cross section represents cortical midline. (**b**–**e**) Expression of NFIA and Sox10 in mouse oligodendrogliomas. (**f**–**m**) E16.5 embryos were injected and subjected to IUE with *GLAST-pBase*, *Pb-MBP-GFP-Ras* and either *Pb-CAG-empty* (**f**–**i**) or *Pb-CAG-HA-NFIA* (**j**–**m**). Representative oligodendroglioma tumors harvested from P14 mice that were electroporated with *Pb-CAG-empty* are shown in **f**–**i**. Representative astrocytoma-like tumors harvested from P14 mice that were electroporated with *Pb-CAG-HA-NFIA* are shown in **j**–**m**. Arrow in **g** denotes perineuronal satellitosis, a hallmark of oligodendroglioma tumors (**Supplementary Fig. 7**). Images are representative of 10 mice and 10 tumors for each condition, derived from three independent IUE experiments and litters for each condition. Scale bars represent 100 μm (**b**) and 250 μm (**f**–**m**).

Together, these results indicate that Olig2 has a polarizing effect on Sox9/NFIA and Sox10/NFIA complexes that exist in pMN, neutralizing the former and reinforcing the latter, thereby functioning to organize these transcriptional relationships that regulate glial sub-lineage fate decisions in the pMN domain during early development.

NFIA regulates generation of glioma subtypes

Our foregoing data indicate that antagonism between Sox10 and NFIA regulates AS and OL fate choice. Given that many developmental programs are reutilized during malignancy, we examined whether this relationship between Sox10 and NFIA is applicable to the generation of glioma subtypes, astrocytoma and oligodendroglioma. Previous studies have found that Sox10 is expressed in all glioma subtypes, whereas NFIA is highly expressed in astrocytoma and nominally expressed in oligodendroglioma[8,22–25]. That NFIA demonstrates very low expression in oligodendroglioma suggests a correlation between its suppression of OL development and its role in glioma formation. This, coupled with our observations that NFIA was highly expressed in astrocytoma and developmentally promoted AS formation, led us to hypothesize that its overexpression in oligodendroglioma promotes a conversion to an astrocytoma-like tumor or fate. To test this hypothesis, we developed a mouse model of oligodendroglioma that combines *in utero* electroporation (IUE) with PiggyBac technology to target the OL lineage with the *RasV12* oncogene in the developing mouse cortex (**Fig. 6** and Online Methods). IUE with these constructs at E16.5 results in the generation of oligodendroglioma tumors by P14 that bear many of the pathological hallmarks of these tumors, including, small monomorphic nuclei lacking processes, perineuronal satellitosis, and invasion into perivascular and subpial spaces (**Fig. 6f**,**g** and **Supplementary Fig. 8**). Furthermore, this mouse model recapitulates the patterns of NFIA and Sox10 expression found in human oligodendroglioma, where Sox10 is highly expressed and NFIA has nominal expression (**Fig. 6b**–**e**).

To examine whether NFIA overexpression influences the generation of glioma subtypes in this mouse model of oligodendroglioma, we combined IUE-mediated PiggyBac overexpression of RasV12 with HA-NFIA and harvested mouse brains at P14. After confirmation of ectopic NFIA expression through immunostaining for HA (**Fig. 6j** and **Supplementary Fig. 7**), analysis of these tumors was performed using molecular and pathological criteria. Molecular analysis revealed a marked increase in the expression of the AS marker GFAP and the endovascular marker PECAM/CD31, indicating that overexpression of NFIA promotes both astroglial and vascular properties of these tumors (**Fig. 6h**,**i**,**l**,**m**). At the pathological level, numerous nodular foci were identified containing tumor cells with more elongated and pleomorphic nuclei consistent with astrocytic differentiation and astrocytoma (**Fig. 6g**,**k**). Together, these molecular and pathological criteria indicate that overexpression of NFIA promotes the conversion of oligodendroglioma to an astrocytoma-like subtype of glioma.

