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Regulation of the production and proliferation of oligodendrocyte progenitors in
development and injury

by

Abraham Langseth

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Copyright 2011

By

Abraham Langseth

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This work includes two published manuscripts. Their full citations are included here.

Langseth A.J., Munji R.N.*, Choe Y.*, Huynh T., Pozniak C.D., Pleasure S.J. (2010) Wnts Influence the Timing and Efficiency of Oligodendrocyte Precursor Cell Generation in the Telencephalon. *J Neurosci* 30:13367-13372.

* These authors contributed equally.

Pozniak C.D., **Langseth A.J.***, Dijkgraaf G.J.P.*, Choe Y., Werb Z., Pleasure S.J. (2010) Sox10 Directs Neural Stem Cells Toward the Oligodendrocyte Lineage by Decreasing Suppressor of Fused Expression. PNAS 107(50): 21795-21800

* These authors contributed equally.

Regulation of the production and proliferation of oligodendrocyte progenitors in development and injury

Abraham Langseth

Abstract

Oligodendrocytes are abundant cells in the central nervous system (CNS) with the important dual role of increasing the speed of action potential conduction by myelinating axons and providing metabolic support for the axons they ensheath. They are damaged in multiple diseases including Multiple Sclerosis and families of leukodystrophies and their replacement in injury and disease is an area of great interest. Oligodendrocytes are produced from a lineage-restricted oligodendrocyte progenitor cells (OPCs). OPCs are distributed throughout the CNS and are of interest, both as the source of oligodendrocytes, and as potential synaptic signaling partners with neurons. Little is known about the signals that control the production of OPCs from neural progenitor cells during development or the signals that regulate OPC proliferation and the maintenance of a pool of OPCs in the nervous system throughout life. For my thesis work I have shown that the canonical Wnt pathway inhibits the production of OPCs from intermediate progenitor cells and a decrease in Wnt signaling is necessary to allow OPC production. I have also shown that Suppressor of Fused (Sufu), a direct target of SoxE transcription factor regulation, is an inhibitor of OPC proliferation and that decreasing Sufu expression in the CNS increases the number of OPCs found in the CNS during development and in a demyelinating lesion at adult ages. My findings shed new light on the regulation of these early steps in the oligodendrocyte lineage.

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Chapter 1: Introduction

Oligodendrocytes

Oligodendrocytes are a highly abundant cell type in the central nervous system (CNS). They ensheath the axons of neurons with multiple wraps of specialized membrane called myelin and this allows for enhanced velocities of action potentials through salutatory action potential conduction (K. R. Jessen and R. Mirsky, 2005; D. L. Sherman and P. J. Brophy, 2005; M. Simons and J. Trotter, 2007; J. L. Salzer et al., 2008). The relevance of oligodendrocytes in myelination has been recognized for almost 100 years (B. Emery).

Myelin

Myelin seems to have first evolved in placoderms (fish with a hinged jaw). Invertebrates and lower vertebrates generally rely on increasing the diameter of axons to allow for increased action potential velocity, but placoderms and more highly evolved vertebrates achieved this effect by insulating small-diameter axons with myelin (B. Zalc et al., 2008). In vertebrates generally smaller diameter axons are left unmyelinated while larger diameter axons are myelinated. Signals that regulate myelination of axons in the peripheral nervous system (PNS) by Schwann cells include the axonally-derived signal neuregulin-1, which may determine which axons receive myelin in the PNS (K. A. Nave and J. L. Salzer, 2006). However, neuregulin-1 does not play an equivalent role in the CNS and the signals that regulate CNS myelination remain largely unidentified (B. G. Brinkmann et al., 2008), although several genes (myelin gene regulatory factor, Zfp191)

and signaling molecules (Notch, PSA-NCAM, LINGO-1, and Wnt) have been identified that are involved in the process of myelination (B. Emery).

Signals from the myelin (such as myelin-associated glycoprotein) can regulate axon diameter (K. A. Nave). In combination with regulation of myelin sheath thickness and node of Ranvier spacing between myelin bundles, this may allow for extremely precise regulation of action potential conduction velocities. Myelin also provides metabolic support for axons to maintain the health of the ensheathed neurons (K. A. Nave, ; S. Quintes et al.).

Oligodendrocyte progenitor cells

Oligodendrocytes are derived from oligodendrocyte progenitor cells (OPCs). This potential was first observed in vitro approximately 30 years ago (M. C. Raff et al., 1983). OPCs are lineage-restricted mitotic progenitor cells. During embryonic development, OPCs are produced by subventricular progenitor cells in the brain and spinal cord. This production occurs in waves from spatially and temporally-restricted domains of progenitor cells (N. Kessaris et al., 2006; W. D. Richardson et al., 2006). Following specification, OPCs migrate, proliferate, and distribute themselves uniformly throughout the CNS (S. Y. Chong and J. R. Chan, ; W. D. Richardson et al., 2006). A population of OPCs (also termed NG2 cells) is maintained in the adult CNS. They are capable of responding to injury and may also receive synaptic communication with neurons. This synaptic signaling from neurons in developing and adult brains may be involved in the regulation of OPC proliferation, migration, and differentiation into oligodendrocytes (D. E. Bergles et al., ; S. Y. Chong and J. R. Chan, ; L. M. De Biase et al., ; S. H. Kang et al., ; W. D. Richardson et al., ; M. Paukert and D. E. Bergles, 2006; L. E. Rivers et al., 2008). Other OPCs received the right combination of extrinsic and

intrinsic signals to cause maturation into oligodendrocytes (S. Y. Chong and J. R. Chan, ; B. Emery). Further signals are required to cause these oligodendrocytes to ensheath axons with myelin (B. Emery, ; S. Y. Howng et al., ; C. Birchmeier and K. A. Nave, 2008).

Production of OPCs

A key step in the oligodendrocyte lineage is the production of OPCs from neural progenitor cells. This step is tightly controlled both spatially and temporally in the developing CNS (N. Kessaris et al., 2006; W. D. Richardson et al., 2006). OPCs are generated in a ventral to dorsal pattern. In the spinal cord OPCs are first produced from the ventral pMN domain at E12.5 and then later from a dorsal Mash1-positive source at E15.5 (W. D. Richardson et al., 2006). In the telencephalon production begins from the medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) at E12.5. A second wave of OPCs is generated from the lateral ganglionic eminence (LGE) at E15.5. A final wave of OPC production occurs from the cortical progenitor zone at approximately P0 (N. Kessaris et al., 2006).

The generation of the progenitor domains which give rise to OPCs is tightly regulated by the interaction between diffusible signaling molecules from signaling centers and their concentration-dependent activation of transcription factors. One of the best understood examples is the patterning of the pMN domain of the spinal cord which first gives rise to motor neurons and then later to OPCs during development. In the developing spinal cord, Wnts and brain morphogenic proteins (BMPs) are expressed dorsally from the roof plate and Sonic Hedgehog (Shh) is expressed ventrally from the floor plate. As distance from the floor plate increases, the concentration of Shh decreases. Different levels of Shh activate different homeodomain transcription factors

at different dorso-ventral locations along the spinal cord. These transcription factors then cross-repress each other to sharpen the boundaries between different domains. In the pMN domain that gives rise to OPCs, Shh in combination with retinoic acid and fetal growth factor signaling induce the specification of pMN domain-specific transcription factors. Shh signaling directly activates the expression of Olig1 and Olig2, transcription factors that are known to be vital for the production of OPCs (M. Fuccillo et al., 2006; G. Lupo et al., 2006).

Canonical Wnt signaling and the oligodendrocyte lineage

The signals that regulate this spatially and temporally restricted process had not been clearly elucidated and were a major focus of my thesis work. I have shown that decreasing canonical Wnt signaling is necessary for the production of OPCs from neural progenitor cells. Canonical Wnt signaling is a well-characterized process. In the absence of Wnt ligand β -catenin is ubiquitinated and degraded by the proteasome. This leaves the TCF/LEF transcription factors bound to co-repressors and suppressing expression of target genes. Extracellular Wnt ligands interact with frizzled and LRP5/6 co-receptors and this results in the stabilization of β -catenin which enters the nucleus, binds to TCF/LEFs, and activates target gene expression (R. T. Moon et al., 2004). Wnt signaling is known to regulate the proliferation and maturation of OPCs into mature oligodendrocytes both in development and injury (K. Azim and A. M. Butt, ; M. Tawk et al., ; T. Shimizu et al., 2005; S. P. Fancy et al., 2009; B. D. White et al., 2009). A recent report has shown that β -catenin is involved in OPC production and maturation (F. Ye et al., 2009), but this study did not differentiate between the many roles of β -catenin in cell polarity, cell adhesion, and signaling pathways other than Wnt which involve β -catenin. By careful dissection of the Wnt signaling pathway using both intracellular and

extracellular regulators I have shown that Wnt signaling regulates the timing and extent of OPC production from the cortical progenitor domain during development.

SoxE's and Sufu

The SoxE (Sox8, 9, and 10) transcription factor family is an important regulator of the oligodendrocyte lineage, controlling the production of OPCs from neural progenitors and the maturation of OPCs into oligodendrocytes (C. C. Stolt and M. Wegner, ; C. C. Stolt et al., 2002; C. C. Stolt et al., 2003; C. C. Stolt et al., 2004; C. C. Stolt et al., 2005; S. Kellerer et al., 2006; C. C. Stolt et al., 2006; M. Finzsch et al., 2008). Expression of PDGFR α and MBP are known to be regulated by SoxE transcription factors (C. C. Stolt et al., 2002; M. Finzsch et al., 2008) but other genes regulated by these transcription factors in the oligodendrocyte lineage have not been identified.