DISCUSSION

Our results describe for the first time, to the best of our knowledge, the relationship between Sox10 and NFIA during glial development in the CNS. We found that NFIA antagonized the ability of Sox10 to induce the expression of myelin genes. An analogous relationship existed during AS development, as Sox10 directly antagonized NFIA induction of AS-related genes. Moreover, loss of Sox10 resulted in enhanced differentiation of white matter AS and increased generation of gray matter AS, suggesting that Sox10 functions early in OLP development to suppress AS developmental programs. Application of these findings to glioma revealed that overexpression of NFIA in a mouse model of oligodendroglioma resulted in the conversion to astrocytoma-like tumors. These observations provide evidence that transcriptional regulators of glial fate oversee the generation of glioma subtypes.

NFIA and Sox genes: gliogenic partners

Our previous studies revealed that NFIA suppresses OLP differentiation by directly repressing myelin gene expression[4]. Here we found that NFIA associated with Sox10 and antagonized its ability to promote myelin gene expression. This mode of antagonism is likely to occur through direct association of these proteins on the promoter regions of these myelin genes, as both NFIA and Sox10 immunoprecipitated these regions at E12.5 in the spinal cord. Subsequent downregulation of NFIA before OLP differentiation (between E16.5–18.5) frees Sox10 of this repression and allows it to drive myelin gene expression in a timely manner. These observations suggest that downregulation of NFIA is a key event regulating the timing of OLP differentiation, as it triggers Sox10 induction of myelin gene expression. Notably, Sox9 directly induces NFIA expression and is similarly downregulated in OLP populations, implicating this regulatory axis in the timing of OLP differentiation[1]. In the future, it will be important to fully dissect the mechanism of NFIA downregulation during OLP differentiation.

Understanding the assembly and regulation of activator complexes that control the timely induction of myelin genes is crucial because their constituents are often expressed in OLPs before their differentiation.

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Our results indicate that NFIA is important for regulating these complexes by antagonizing Sox10 function in OLPs. This relationship between Sox10 and NFIA is further reinforced by Olig2, which facilitates Sox10/NFIA complex formation. The involvement of Olig2 with this complex serves two key functions: it ensures timely myelin gene expression by promoting NFIA association with Sox10 and it inhibits NFIA from promoting AS fate. The inhibition of AS fate in pMN domain is a key function of Olig2 that is poorly defined. Our mechanistic studies on this matter have revealed that, in addition to facilitating Sox10/NFIA interactions, Olig2 also neutralizes Sox9/NFIA cooperativity. Thus, Olig2 interaction with the Sox family/NFIA complexes represents a new layer to the intricate transcriptional interplay that regulates both patterning and cell fate decisions during early gliogenesis.

The premise for examining the relationship between Sox10 and NFIA is that we previously found that Sox9 and NFIA form a complex and cooperate to regulate a set of genes expressed during the initiation of gliogenesis[1]. Although NFIA associates with both Sox9 and Sox10, two closely related members of the SoxE subfamily, these relationships have markedly different consequences: NFIA and Sox9 have a cooperative relationship, whereas the NFIA and Sox10 relationship is antagonistic. Given that Sox9 and Sox10 generally function in a pro-glial manner, coupled with the redundant nature of many Sox genes, it is surprising that their association with NFIA results in such contrasting outcomes. These vastly different outcomes are mediated in part by interaction with Olig2; however, it is likely that Sox10/NFIA complexes have different constituents than Sox9/NFIA complexes and that these other factors also contribute to these contrasting effects. Indeed, another mode of Sox10 regulation involves sequestration and subsequent inhibition of myelin gene activation by Hes5 and Sox5/6 (ref. 26). These observations, coupled with our findings, point to a more nuanced role for Sox genes and NFIA in glial specification and the compartmentalization and differentiation of glial sub-lineages. Moreover, given that several other members of the Sox and NFI families are expressed during gliogenesis in the developing spinal cord and other regions of the CNS, a Sox/NFI transcriptional regulatory code for glial development may exist and warrant further investigation[27].