Christine Pozniak, a post doc in the Pleasure Lab, identified Suppressor of Fused (Sufu) as a novel target of SoxE regulation (C. D. Pozniak et al.). Sufu is an inhibitor of the Sonic Hedgehog pathway and acts by promoting the nuclear export of the Gli transcription factors as well as regulating the processing of Gli's into activator and repressor forms (Y. Chen et al., ; E. W. Humke et al., ; H. Tukachinsky et al., ; C. Wang et al., ; M. H. Chen et al., 2009; J. Jia et al., 2009; L. Ruel and P. P. Therond, 2009). Sufu has also been reported to inhibit canonical Wnt signaling through the nuclear export of beta-catenin (X. Meng et al., 2001). Sufu has been shown to play several roles in the nervous system including patterning during early embryonic development (J. J. Kim et al., ; A. F. Cooper et al., 2005; J. Svand et al., 2006) and maintenance of multipotent progenitor cells (M. A. Cwinn et al.). I collaborated with Christine and we determined that Sufu inhibits production of OPCs from neural progenitor cells in culture and that part of the mechanism by which SoxE transcription factors enhance OPC

production is by the suppression of Sufu expression. We also showed that mice heterozygous for Sufu have increased numbers of OPCs in the forebrain at embryonic and early postnatal ages.

I am extending these studies to determine the mechanism by which Sufu increases OPC numbers. I have ruled out changes in progenitor zone patterning, specification of OPCs from subventricular progenitor cells, OPC apoptosis, and OPC maturation. My current hypothesis is that Sufu regulates the proliferation of OPCs and this is supported by data from mice lacking Sufu specifically in Olig2-positive cells. I have also show that Sufu heterozygous mice have increased numbers of OPCs in a demyelinating lesion but no change in the number of mature oligodendrocytes that result in the lesioned area, paralleling the increased number of OPCs observed in these mice in development with no change in the onset or extent of myelination.

A signaling pathway regulated by Sufu to cause these effects is of great interest, as is the role of Sufu in the proliferation of neural progenitor cells. These are active areas of investigation in the Pleasure Lab and we will continue and extend the studies presented here.

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Chapter 2: Published Manuscript 1

Development/Plasticity/Repair

Wnts influence the timing and efficiency of OPC generation in the telencephalon

Wnts influence OPC generation

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Abstract

Oligodendrocyte precursor cells (OPCs) are generated from multiple progenitor domains in the telencephalon in developmental succession from ventral to dorsal. Previous studies showed that Wnt signaling inhibits the differentiation of OPCs into mature oligodendrocytes. Here we explored the hypothesis that Wnt signaling limits the generation of OPCs from neural progenitors during forebrain development. We manipulated Wnt signaling in mouse neural progenitor cultures and found that Wnt signaling influences progenitors cell autonomously to alter the production of OPCs, and that endogenous Wnt signaling in these cultures limits the efficiency of generating OPCs from neural progenitors. To examine these events *in vivo*, we electroporated a soluble Wnt inhibitor or a dominant-negative transcriptional regulator into embryonic mouse neocortical ventricular zone before the usual onset of OPC production and showed that decreasing Wnt signaling in cortical progenitors results in early production of OPCs. Our studies indicate that Wnt signaling influences the timing and extent of OPC production in the developing telencephalon.

Introduction

Oligodendrocyte precursor cells (OPCs) are generated from progenitor zones in the forebrain beginning at various times during embryonic development (N. Kessaris et al., 2006). The mechanisms regulating the spatial and temporal production of OPCs have not been clearly elucidated; in particular, it is not clear why OPCs appear at later stages dorsally than ventrally. Sonic Hedgehog regulates the production of OPCs from

ventral progenitor zones, but the signaling pathways that regulate the dorsal generation of OPCs are only beginning to be elucidated (W. D. Richardson et al., 2006).

Wnts are secreted glycoprotein ligands that regulate development of dorsal CNS structures (K. J. Lee and T. M. Jessell, 1999). Wnts have prominent roles in progenitor cell proliferation, cell lineage and differentiation decisions (J. L. Freese et al., 2009). In the telencephalon Wnt signaling plays a prominent dorsalizing role (S. M. Lee et al., 2000; A. S. Kim et al., 2001; M. Backman et al., 2005; C. J. Zhou et al., 2006; O. Machon et al., 2007; A. Chenn, 2008; A. A. Gulacsi and S. A. Anderson, 2008; J. L. Freese et al., 2009). Some have suggested that the dorsal-ventral gradient of Wnt signaling in the cortex is related to the gradient of cortical neuronal differentiation (O. Machon et al., 2007). Studies have shown that Wnts inhibit the differentiation of OPCs into oligodendrocytes and thereby regulate myelination and remyelination following injury (T. Shimizu et al., 2005; S. P. Fancy et al., 2009; F. Ye et al., 2009).

These factors raise the possibility that Wnt ligands in the dorsal forebrain influence the generation of OPCs from neural progenitors, thereby controlling the timing of OPC production. A recent report (F. Ye et al., 2009) demonstrated that disrupting β -catenin changes the number of OPCs in the developing CNS and that Wnts work with histone deacetylases to regulate oligodendrocyte differentiation. However, this study leaves a number of unanswered questions since it relied on the Olig1-credriver line expressed very early in the forebrain and on manipulating β -catenin directly. Reliance on loss-of-function alleles of β -catenin does not allow exclusion of non-Wnt functions of β -catenin in the ventricular zone such as cell adhesion. Also, reliance on dominantly active β -catenin can lead to effects that are not normally regulated by Wnt signaling *in vivo*.

We wanted to determine whether Wnt signaling influences the generation of OPCs from neural progenitor cells and whether it plays a role in the delayed appearance of OPCs from dorsal telencephalic regions. We regulated the levels of extracellular Wnt ligand and cell-autonomously altered Wnt signaling. We show that Wnt signaling negatively regulates the specification of OPCs from neural progenitors and that inhibition of Wnt signaling drives a robust pre-scheduled increase in the production of OPCs in the cortex. This indicates that Wnts influence the timing and balance of OPC production from ventricular zone progenitors.

Methods

Animals. All animal protocols were approved by the UCSF IACUC. P5 CD1s (Charles River) were used for cultures. BAT-gal mice (S. Maretto et al., 2003) were used for β -galactosidase (β -gal) expression analysis and for DNLe1 electroporations. C57Bl6 mice (Jackson) were used for Dkk1 electroporations. Males and females were used for all experiments.

Retroviral production. pLNCX2 (Clontech) was modified to include a new multiple cloning site and histone H2B fused to monomeric red fluorescent protein (pLH2BmRFP). A dominant-negative Lef1 (DNLe1) construct (Lef1 (Clontech) with a truncated N-terminus) was inserted. Retrovirus was produced in GP2-293 cells (Clontech) and concentrated using standard methods.

In utero electroporations (EP). See (G. Li et al., 2008) for method. EPs were targeted to the lateral cortex at E13.5 or E15.5 and collected 4 or 6 days later. The DNLe1 construct was inserted into pCAGGS (chicken β -actin cytomegalovirus promoter-driven

expression). pCIG (GFP) (from A. McMahon (S. G. Megason and A. P. McMahon, 2002)) was used as a control. pCIG-Dickkopf-1 was provided by L.W. Burrus.

Immunohistochemistry and X-Gal staining. Brains were processed using standard methods. Cells and tissue sections were stained using standard protocols with: rat anti-PDGFR α (BD Biosciences Pharmingen, 1:500 for tissue and 1:800 for cells), rabbit anti-Olig2 (Millipore, 1:1000 for tissue), mouse anti- β -gal (Promega, 1:2000 for tissue and 1:6000 for cells), and mouse anti-O4 (Sigma, 1:30 for cells). Fluorescent secondary antibodies (1:1000, Invitrogen) and DAPI (1:3000, Sigma) were used. For X-gal staining, floating sections were stained with X-gal substrate (Invitrogen).

Cell Culture. Neural progenitor cells (NPCs) were from lateral ventricle SVZ tissue of P5 CD1s. Tissue was dissociated with 0.1% Trypsin (Worthington) and 0.1% DNase1 (Roche). DMEM/F12 (50:50, Gibco) was supplemented with 10X hormone mix (40 mg transferrin, 10 mg insulin, 3.86 mg putrescine, 4.0 ml 3mM selenium, 4.0 ml 2mM progesterone, 10 ml 2 mg/ml Heparin [all from Sigma]), 0.8 ml 30% glucose, 0.6 ml 7.5% NaHCO₃, 10 ml 30% glucose, 7.5 ml 7.5% NaHCO₃, 2.5 ml 1 M HEPES, 5 ml 200 mM glutamine, 5 ml Pencillin-Streptomycin, 2 ml Fungizone). Media containing EGF (Sigma, 10 ng/ml), FGF (Sigma, 20 ng/ml) and B27 (Invitrogen) was added. Cells were plated at 80,000 cells per 25 cm² flask (Corning), grown in 5 ml of complete media plus growth factors and B27. Passage 1 or 2 NPCs were used.

Cell culture – Wnt treatments. NPCs were dissociated with 0.05% trypsin-EDTA, washed with PBS, pelleted and resuspended in 500 μ l of complete media with 2% FBS. The cells were plated on laminin-coated (1 mg/ml, Invitrogen) chamber slides (Nunc) at 37,500 cells/well. Mouse Wnt3a (0.15ng/ μ L), Dickkopf1 (Dkk1, 0.03ng/ μ L), or an

equivalent volume of PBS were added to each well. One half of the media was replaced each day. The cultures were analyzed after 4 days *in vitro* (DIV).

Cell culture – Retroviral infection. NPCs were dissociated, washed with PBS, pelleted and resuspended in 500µl complete media containing EGF, FGF, B27 and 5 µg/ml polybrene (Sigma). Concentrated virus (50-100 µl; approx 1×10^5 transducing units) was added to approximately 300,000 cells, which were spin infected for 90-120 min at 170 x *g*. The cells were plated in complete media containing growth factors in a 25cm² flask for 24 h to allow integration and expression. Cells were resuspended in complete media with 2%FBS and plated on chamber slides at 37,500 cells/well and analyzed after 4 DIV.

Image analysis and quantification. Three to four biological replicates of each experiment were performed. Ten 20X magnification images were quantified for each replicate of each cell culture condition. Cell counts from 3-4 coronal sections of each EP brain were averaged.

Statistical analysis. Results are expressed as mean $\pm$ SEM. Data were analyzed using two-tailed Student's *t* test with unequal variance. Multiple comparisons were made using ANOVA with a post-hoc Holm-Sidak test. Any value of $p \leq 0.05$ was considered significant.

Results

Wnt signaling regulates OPC generation from telencephalic progenitor cells

To test the role of Wnt signaling in the generation of OPCs, we cultured neural progenitors from the telencephalons of perinatal mice. We plated the progenitors and exposed them to FBS to induce differentiation along with soluble mouse Wnt3a ligand

(Fig. 1C,D), soluble mouse Dickkopf1 (Dkk1) Wnt inhibitor (Fig. 1E,F), or an equivalent volume of PBS as a control (Fig. 1A,B) for 4 days *in vitro* (DIV) and then labeled the cells with the OPC markers PDGFR α and O4. Treatment with Wnt3a caused a 25% decrease in the percentage of OPCs and inhibition of Wnt signaling by Dkk1 caused a 54% increase in the percentage of OPCs compared to controls (Fig. 1K). This was not a result of changes in the proliferation or apoptosis of OPCs (Fig. 1L). These results indicate that Wnt signaling influences the generation of OPCs from progenitor cells.

To determine whether this is a cell-autonomous effect, we infected progenitors with a retrovirus encoding a dominant-negative form of Lef1 (DNLef1-mRFP) (Fig. 1I,J, arrowheads indicate infected OPCs) or a nuclear mRFP control vector (Fig. 1G, H). PDGFR α and O4 labeling revealed a doubling in the percentage of infected OPCs 4 days post infection (Fig. 1M). To ensure that this effect was actually autonomous, we counted the OPCs among uninfected cells and found no difference between control and experimental conditions (Supplemental Fig. 1).

Our data indicate that Wnt signaling influences the production of OPCs from neural progenitors, but we wanted to determine whether high levels of Wnt signaling are inconsistent with the decision of neural progenitors to generate OPCs. To examine this, we cultured neural progenitors from a Wnt signaling reporter mouse (BAT-gal). These cultures were differentiated for 4 DIV and then labeled with antibodies for β -gal (Fig. 1N, arrows) and PDGFR α (arrowheads). Similar to previously published reports (S. P. Fancy et al., 2009; B. D. White et al., 2009), we found that β -gal was expressed in approximately 6% of the OPCs in our cultures. This indicates that part of the normal differentiation program for OPCs involves a lack of significant Wnt signaling at the time of OPC specification.

Wnt signaling regulates the timing of OPC generation during development

A previous report (N. Kessaris et al., 2006) showed that there are multiple waves of OPC production beginning ventrally and that cortical production does not begin until ~P0. We wanted to assess global levels of Wnt signaling in the cortical progenitor zone before and after the onset of cortical OPC production. X-gal staining of BAT-gal brains revealed that Wnt signaling is very high in cortical progenitors at E17.5 (before cortical OPC production) and decreased by P5 when OPC production is prominent (Fig. 2A,B). β -gal and PDGFR α labeling (Fig. 2C,F) at E17.5 showed many cells in the cortical VZ/SVZ/IZ and many presumptive neurons in the cortex that have strong Wnt signaling (Fig. 2D,E), but at P5 the number of cells near the ventricle with strong Wnt signaling was decreased and the OPCs in the cortex do not show evidence of strong Wnt signaling (Fig. 2G,H). At E17.5 many OPCs that were generated from ventral progenitor domains had migrated to the cortex and were within the region of strong Wnt signaling (Fig. 2C,D) (N. Kessaris et al., 2006), but no OPCs are generated from the cortical progenitor zone at this age (Fig. 2E) (N. Kessaris et al., 2006). We replicated the lineage tracing of cortically derived OPCs performed previously (N. Kessaris et al., 2006) using Emx1-Cre and found that few OPCs in the cortex at P0 are derived from the cortical progenitor domain but that by P4 cortically derived OPCs were abundant (data not shown). At P5 OPCs were throughout the cortex (Fig. 2F), including in the progenitor zone, which showed reduced Wnt signaling (Fig. 2H). At this stage, many of the cells that showed the strongest Wnt signaling are associated with presumptive blood vessels (Fig. 2G arrowhead) (C. A. Franco et al., 2009).

Manipulation of Wnt signaling in progenitors changes the timing of OPC generation

The timing of decreasing Wnt signaling in cortical progenitors raises the possibility that OPC generation from the cortex is limited by the strong Wnt signaling present prenatally and that this is a factor in determining the onset of OPC production. To test this idea we performed *in utero* electroporations (EP) of a DNLeF1 or control mRFP construct into neocortical progenitors at E15.5 and collected the embryos 4 days later at P0.5 (Fig. 3A,B, arrowheads indicate EP OPCs). This approach alters the level of Wnt signaling in primary multipotent cortical progenitors and addresses whether Wnt signaling influences production of OPCs from these cells. PDGFR α and Olig2 antibody labeling showed that there was a 66% increase in the percentage of EP OPCs after DNLeF1 electroporation compared to controls (Fig. 3E). No changes in the mitotic fraction or apoptosis of EP OPCs were observed (data not shown). This indicates that shutting off the transcriptional output of the Wnt pathway in progenitors drives an increase in the production of OPCs.

To determine whether the endogenous levels of Wnt ligand present in the brain limit early OPC production, we performed EPs of Dkk1 or control GFP (Fig. 3C, D, white boxes indicate region of quantification) at E15.5 and collected the brains 6 days later. PDGFR α and Olig2 antibody labeling showed a 61% increase in the number of OPCs in the EP region (Fig. 3F). This manipulation is not cell-autonomous and influences both the neural progenitors and the OPCs already present in the cortex. Since others have shown that Wnt signaling is observed in a subset of cortical OPCs and may drive their proliferation (S. P. Fancy et al., 2009; B. D. White et al., 2009), the Dkk1 EP may decrease proliferation of OPCs already present in the cortex. Therefore, the decrease in Wnt signaling in the progenitor domain must cause an increase in the number of OPCs

produced which overcomes their decreased drive to proliferate. This indicates that decreasing Wnt signaling in progenitors or decreasing their exposure to endogenous levels of Wnt ligand in the environment leads to an early onset of cortical OPC generation.

Discussion

Our study shows that Wnt signaling in progenitor cells influences the generation of OPCs in a cell-autonomous manner. Strong Wnt signaling inhibits OPC generation and low levels of Wnt signaling both accompany and drive OPC generation. This mechanism is used during development to influence the timing of OPC generation and is a significant factor influencing the efficiency of OPC production from neural precursor cells.

Decreased Wnt signaling accompanies the appearance of and drives production of OPCs

We have shown that Wnt signaling in the cortical progenitor domain is strong at embryonic ages when mostly neurons are produced and that there is a significant decrease in Wnt signaling when OPCs are generated. The addition of Wnt3a ligand to neural progenitor cultures causes a significant decrease in the percentage of OPCs produced from these progenitors, whereas addition of Dkk1 strongly increases the percentage of OPCs produced. This indicates that Wnt ligands and inhibitors are capable of acting directly on neural progenitors and that changes in the extracellular concentrations of Wnts and inhibitors can significantly influence the lineage decisions of

progenitors. The lesser effect of Wnt3a compared to Dkk1 implies that significant Wnt ligand levels natively found in these culture systems are one inhibitor of OPC production.

Retroviral infection of DNLeF1 into neural progenitors drove a significant increase in the percentage of OPCs produced from these infected progenitors without influencing the uninfected cells. This indicates that Wnt signaling acts autonomously to influence lineage decisions of individual progenitor cells, and that lower Wnt signaling levels favor the production of OPCs. Previous reports indicate that Wnt signaling drives proliferation in OPCs (S. P. Fancy et al., 2009; B. D. White et al., 2009) and blocks terminal differentiation during development and remyelination (T. Shimizu et al., 2005; S. P. Fancy et al., 2009; F. Ye et al., 2009). We did not see these effects in our neuronal progenitor cultures when exposed them to Wnt ligand or inhibitors for short periods of time. However, when we exposed our cultures to Wnt3a ligand for 8 days we observed a 46% increase in the percentage of OPCs in the Wnt3a condition caused by a 257% increase in the proportion of proliferating OPCs (apoptosis of OPCs does not explain these changes) (Supplemental Fig. 2).

To test the influence of Wnt signaling on the timing of OPC production from cortical progenitors, we electroporated a Dkk1-expressing construct into the progenitor zone of the lateral embryonic cortex at E15.5, before the time of cortical OPC production. At P2 the number of OPCs observed in the electroporated region was increased despite the potential for decreased proliferation of the earlier born OPCs caused by decreased Wnt signaling (S. P. Fancy et al., 2009; B. D. White et al., 2009). This indicates that Wnts act on the cortical progenitors and that decreasing Wnt signaling in a physiologically relevant manner, without manipulating intracellular Wnt signaling components, influences the cell types produced from cortical progenitors.

We also determined that Wnt signaling acts on the cortical progenitors in a cell-autonomous manner by electroporating a DN Lef1 -expressing construct into the lateral cortical progenitor zone at E15.5 and examining OPC production 4 days later, at P0.5 when cortical production of OPCs normally begins (N. Kessaris et al., 2006). These studies show that shutting off the transcriptional output of the Wnt pathway in the primary progenitors of the embryonic cortex and their progeny significantly increases the percentage of electroporated cells becoming OPCs. This indicates that Wnt signaling acts cell-autonomously on cortical progenitors to influence the production of OPCs. Whether Wnts also act to regulate migration of OPCs to the cortex is not something we explicitly examined in our studies, however, our electroporation experiments with DKK1 did not show any change in the overall distribution of OPCs. Further studies might be warranted to examine possible roles of Wnts in OPC migration.