Sox10 and the suppression of astrocyte fate

Our studies of the relationship between Sox10 and NFIA in OLP development led us to investigate the effect of this relationship on AS development programs, where we found that Sox10 antagonized the ability of NFIA to promote AS development. NFIA is expressed in the pMN domain and in OLP populations; thus, its ability to promote the AS fate must be suppressed in these populations. Our findings indicate that Sox10 is important for suppressing the ability of NFIA to promote AS development programs. Previously, we found that Olig2 suppresses NFIA induction of AS fate; thus, it seems likely that Sox10 and Olig2 collaborate to suppress global AS programs in OLPs[28]. Suppression of AS fate represents a new function for Sox10 and may resolve a long-standing paradox surrounding its role in OLPs. Sox10 is expressed in pMN domain, yet its function has been primarily linked to myelination, which occurs several days after specification. Given that NFIA is also expressed in pMN, our data indicate that Sox10 functions early in OLP development to suppress AS development and preserve the integrity of the OLP lineage. The mutually cross-repressive nature of Sox10/NFIA on OL and AS development programs is a form of transcriptional checks and balances that ensures proper compartmentalization of the sub-lineages early in glial development (**Supplementary Fig. 9**). Indeed, the dynamics of these relationships may explain why small subsets of pMN-derived cells generated AS (**Supplementary Fig. 5**).

To further investigate this role of Sox10, we examined how genetic deletion affects AS development, finding that its loss resulted in increased GFAP expression in WMAs and increased production of Id3-, AldoC- and GS-expressing GMAs. Our data indicate that the increased GFAP expression in WMA was not a result of a conversion of Sox10-expressing cells to WMAs or increased production of WMAs. This argues that the increase in GFAP expression occurs in individual WMAs and is likely a result of accelerated differentiation from pMN populations, where NFIA is left unchecked by the absence of Sox10. The effects are different for GMA AS, where the numbers of Id3- and AldoC-expressing AS are increased as a result of a conversion of Sox10-LacZ cells to the AS fate. These results indicate that WMAs and GMAs have different requirements for Sox10 function, suggesting that these populations have unique transcriptional requirements. This notion is supported by previous studies showing that GMA and WMA in the cortex have differential requirements for Olig2, with GMAs demonstrating increased GFAP expression in its absence[29,30]. Because Sox10 and Olig2 both suppress NFIA function and GMA development, it will be important to uncover how their functions are coordinated and identify the associated transcriptional networks supporting this function.

The fact that Sox10-LacZ cells convert to GMAs raises the question of which populations undergo this conversion. One possible source is NG2 cells, which are OLP populations that generate OL and GMAs in the ventral forebrain and spinal cord in the adult[31,32]. Given this link between NG2 cells and GMAs, it may be interesting to investigate whether Sox10 also suppresses GMA production from NG2 cells in the adult and whether its relationship with NFIA regulates the balance between OL and AS generation.

Linking glial diversification and glioma subtypes

Our observations that NFIA function and expression is strongly correlated with AS formation and astrocytoma, respectively, and not OLPs and oligodendroglioma, led us to hypothesize that it can influence the generation of glioma subtypes. Using a mouse model of oligodendroglioma, we found that overexpression of NFIA converts an oligodendroglioma to an astrocytoma. Together, these results provide, to the best of our knowledge, the first evidence that developmental paradigms regulating glial sub-lineage diversification similarly regulate the generation of glioma subtypes.

It is becoming increasingly clear that tumorigenesis is the convergence of genetic mutation and developmental context. This tenet was established at the cellular level in glioma using the RCAS-tva system, where targeting Ras and Akt to neural progenitors resulted in glioblastoma, whereas targeting these same oncogenes to AS resulted in decreased tumor penetrance and malignancy[33]. Furthermore, targeting these same lineages with PDGF-B resulted in the generation of oligodendroglioma[34]. Although targeting different oncogenic stimuli to distinct cell populations results in specific subtypes and grades of glioma, the molecular basis for how developmental and cellular context influences the generation of tumor subtypes remains poorly defined. The longstanding view on developmental contributions to tumorigenesis has correlated differentiative status with proliferation and malignancy. Our results provide a new perspective on the role of developmental processes in tumorigenesis, where the transcriptional processes that govern lineage diversification similarly regulate the generation of tumor subtypes. Here, we found that overexpression of the AS fate determinant NFIA alters glioma subtype independently of cell context or oncogenic stimuli, suggesting that specific interactions between oncogenes and developmental regulators of glial sub-lineages influence the generation of glioma subtypes. More broadly, such