Wnt signaling is not the only mechanism influencing the production of OPCs. We electroporated DN Lef1 into the lateral cortical progenitor zone at E13.5 and analyzed the brains at E17.5, several days before the reported onset of cortical OPC production (N. Kessaris et al., 2006). We observed no significant difference in the percentage of electroporated cells positive for OPC markers (data not shown). This indicates that there are additional mechanisms inhibiting OPC production at earlier stages in development.

Our studies indicate that high Wnt signaling has contrasting effects on the oligodendrocyte lineage at different developmental stages – blocking specification of OPCs but driving the proliferation of already established OPCs (this latter finding in agreement with previous publications (S. P. Fancy et al., 2009; B. D. White et al., 2009)). In addition, Wnt signaling is known to inhibit OPC differentiation during development and remyelination (T. Shimizu et al., 2005; S. P. Fancy et al., 2009; F. Ye et al., 2009). Thus

Wnt signaling plays multiple roles at distinct points in the oligodendrocyte lineage.

Possible mechanisms for the effects of Wnt signaling on OPC specification come from previous studies showing that Wnt signaling down regulates Olig2 gene expression in

HCN cells (F. Ye et al., 2009) and induces the expression of the neurogenic gene

NeuroD1 in adult hippocampal sub-granular zone progenitors (Z. Gao et al., 2009; T.

Kuwabara et al., 2009). If a similar mechanism exists in the SVZ during development, it could strongly favor neuronal production.

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Figure Legends

Figure 1. High Wnt tone decreases OPC production from neural progenitors while low Wnt tone enhances production. Application of Wnt3a to neural progenitor cultures for 4 days decreased the number of PDGFR α - (**C**, 11.34 $\pm$ 0.93%, mean percent of total cells $\pm$ standard error) and O4-positive cells (**D**, 8.40 $\pm$ 0.53%) and application of Dkk1 antagonist increased the number of OPCs (**E**, 23.14 $\pm$ 1.88%; **F** 15.48 $\pm$ 1.56%) compared to control cultures (**A**, 15.06 $\pm$ 0.89%, **B**, 11.38 $\pm$ 0.75%). This is graphed in (**K**). This is not a result of proliferation (Ki-67) or apoptosis (activated Caspase3) of OPCs as the percentage of proliferating OPCs was greatest in the Wnt3a group (3.19 $\pm$ 0.65%, Control = 1.89 $\pm$ 0.46%, Dkk1 = 2.30 $\pm$ 0.27%) and the percentage of apoptotic OPCs was greatest

in the Dkk1 group and significantly greater than control (Dkk1 = $1.78 \pm 0.49\%$, Control = $0.15 \pm 0.08\%$ Wnt3a = $0.20 \pm 0.09\%$) (**L**). Retroviral infection of neural progenitors with DNLe1-mRFP results in a significant increase in PDGFR α -positive cells (**I**, $23.74 \pm 3.26\%$) and O4-positive cells (**J**, $20.07 \pm 1.99\%$) by 4 days post infection compared to mRFP control infection (**G**, $8.18 \pm 1.18\%$; **H**, $10.12 \pm 1.62\%$). Arrowheads indicate infected OPCs. This is graphed in (**M**). N = 3 for all groups. Neural progenitor cells were cultured from BAT-gal mice. (**N**) $6.32 \pm 1.13\%$ of PDGFR α -positive BAT-gal cells (arrowheads) show detectable Wnt signaling (arrows). Scale bars = 10 μ m. (*) indicate $p \leq 0.05$ for ANOVA in (**K**) and Student's *t* test in (**L** and **M**).

Figure 2. Wnt tone decreases in progenitor zones before they produce OPCs. **A, B**, BAT-gal sections were treated with Xgal. **A**, Cortical cells have high Wnt tone during neurogenesis (E17.5). **B**, Wnt tone in the cortex and SVZ is significantly decreased by the time of OPC production (P5). **C-H**, β -galactosidase and PDGFR α immunohistochemistry of BAT-gal forebrain. **C**, At E17.5, cells near the ventricle and in the cortex show strong Wnt signaling. Higher magnification images (**D, E**). **F**, By P5 only cortical blood vessels and scattered cortical cells show strong Wnt tone. Higher magnification images (**G, H**). Most PDGFR α -positive cells do not have strong Wnt signaling. Lines indicate lateral ventricle. Arrowhead indicates blood vessel. Scale bars = 500 μ m.

Figure 3. Decreasing Wnt tone in cortical progenitors results in a pre-scheduled increase in cortical OPC production. E15.5 EP of mRFP control (**A**) or DNLe1-mRFP

(**B**) into the lateral cortex were collected at P0. DNLeF1 EP caused an increase in the percentage of EP PDGFR α (DNLeF1 = $9.87 \pm 1.66\%$, RFP = $4.86 \pm 0.75\%$) and Olig2-positive cells (not shown, DNLeF1 = $9.62 \pm 1.13\%$, RFP = $3.24 \pm 1.11\%$). Arrowheads indicate EP cells that are PDGFR α -positive. This is graphed in **E**. E15.5 EPs of GFP control (**C**) or Dkk1-GFP (**D**) into the lateral cortex were collected at P2. A region of interest was selected in the center of the EP area (white box) and the total number of PDGFR α or Olig2-positive cells within the region were quantified. Scale bar = 500 μ m. **F**, Dkk1 EP resulted in a significant increase in the number of PDGFR α (205.25 ± 3.26) or Olig2-positive cells (346.50 ± 28.83) in the EP region compared to control (116.75 ± 9.92 PDGFR α and 215.50 ± 16.25 Olig2). N = 3-4 for all groups. (*) indicates $p \leq 0.05$ for Student's *t* test in (**E** and **F**).

Supplemental Figure 1. DNLeF1 infection of neural progenitors does not alter OPC production from uninfected cells. Uninfected cells from DNLeF1 or mRFP infected cultures were quantified for PDGFR α expression at 4 DIV. There was no difference in the percentage of uninfected OPCs (mRFP = $8.56 \pm 4.23\%$, DNLeF1 = $10.13 \pm 1.01\%$).

Supplemental Figure 2. Wnts induce OPC proliferation. Application of Wnt3a to neural progenitor cultures for 8 days significantly increased the number of PDGFR α -positive cells ($27.28 \pm 2.09\%$) compared to control cultures (**A**, $18.66 \pm 1.19\%$). This is a result of increased proliferation of OPCs as the percentage of proliferating OPCs was greatest in the Wnt3a group ($13.15 \pm 3.11\%$, Control = $3.68 \pm 0.38\%$) and was not a result of apoptosis (**B**, Wnt3a = $3.11 \pm 0.79\%$, Control = $0.35 \pm 0.13\%$). (*) indicate $p \leq 0.05$ for Student's *t* test.

Figure 1.

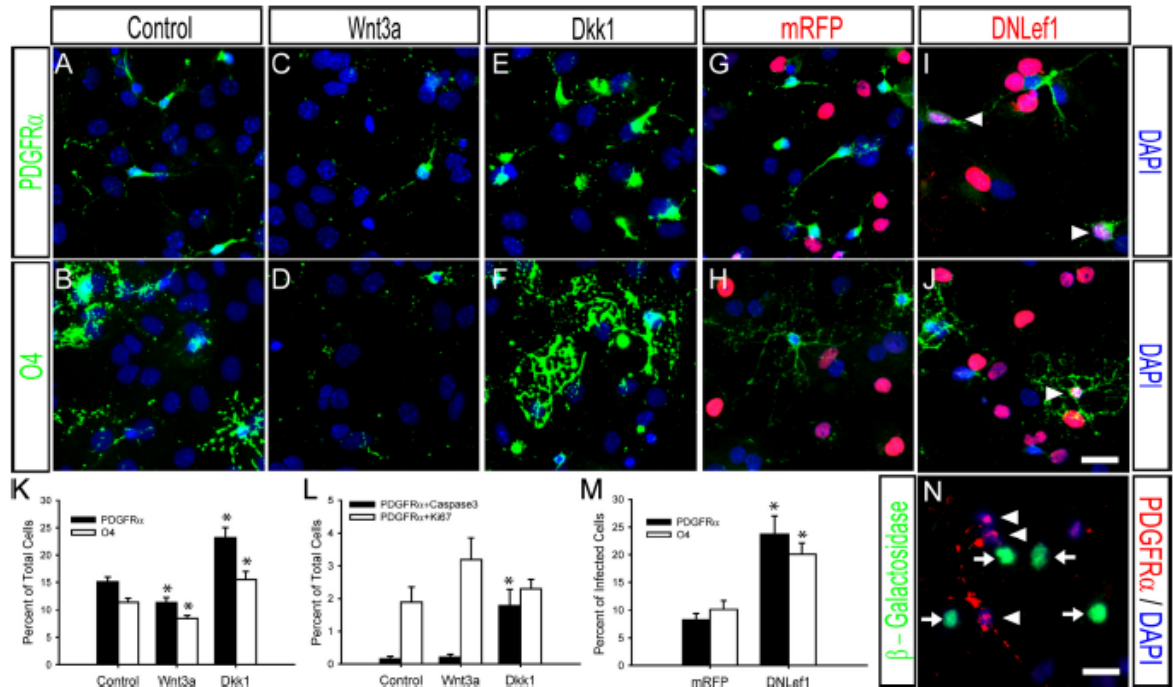


Figure 2.