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specific relationships likely toggle oncogenic (or tumor suppressor) signaling pathways and are key components of how developmental context regulates tumorigenesis. In glioma, analogous relationships have been established for STAT3: in the absence of PTEN, STAT3 suppresses tumorigenesis, whereas, in the presence of EGFR-vIII, overexpression STAT3 promotes tumorigenesis[6].

Previous studies have established that transcriptional regulators of developmental gliogenesis also contribute to glioma tumorigenesis; these include Olig2, STAT3 and NFIA[6–8]. In each of these studies, the given factor was found to contribute to gross tumorigenesis (see above); whether it influenced the generation of specific glioma subtypes was not determined, reflecting perhaps the limits of the model system used or the mode of gene manipulation. Our studies are unique in that we combined overexpression with a developmental model of glioma, revealing previously unknown roles and new insight into NFIA function during glioma formation. It is likely that employing analogous experimental approaches for STAT3 and Olig2 will also reveal new insights into their respective functions during glioma formation. Finally, prior studies on Sox10 using the RCAS-tva model revealed that its overexpression did not alter glioma subtypes and only modestly increased tumor penetrance in the PDGF-B model of oligodendroglioma[24]. Analogous experiments in our IUE/MBP-Ras model gave similar results, indicating that increased Sox10 expression in oligodendroglioma models does not influence the generation of glioma subtypes.

Our results identified a previously unknown developmental relationship regulating the diversification of glial sublineages during CNS development that also manages the generation of glioma subtypes. The conservation of glial developmental relationships in tumorigenesis has important implications in the treatment of glioma, as the ability to manipulate cell fates in tumors to provoke more differentiated or less malignant phenotypes is a potential therapeutic approach to this largely fatal disease. A deeper understanding of how NFIA and Sox10 function is coordinated with other determinants of glial fate to orchestrate glial diversification during development and tumorigenesis will be important in determining whether malignant gliomas can be managed through conversion to less malignant subtypes.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.D. and S.M.G. conceived the project, designed the experiments and wrote the manuscript. S.M.G. and W.Z. performed all of the experiments. B.D., S.M.G., W.Z. and C.M. analyzed the data. C.C.S., M.W., T.-W.H., J.L.N., J.J.L. and F.C. provided essential reagents.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Chick and mouse experiments. Expression constructs were cloned into the RCAS(B)[35] or pCIG vectors and injected into the chick spinal cord at stage HH13-15 (~E2). Harvested embryos were fixed in 4% paraformaldehyde at ten volumes per tissue weight for 2–4 h, depending on stage. Electroporation was carried out with a BTX Electro Square Porator[36]. The *Sox10-LacZ*[20] and *Sox10-Cre*[19] mouse lines were used in these studies. *In situ* hybridization and immunohistochemistry analysis was performed as described[1].

ChIP, immunoprecipitation and reporter assays. Mouse E12.5 spinal cords were dissected, dissociated and processed for ChIP assays. The samples were pre-cleared with protein G beads and immunoprecipitated using 2 μg of NFIA antibody (Abcam, AB11988), Sox10 (rabbit polyclonal) or control IgG (Santa Cruz). The DNA was purified and PCR was performed using region-specific primers. HEK293 or HEK293T cell lines cells were transfected with pGL3-reporter constructs and a CMV-β-galactosidase vector using Superfect transfection reagent (Qiagen). Cells were harvested and analyzed for luciferase activity; β-galactosidase was used to normalize for transfection efficiency.

Co-immunoprecipitation was performed by transfecting P19 or Oli-Neu cells with Flag-NFIA and/or HA-Sox10; harvested cell lysates were subject to immunoprecipitation using a specific antibody or IgG control and protein G agarose beads.

GST pulldown experiments. For GST pulldown experiments, GST or GST fusion proteins with portions of Sox10 were produced in the *Escherichia coli* strain Rosetta (Novagen) following induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h of induction at 25 °C, cells were collected by centrifugation, resuspended in lysis buffer supplemented with 1 mM dithiothreitol and protease inhibitors, and sonicated for two 30-s pulses at 4 °C. Bacterial debris was removed by centrifugation at 15,000 r.p.m. for 20 min. Equal amounts of GST or GST fusions were immobilized on glutathione agarose beads (Life Technologies). GST bound beads were incubated for 2 h at 4 °C with *in vitro*–translated 35S-labeled proteins (Promega) or non–radio-labeled 'cold' proteins in binding buffer (1× phosphate-buffered saline, 1% Triton X-100 (vol/vol), 25 mg ml^{-1} BSA, 1 mM DTT and protease inhibitors). After extensive washing, resin-bound proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The Sox10 plasmids used in these studies were cloned into the PGEX-KG vectors[37].

OLP and cortical culture. Oligosphere cultures were performed as previously described[4,38]. For viral infection of OLPs, cells were dissociated and plated on poly-D-lysine (PDL)-coated coverslips at a density of 1.5×10^4 cells per cm^2 in OPM media subsequently infected with NFIA-FUIGW, FUIGW-Sox10 or control GFP virus for 14 h. Rat cortical progenitor cells were isolated via dissection of E13.5 rat embryonic cortex and dissociation with papain. Cells were grown in DMEM/F12 supplemented with N2 and B27 (GIBCO) and 20 ng/ml bFGF (R&D Systems), as described previously[2,16]. Cells were transfected with Lipofectamine 2000 using pCS+ or pcDNA plasmids containing GFP, Myc-mSox10, HA-mNFIA and/or myc-mOlig2. In experiments that required the removal of bFGF to promote AS differentiation, bFGF was removed 24 h post-transfection and replaced with media described above supplemented with 2% FCS[16] (vol/vol).

Oligosphere differentiation. Mouse embryonic E14.5 cortex were dissected, dissociated and plated in neurosphere proliferation media (NPM), which consisted of DMEM/F12, B27 supplement (Gibco), and 10 ng ml^{-1} EGF and 10 ng ml^{-1} bFGF. Neurospheres were allowed to form for 4–6 d. To generate oligospheres, whole neurospheres were then plated on (PDL-coated coverslips in oligosphere proliferation media (OPM) that was composed of NPM supplemented with 10 ng ml^{-1} platelet-derived growth factor (PDGF), but without EGF. After 2 d, oligospheres were induced to differentiate by replacing OPM with basal chemically defined medium supplemented with 15 nM triiodthyronine, 10 ng ml^{-1} CNTF and 5 mg ml^{-1} NAC. For viral infection of OL precursors, neurospheres were dissociated and plated on PDL-coated coverslips at a density of 1.5×10^4 cells per cm^2 in OPM media. Cells were allowed to attach for 3 h before exposure to virus. Cells were transduced with either NFIA-FUIGW or control GFP virus for 14 h, followed by replacement of media with OPM. After 48 h, differentiation was induced as described above.

***In utero* electroporation and glioma formation.** *In utero* electroporation was performed as previously described[39]. Electroporation was performed at embryonic day 16 and gestation age was confirmed during surgery. All plasmids were used at the final concentration of 2.0 μg μl^{-1}. Selective targeting of OL is achieved by co-electroporating a 'helper plasmid' that regulates *pBase* transposase expression by an upstream *Glast* promotor (*pGlast-PBase*) while a 'donor plasmid' carries a bicistronic *GFP-t2a-RasV12* transgene driven by the *MBP* promoter flanked by terminal repeat (TR) sequences, which transposase recognizes (*MBP-GFPt2aRas*). For the IUE/oligodendroglioma-NFIA overexpression studies, HA-NFIA was cloned downstream of a constitutive CAG-HA-NFIA promoter, flanked by TR sequences. This plasmid (or the empty control) was co-electroporated along with the *pGlast-PBase* and MBP-*GFPt2aRas*. Animals were harvested at P14 and brains were fixed in 4% paraformaldehyde. After fixation, brains were embedded in paraffin, sectioned and subjected to molecular and pathological analysis via immunostaining or hematoxylin and eosin staining. All mouse experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Antibodies. The following antibodies were used for ChIP, western blot and immunofluorescence: GFAP (Chemicon, MAB360, 1:1,000), GFAP (DAKO, Z0334, 1:1,000), HA (Roche, 1867423; Covance, 16B12; SCBT, SC805), LacZ (Abcam, AB9361, 1:1,000), LacZ (MP, AB3403, 1:1,000), MBP (Covance, SMI-94R, 1:500), NFIA (1:2,000), Nkx6.1 (DSHB, F55A10, 1:5), Olig2 (R&D, AB9610, 1:2,000), Pax6 (Abcam, AB5790, 1:500). PLP (MP, MAB388, 1:200), S100 (DAKO, Z0311, 1:1,000), Sox10 (1:10,000), Sox10 (SCBT, SC17342, 1:100).