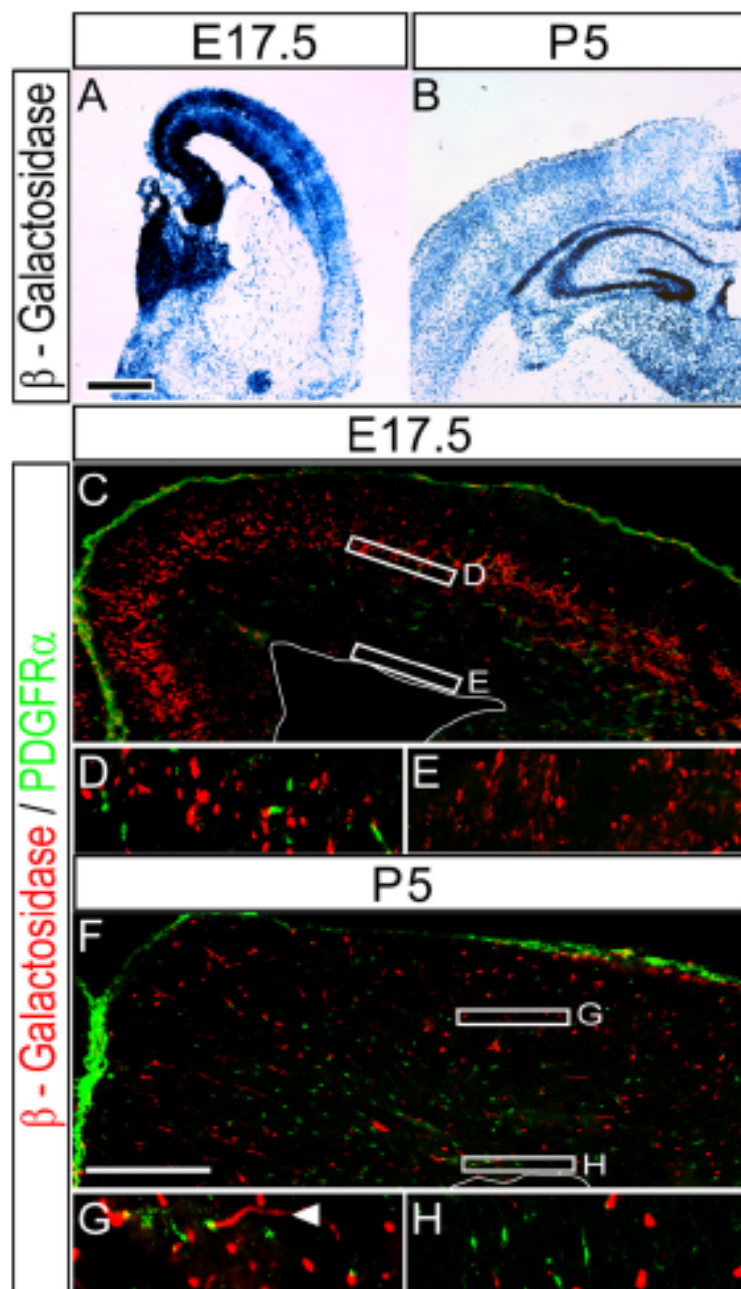
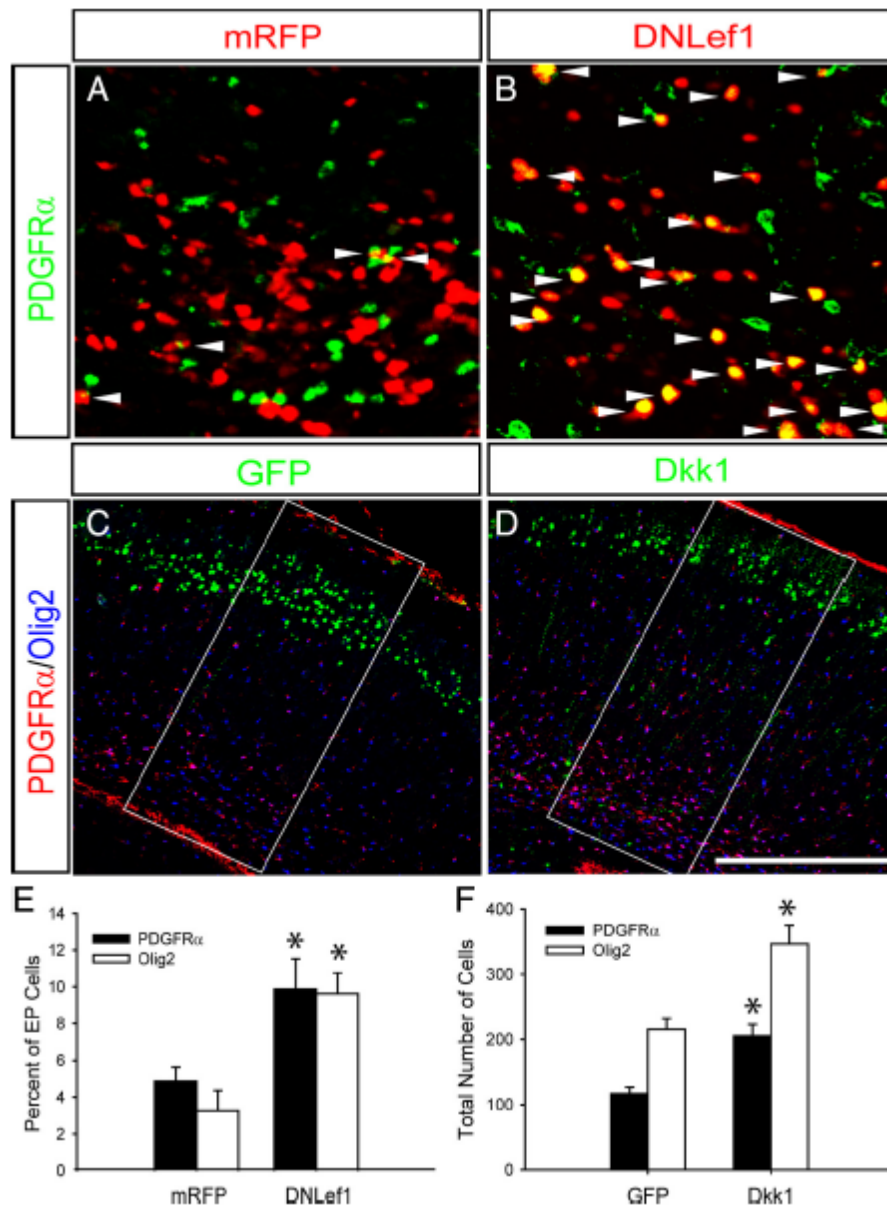
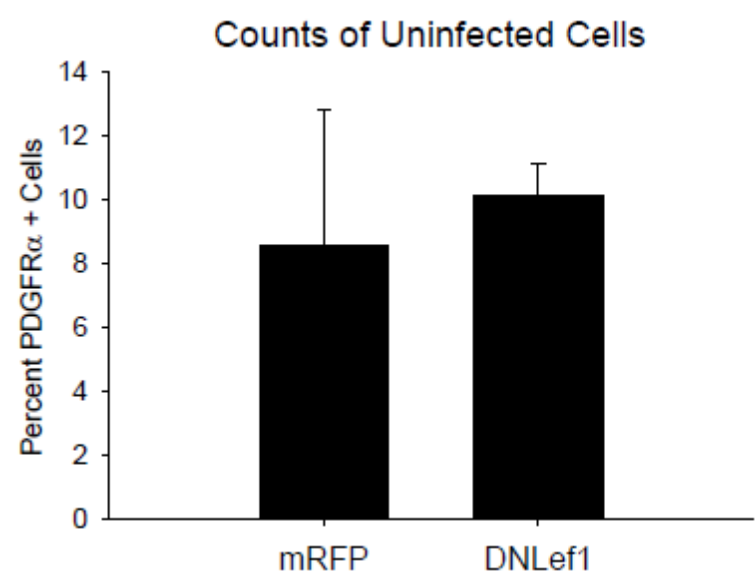


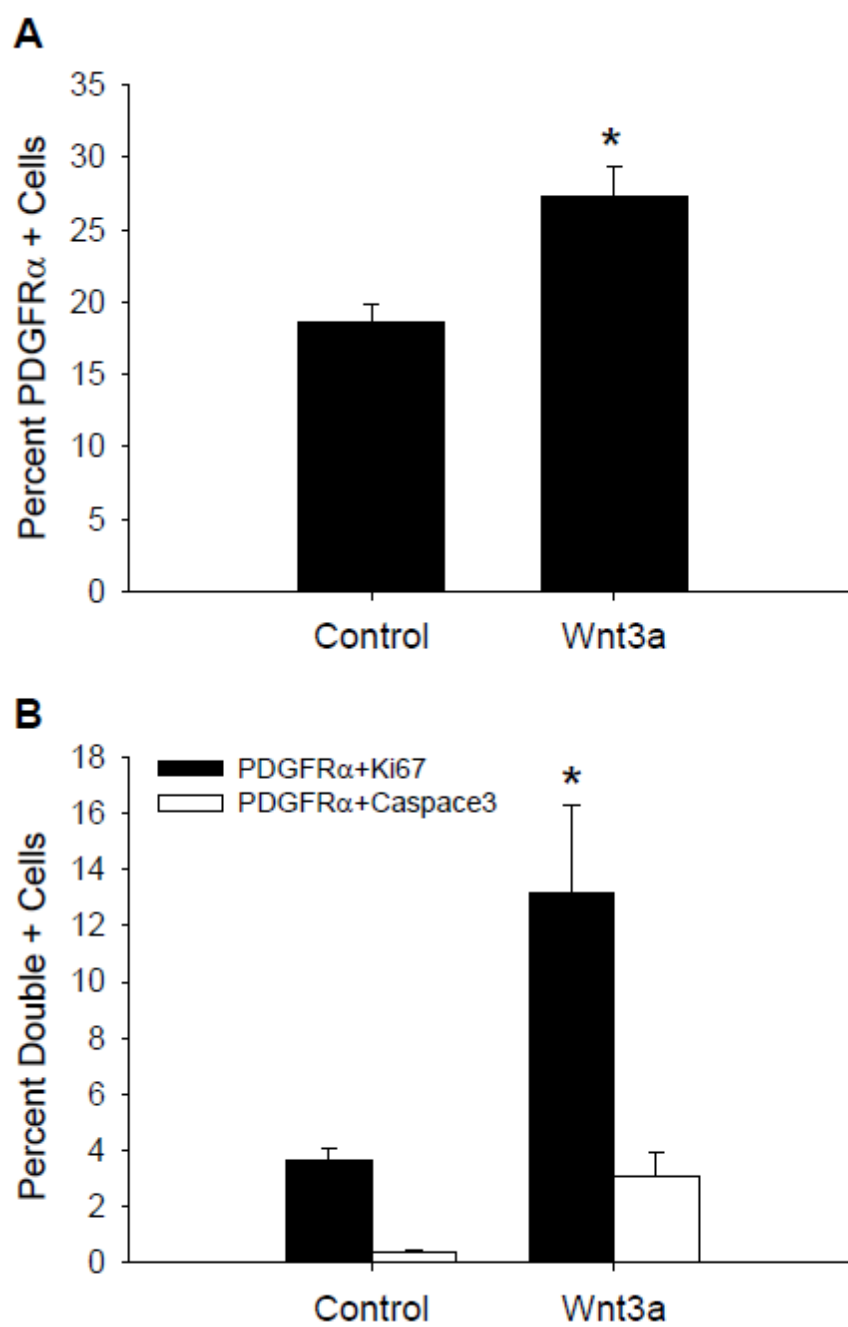
Figure 3.