ChIP assay. Mouse E12.5 spinal cords were dissected, dissociated and processed for ChIP assays. Harvested cells were fixed with 1% formaldehyde (vol/vol) for 10 min. Cross-linked chromatin was then sheared by sonication and cleared by centrifugation. The samples were pre-cleared with protein G beads and immunoprecipitated using appropriate antibody or control IgG (Santa Cruz Biotechnology). Immunoprecipitated complexes were isolated, the cross-links reversed, and proteins digested with proteinase K. The DNA was purified and PCR was performed using region-specific primers. The following primers were used: MBP Forward 5′- TACAGGCCCACATTCATATCTC, Reverse 5′-TTCTTGGATGGTCTGAAGCTC; MBP-Control Forward 5′-CACAAC ACACAAGGAAAAGGAT, Reverse 5′-GGGGAAGAATGCTTCACTTAAT; PLP Forward-5′-TGGTCACACACAGTCTGTTCAT, Reverse 5′-GGGTCTG AATCAAAAGCCTACT; PLP-Control Forward 5′-TCTGTAAACACGGC TATTCAGC, Reverse 5′-TCAGGCCTCTTTTCTCAACATA; MAG Forward 5′-TTGATTCCTGGGTCCTACTAGC, Reverse 5′- AACTAGGAGAGGGTGT GTTTCC; MAG-Control 5′-ATGCCAGTCTAGACCCATTCTT, Reverse 5′-C GTGCAAAGCACATATACACAT; GFAP Forward 5′-CAGGGCCTCCTC TTCATG, Reverse 5′-TAGAGCCTTGTTCTCCACC; GFAP Control Forward 5′-AGTTACCAGGAGGCACTTGC, Reverse 5′-CGGTTTTCTTCGCCCTCCA; APCDD1 Forward 5′-ATTAAAGAAAGGCAGGACAGGA, Reverse 5′-ATG CCTCCAAAATATCCAGCTA; APCDD1-Control Forward 5′-GGTTCATG ATTCTGCACTCTGT, Reverse 5′-AGAGACAACCCTGTGAAGACAA.

Culture and transfection of cortical progenitors. Rat embryonic E14.5 cortex were dissected, dissociated, then plated at a density of 2×10^4 cells per cm^2 on plates coated with 15 μg ml^{-1} of polyornithine (Sigma) and 1 μg ml^{-1} human fibronectin (Biochemical Technologies). Cells were grown in DMEM/F12 supplemented with N2 and B27 (GIBCO) and 20 ng ml^{-1} bFGF (R&D Systems). Cells were expanded for 4 d followed by transfection with Lipofectamine 2000. In experiments requiring removal of bFGF to promote AS differentiation, bFGF was removed 24 h post-transfection and replaced with media described above supplemented with 2% FBS (vol/vol).

Statistics. ANOVA was used to analyze the luciferase reporter assays to determine the differences between group means; *t* test was used to compare individual means. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications[1,2,12,20]. Data distribution was assumed to be normal, but this was not formally tested. Blinding and randomization of samples was not used in the data analysis.

A **Supplementary Methods Checklist** is available.

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