Supplemental Figure 1.



Supplemental Figure 2.



Chapter 3: Published Manuscript 2

Sox10 directs neural stem cells toward the oligodendrocyte lineage by decreasing Suppressor of Fused expression

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4 Figures 1 Supplemental table, 1 Supplemental Materials

Author Contributions: CDP, AJL, GJPD, SJP – Designed research; CDP, AJL, GJPD, YC – Performed research; CDP, AJL, SJP – Analyzed data; CDP, AJL, GJPD, YC, ZW, SJP – Wrote the paper

Abstract

Oligodendrocyte precursor cells (OPCs) are lineage-restricted progenitors generally limited *in vivo* to producing oligodendrocytes. Mechanisms controlling genesis of OPCs are of interest due to their importance in myelin development and their potential for regenerative therapies in multiple sclerosis and dysmyelinating syndromes. We show here that the SoxE transcription factors (comprising Sox8, 9, and 10) induce multipotent neural precursor cells (NPCs) from the early postnatal subventricular zone (SVZ) to become OPCs in an autonomous manner. We performed a chromatin immunoprecipitation-based bioinformatic screen and identified Suppressor of Fused (Sufu) as a direct target of repression by Sox10. *In vitro*, overexpression of Sufu blocked OPC production, while RNAi-mediated inhibition augmented OPC production. Furthermore, mice heterozygous for Sufu have increased numbers of OPCs in the telencephalon during development. We conclude that Sox10 acts to restrict the potential of NPCs toward the oligodendrocyte lineage in part by regulating the expression of Sufu.

Introduction

Neural tube patterning is controlled by the interplay between homeobox genes and basic helix-loop-helix (bHLH) transcription factor families regulating cell fate, positional identity and the secreted morphogens that regulate them (S. W. Wilson and J. L. Rubenstein, 2000; J. Briscoe and J. Ericson, 2001). The transcriptional regulation of oligodendrocyte development is of increasing interest as they are the primary sources of CNS myelin and a target of attack in multiple sclerosis (W. D. Richardson et al., 2006). Early oligodendrocytes are produced from the ventral progenitor domain in the spinal cord that also produces cholinergic motor neurons, and from a ventral telencephalic domain that

produces GABAergic interneurons and cholinergic projection neurons (W. D. Richardson et al., 2006), while later waves of oligodendrocytes are specified from the dorsal neural tube (both in the spinal cord and cortex) (N. Kessaris et al., 2006; W. D. Richardson et al., 2006). Thus, progenitor cells from several distinct dorsal-ventral domains have the ability to adopt oligodendrocyte fate despite dramatic differences in regional specification by morphogenic signals.

Two transcription factor families: the bHLH proteins Olig 1 and 2 (N. Tekki-Kessaris et al., 2001; Q. R. Lu et al., 2002; H. Takebayashi et al., 2002; Q. Zhou and D. J. Anderson, 2002; M. A. Petryniak et al., 2007) and the SoxE family of transcription factors are implicated in the transcriptional control of oligodendrocyte development. Sox proteins contain a high-mobility-group (HMG) domain essential for interaction with and recruitment of other transcription factors (M. Wegner, 1999; S. Wissmuller et al., 2006) and have a fundamental role in oligodendrocyte genesis. Upon commitment to the oligodendrocyte lineage, expression of Sox1-3 is turned off and Sox8, 9 and 10 (classified as the SoxE group) are induced. Sox9 is important for oligodendrocyte specification, as there are reduced oligodendrocytes and increased numbers of motor neurons in the Sox9^{-/-} ventral spinal cord (C. C. Stolt et al., 2003). Sox10 is only expressed by OPCs and oligodendrocytes in the CNS (K. Kuhlbrodt et al., 1998), and essential for the generation of mature oligodendrocytes (C. C. Stolt et al., 2002; C. C. Stolt et al., 2004). Overexpression of Sox10 in the chick spinal cord induces ectopic oligodendrocytes (Z. Liu et al., 2007), furthermore, neurospheres isolated from Sox10 mutants do not myelinate axons after injection into the brains of dysmyelinating mice (C. C. Stolt et al., 2002). Sox10 also directly regulates the expression of myelin basic protein cooperatively with Olig1 (H. Li et al., 2007) and may regulate the expression of PDGFR α , another marker of the oligodendrocyte lineage (M. Finzsch et al., 2008c).

Thus, SoxE transcription factors are superb candidates for regulating oligodendrocyte fate (M. Wegner and C. C. Stolt, 2005) but whether they work to instructively induce oligodendrocyte fate and restrict the ability of multipotential cells to produce alternate fates remains unclear.

Here we demonstrate that expression of SoxE transcription factors drives multipotential NPCs to become OPCs *in vitro*. Moreover, an important Sox10 transcriptional effect is decreased expression of Sufu, a newly identified modulator of oligodendrocyte production.

Results

Expression of Sox8, 9 and 10 induce oligodendrocyte lineage markers in SVZ

progenitors Multipotential NPCs from the postnatal cortical SVZ produce OPCs *in vivo* (B. Menn et al., 2006). These progenitors should pass through a similar OPC-like state during differentiation into oligodendrocytes *in vitro*, and this transition should allow for a study of factors that can influence OPC specification. Undifferentiated NPC cultures did not express the OPC marker O4 (**Fig 1A**). However, within 2 days after induction to differentiate with serum, cells with all of these markers were present as a significant, but minority population (12% O4+ cells indicative of OPCs, 17% Tuj1+ cells indicative of neurons, 38% GFAP+ cells indicative of astrocytes). To determine whether forced overexpression of the SoxE or Olig transcription factors is sufficient to drive multipotential NPCs toward oligodendrocyte fate retroviruses expressing Sox8, Sox9, Sox10, Olig1 or Olig2 (together with a monomeric red fluorescent protein fused to histone H2B to track infected cells) were used to infect dissociated neurospheres. These cells were plated onto laminin 24 hours after viral infection and maintained in EGF/FGF growth conditions for a further 48 hours with no differentiation stimulus added (**Fig. 1A**).

Olig1 or Olig2 overexpression was insufficient to induce expression of OPC markers (**Fig. 1A**; Empty virus 0.3% +/- 0.08 O4+ cells, Olig1 virus 0.04% oligos +/- 0.03 O4+ cells, and Olig2 to 0.04% +/- 0.08% O4+ cells). By contrast, expression of any of the three SoxE transcription factors induced O4 glycolipid antigen expression, despite the continued presence of EGF and FGF (**Fig. 1A**). Sox10 overexpression consistently generated the greatest number of O4+ infected cells (usually >40% of infected cells became O4+) (**Fig. 1A-B**), however, Sox9 ($\approx$ 20% O4+ cells) and Sox8 ($\approx$ 15% O4+ cells) overexpression also led to a significant increase in the number of cells expressing O4 ($p < 0.005$). Thus, SoxE transcription factor expression is sufficient to induce rapid expression of markers of the OPC/oligodendrocyte lineage in neural progenitors without additional differentiation stimuli.

To determine whether Sox transcription factors played a role in increasing OPC specification and maturation we also took cultures separately infected with either the SoxE factors or Empty retrovirus and induced them to differentiate using two stimuli known to drive differentiation of neural stem cells: fetal bovine serum (FBS) or thyroid hormone (TH). The percentage of Sox8, 9 and 10 infected cells expressing either O4 or CNPase (a more mature OPC marker) increased dramatically by 2 days post differentiation in both FBS and TH and morphologically resembled oligodendrocytes (**Fig. 1C-E**). Therefore, in the presence of a differentiation signal Sox8, 9 or 10 all effectively increase OPC production and maturation.

Sufu is a predicted direct target of Sox 10 and is downregulated in Sox10 expressing NPCs.

To identify novel molecular targets involved in oligodendrocyte specification we combined Chromatin Immunoprecipitation (ChIP) with hybridization to a promoter array

after infecting SVZ progenitors with Sox10 retrovirus. We performed ChIP after infection with an equally efficient Sox10 retrovirus expressing 3 copies of the hemagglutinin (HA) epitope (Sox10-3xHA) and shearing of the chromatin to generate on average 1kb fragments of genomic DNA. The immunoprecipitated DNA-protein complexes were dissociated and the purified DNA fragments were randomly amplified by PCR and used to probe 50 bp tiled arrays made up of 5 kb of predicted upstream promoter regions for every annotated mouse gene (NimbleGen). From this analysis, we obtained a panel of genes with potential Sox10 binding sites that were statistically significant at $P < 1 \times 10^{-4}$ (**Suppl. Table 1**). We focused on genes that appeared in both the ChIP-on-chip screen and an ongoing profiling study comparing the mRNA expression changes in NPCs forced to express Sox10, Mash1 or Neurogenin2. Among the genes found to have both predicted Sox10 binding sites and significant changes in mRNA levels after Sox10 expression was a well-established regulator of multiple morphogenic signaling pathways – Suppressor of Fused (Sufu) (Q. Ding et al., 1999; X. Meng et al., 2001; M. D. Taylor et al., 2004; P. C. Barnfield et al., 2005; T. Osterlund and P. Kogerman, 2006) (**Fig. 2**).

In addition to the reduction in Sufu mRNA after Sox10 expression (**Fig. 2B**), Sufu protein levels were also significantly reduced 24 hours after expression of Sox10 in NPCs (**Fig. 2C**). Directed ChIP confirmed the identification of a Sox10 binding site in the first intron of the Sufu gene (**Fig. 2A, D**). The Trim8 gene, which is adjacent to Sufu on the same chromosome was used as a control and we found no evidence of nonspecific Sox10 binding to this gene, consistent with Sox10 modulating the expression of this important regulatory protein directly and rapidly after expression in NPCs.

Manipulation of Sufu expression regulates specification of OPCs

We asked whether increased or decreased expression of Sufu in NPCs alters the specification of OPCs by Sox10. Retroviruses expressing either HA-tagged mouse Sufu or shRNAs targeting Sufu (M. Varjosalo et al., 2006)) were used to regulate expression (**Fig 3A**). To determine whether manipulation of Sufu expression affects the ability of Sox10 to drive OPC specification, we infected cells with Sox10 virus combined with one of three additional viruses: 1) a control shRNA virus (directed against luciferase) to address non-specific off target shRNA effects; 2) HA-Sufu virus; or 3) Sufu shRNA1 virus. Sufu overexpression dramatically decreased the production of double infected O4+ cells by Sox10 to approximately 7.5% (**Fig. 3B**). Conversely, a 1.5 fold increase in the production of OPCs in EGF/FGF was observed with Sox10 and Sufu shRNA co-infection of NPCs (**Fig. 3B**). Infection with Sufu shRNA viruses alone in EGF/FGF was insufficient to drive OPC specification, indicating that there are other targets of Sox10 that are required for OPC specification and may include PDGFR α and MBP genes (H. Li et al., 2007; M. Finzsch et al., 2008c).

Whether Sufu overexpression is also able to block the effects of Sox10 on oligodendrocyte production after exposure to serum was determined by co-infecting cells with either Sox10 + Empty virus or Sox10 + Sufu virus and switching them into serum. Sufu dramatically blocked the ability of Sox10 induced oligodendrocyte lineage (**Fig. 3C**) and inhibited the ability of NPCs to adopt an oligodendrocyte fate without Sox10 overexpression (**Fig. 3D**). Hence, Sufu is a critical negative regulator of oligodendrocyte differentiation from NPCs.

Mice with reduced Sufu expression have enhanced developmental production of OPCs

Sufu knockout mice die embryonically before OPCs are generated, however, Sufu +/- mice are viable and fertile (Y. Lee et al., 2007) and have significantly reduced levels of Sufu protein (**Fig. 4A**). Coronal sections of embryonic day 15.5 or postnatal day 2 Sufu +/- or Sufu +/- forebrains were stained with antibodies against Olig2 and PDGFR α to detect OPCs (**Fig. 4B**). There appeared to be a substantial increase in the numbers of OPCs (**Fig. 4B**) and we counted PDGFR α (a more stage specific marker for OPCs than Olig2) cells in brain sections at matched levels in mutant and control mice. At two developmental ages (E15.5 and P2) Sufu +/- brains had higher average numbers of PDGFR α -positive cells in the forebrain indicative of increased OPCs (E15.5, Sufu +/- 1359 $\pm$ 164; Sufu +/-, 2467 $\pm$ 257; P2, Sufu +/- 7535 $\pm$ 202; Sufu +/- 10921 $\pm$ 550) (**Fig. 4C-D**). These data indicate that *in vivo*, as *in vitro*, Sufu is a physiologic regulator of OPC development.

Discussion

We have shown SoxE transcription factors strongly influence multipotential neural stem cells toward an OPC fate and the oligodendrocyte lineage. Sox8 was less effective at driving cells to adopt OPC markers than either Sox9 or Sox10. These distinctions within the SoxE family are consistent with *in vivo* studies suggesting that Sox9 and Sox10 are redundant, while Sox8 is incapable of fully compensating for either Sox9 or 10 (K. Kuhlbrodt et al., 1998; C. C. Stolt et al., 2002; C. C. Stolt et al., 2003; C. C. Stolt et al., 2004; M. Wegner and C. C. Stolt, 2005; Z. Liu et al., 2007; M. Finzsch et al., 2008a). We also found that Sox10 regulates the expression of Sufu, which we show is an important determinant of OPC production from forebrain precursors.

SoxE expression in the CNS is carefully orchestrated beginning with Sox9 expression throughout the spinal cord neuroepithelium, persisting in OPCs and

maintained in adult astrocytes (C. C. Stolt et al., 2003), followed by Sox8 expression, which persists in maturing oligodendrocytes and astrocytes (E. Sock et al., 2001; C. C. Stolt et al., 2004; C. C. Stolt et al., 2005). Consistent with this, Sox8 knockouts have delayed oligodendrocyte terminal differentiation (C. C. Stolt et al., 2004) and mice lacking Sox9 have significant reductions in both OPC and astrocyte precursors (C. C. Stolt et al., 2003). Sox10 is expressed as OPCs are specified and is required for terminal differentiation of OPCs into oligodendrocytes (C. C. Stolt et al., 2002). More recently, studies using combinations of SoxE knockouts demonstrates significant redundancy between the SoxE members. For example Sox8 and 9 double knockout animals lack oligodendrocytes entirely suggesting a redundant role for Sox8 in early specification of OPCs. Sox9 conditional knockout mice crossed with mice lacking Sox10 display increased OPC apoptosis indicating a role for both proteins in OPC survival (M. Finzsch et al., 2008a). Our in vitro experiments using cultured neurospheres infected with Sox8, 9 or 10 under differentiating conditions such as FBS or TH demonstrate that all of the SoxE factors enhance OPC specification and maturation.

Sufu acts as a powerful negative regulator of the hedgehog signaling pathway in vertebrates (Q. Ding et al., 1999) by binding to Gli1, 2, and 3 and affecting Gli processing or degradation (Q. Ding et al., 1999; P. C. Barnfield et al., 2005; M. H. Chen et al., 2009; J. Jia et al., 2009). Recent studies have indicated Sufu has a complex relationship with hedgehog signaling and appears to both be an inhibitor of Shh signaling but also required for the proper balance of Gli activators and repressors with both cilia dependent and independent aspects of its functions (M. H. Chen et al., 2009; J. Jia et al., 2009). Sufu also interacts with the Wnt signaling pathway (X. Meng et al., 2001) and may act as a general scaffold protein for other signaling molecules (Y. Lee et al., 2007; D. Jenkins, 2009). These complex relationships of Sufu with both the hedgehog, Wnt

and perhaps other pathways make it unsurprising that there have been only moderate effects, after the initial patterning stage, of Shh on OPC production seen in previous reports (N. Tekki-Kessarar et al., 2001; N. Kessarar et al., 2004). The potential role of Sufu as a regulator of Wnt signaling is particularly relevant given recent studies showing that dysregulated Wnt signaling inhibits the progression of oligodendrocyte precursors to mature myelinating oligodendrocytes (S. P. Fancy et al., 2009; K. Feigenson et al., 2009) and that Wnt signaling has a negative regulatory role in oligodendrocyte cell fate acquisition (F. Ye et al., 2009; A. J. Langseth et al., 2010).

We have identified Sufu as a target of Sox10 repression and identified a Sox10 binding site in the first intron of Sufu. Moreover, we have demonstrated that Sufu plays a role *in vitro* and *in vivo* in OPC specification. In the peripheral nervous system Sox10 controls Schwann cell myelination by acting in synergy with other transcription factors such as Krox20, NFATc, and Oct6 to regulate myelin genes (R. I. Peirano et al., 2000; N. Bondurand et al., 2001; J. Ghislain and P. Charnay, 2006). For example, Sox10, Krox20 and NFATc synergistically bind to the first intron of Myelin protein zero and activate transcription (R. I. Peirano et al., 2000). We propose a similar model whereby Sox10, perhaps in combination with a novel transcription factor(s), binds and represses Sufu transcription thereby enhancing OPC differentiation. Whether Sox10 repression of Sufu would result in modulation of the Shh or Wnt pathways that may alter OPC specification is unknown. Finally, it remains unknown if Sox8 or Sox9 also modulate Sufu expression to induce early OPC specification or in the case of Sox9, astrocyte maintenance in the adult. Future investigation of Sufu dysregulation in the SoxE knockouts would help to answer these questions.

Our observations also have important implications for tumorigenesis in the nervous system. Loss of Sufu by mutation leads to deregulated Shh signaling and Wnt

signaling, and is an important cause of medulloblastoma (a cerebellar granule precursor tumor) in children (M. D. Taylor et al., 2002) and mice (Y. Lee et al., 2007). SoxE transcription factors are abundantly expressed in gliomas (S. I. Bannykh et al., 2006; B. Schlierf et al., 2007) and we predict that in these tumors Sufu expression would be reduced due to downregulation by transcriptional effects of SoxE proteins. This may provide a mechanism for altered Shh and Wnt signaling levels in such tumors and may be a key aspect of their degree of aggressiveness.

This work may have important implications for regenerative approaches promoting oligodendrocyte replacement in demyelinating diseases. Demyelination in either experimental animal models or patients with multiple sclerosis leads to brisk up regulation of OPC production in the SVZ (N. Picard-Riera et al., 2002; B. Menn et al., 2006; B. Nait-Oumesmar et al., 2007). However, the ubiquity of this response in patients with demyelination isn't known and it remains unclear whether further augmentation of this response would be beneficial. Our studies indicate that developing methods to downregulate the expression of Sufu in these patients may have therapeutic benefit.

Methods

Retroviral cloning and production, neurosphere infection, clonal analysis, data analysis, Western blotting and immunohistochemistry are described in Supplemental Methods and in published work (A. J. Langseth et al., 2010).

Primary Cell Culture

All animal procedures were performed according to protocols approved by the UCSF Institutional Animal Care and Use Committee (IACUC). SVZ tissue from postnatal day 5

CD1 mice was collected in and dissociated with 0.1% Trypsin (Worthington) at 37°C water bath. Following dissociation by trituration single cells were plated in Complete Media consisting of DMEM/F12 (50:50, Gibco) supplemented with 10X hormone mix (per 50 ml stock added to 450 ml of medium: 40 mg transferrin, 10 mg insulin, 3.86 mg putrescine, 4.0 ml 3 mM selenium, 4.0 ml 2 mM progesterone, 10 ml 2 mg/ml heparin [all from Sigma]), 0.8 ml 30% glucose, 0.6 ml 7.5% NaHCO₃, 10 ml 30% glucose, 7.5 ml 7.5% NaHCO₃, 2.5 ml 1 M HEPES, 5 ml 200 mM glutamine, 5 ml Pencillin-Streptomycin, 2 ml Fungizone), EGF (Sigma, 10 ng/ml), FGF (Sigma, 20 ng/ml) and B27 (Invitrogen). Approximately 80,000 cells were plated in a 25 cm² flask (Corning), grown for 3-4 days until spheres grew large enough to be passaged. Neurospheres were dissociated and infected with retroviruses as described in Supplemental Methods.

ChIP assays and DNA microarrays

Established protocols were used for the chromatin immunoprecipitation experiments (<http://genomics.ucdavis.edu/farnham/protocols/chips.html>), and hybridization to mouse promoter arrays was performed by NimbleGen. Neurosphere cultures infected with either a Sox10-3xHA expressing or an Empty control retrovirus were plated on laminin coated 6 well plates (Falcon) for 48 h before the samples were collected. A monoclonal HA antibody (HA.11 [Covance], 1:150) was used for the immunoprecipitations.

Hybridization intensities were expressed as a ratio of the signals from Sox10-3xHA infected cells and Empty control infected cells. For targeted ChIP, ChIP followed by targeted PCR to examine Sox10 binding to Sufu and the adjacent Trim8 gene, which served as a negative control. The following primers were used in a PCR reaction with 37 amplification cycles (94°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec): Sufu-5'

gcaaattggtgctgaagt and Sufu-3' cccatctccagaggcaataa, product = 128bp; TRIM-5' cccggcagaagttatgttg and TRIM-3' gcggagaattggaagaactg, product = 106bp.

Immunocytochemistry

Cells plated on chamber slides were fixed with 4% paraformaldehyde, washed 3X with PBS and blocked and permeabilized for 30 minutes with PBS containing 0.3% Triton X-100 and 10% lamb serum. Incubation with primary antibodies (O4 (mouse 1:30, Chemicon and Sigma), Nestin (rabbit 1:100, Chemicon), GFAP (rat 1:500, Zymed), Tuj1 (mouse or rabbit 1:500, Covance), PDGFR α (rabbit 1:200, Santa Cruz; rat 1:500, BD Biosciences Pharmingen), Sox10 (rabbit 1:100, ABR), CNPase (mouse 1:500, Sternberger monoclonals), and Olig2 (rabbit 1:1000, D. Rowitch)) were at 4°C overnight. Each experiment was performed multiple times and graphs represent 3 separate experiments for which 6 fields were quantified per condition. Similar protocols were used for histological analysis of brain sections and are described in Supplemental methods.

Supplemental Methods

Neurosphere culturing

Tissue was collected into a tube of sterile PBS containing 1% Penicillin-Streptomycin and 0.4% Fungizone, and dissociated with 0.1% Trypsin (Worthington) for 30 min in a 37°C water bath. Following trypsinization 0.1% DNase1 (Roche) was added directly to the tube and incubated for 1 min at 25°C before washing the tissue twice with sterile PBS, each time allowing the tissue pieces to settle to the bottom of the tube.

Complete DMEM/F12 (50:50, Gibco) was supplemented with 10X hormone mix (per 50 ml stock added to 450 ml of medium: 40 mg transferrin, 10 mg insulin, 3.86 mg putrescine, 4.0 ml 3mM selenium, 4.0 ml 2mM progesterone, 10 ml 2 mg/ml Heparin [all from Sigma]), 0.8 ml 30% glucose, 0.6 ml 7.5% NaHCO₃, 10 ml 30% glucose, 7.5 ml 7.5% NaHCO₃, 2.5 ml 1 M HEPES, 5 ml 200 mM glutamine, 5 ml Pencillin-Streptomycin, 2 ml Fungizone) and was used in all neurosphere experiments. One ml of complete media containing EGF (Sigma, 10 ng/ml), FGF (Sigma, 20 ng/ml) and B27 (Invitrogen) was added to the tissue, which was dissociated into single cells by gentle trituration. Cells were plated at a density of approximately 80,000 cells per 25 cm² flask (Corning), grown in 5 ml of complete media plus growth factors and B27 for 3-4 days and fed every other day before spheres grew large enough to be passaged.

Virus construction

The retroviral transfer vector pLNCX2 (Clontech) was modified in the following manner: A new multiple cloning site (5'-*Bgl*II-*Pac*I-*Xho*I-*Hind*III-*Avr*II-*Hpa*I-*Not*I-*Cl*aI-3') was introduced downstream of the immediate early CMV promoter, and the neomycin resistance gene was replaced with histone H2B fused to either enhanced green fluorescent protein (pLH2BeGFP) or monomeric red fluorescent protein (pLH2BmRFP), so that transduced cells are identifiable by a fluorescent nucleus. Mouse Sox8, Sox9, Sox10, Olig1 and Olig2 cDNA's were obtained from the Mammalian Gene Collection and, with the exception of Olig2, were PCR amplified to flank the open reading frames with a 5' *Xho*I and a 3' *Cl*aI site in order to facilitate their sub-cloning into the above described retroviral vectors. The Olig2 open reading frame was excised as a *Taq*I-*Avr*II fragment that was inserted into the *Xho*I and *Avr*II sites of pLH2BmRFP. The N-terminal HA epitope tagged mouse Sufu open reading frame was excised as a *Hind*III-*Not*I

fragment from pCMV5-HASufu (a kind gift from Dr. Pao-Tien Chuang, UCSF) that was inserted into the *HindIII* and *NotI* sites of pLH2BeGFP. Sox10-3xHA was created by PCR amplification of Sox10 without stop codon to flank the open reading frame with a 5' *XhoI* and a 3' *NotI* site that were used for sub-cloning into pLH2BmRFP. Subsequently, a 111 bp *NotI* fragment containing three copies of the HA epitope, a stop codon and a *BglII* site was inserted. pSIREN-HygEGFP was generated in the following manner: The *EcoRI* site in the dual-function marker of pHygEGFP (Clontech) was mutated and the CMV_{ie}-HygEGFP cassette from this plasmid was used to replace the PGK-Puro cassette in pSIREN-RetroQ (Clontech). Subsequently, a luciferase control siRNA (Clontech) and two previously described Sufu siRNA's³⁹; first nucleotides of the 21 bp target sequences correspond to position 740 [siRNA1] and position 583 [siRNA2] of Sufu mRNA with GenBank Accession # AF134893) were inserted into the *BamHI* and *EcoRI* sites of this self-inactivating retroviral siRNA expression vector. All constructs were sequenced to confirm the absence of undesirable mutations. Cloning details, vector maps and sequence files are available upon request.

Data analysis

The micro-array analysis performed by Nimblegen used a 60mer catalog array of mouse build 5 from UCSC (mm5, May 2004 genome assembly) of 34,062 genes at a minimum of 11probes/gene and also included 5000 random probes. The probe intensities were quantile-normalized and 11+ probes per gene were summarized using the robust multi-array average method (RMA), developed originally for Affymetrix arrays. To normalize the data for each array, the raw intensities were extracted from NimbleGen PAIR files to calculate M, the log₂ ratios of IP-enriched DNA (Cy5) and input total DNA (Cy3), for each probe. M was then loess-normalized to remove the strong signal-

dependent dye imbalance. Qualitative diagnostic plots and several measures of signal to noise strength were used collectively for quality check. The normalized M (referred as M hereafter) was subject to a two-step sliding window approach to identify sites with significant IP-enrichment: (i) Sliding window statistics. Calculate statistics over probes within a sliding window and apply cutoffs to call significant windows (see details below); (ii) Site formation. The significant windows were then merged to form nonredundant sites if they overlap for at least one probe. For each probe location, the following three statistics were calculated over probes that are located within the [-500bp, +500bp] window of that probe: (1) medM: median M of probes in window, (2) pM: p-value of t-test that $M=0$ vs $M \neq 0$, (3) pA: p-value of one-sided t-test that the average Cy5 and Cy3 signals of the probe in window =, vs >, random probe signals. A window is called significant using the following criteria: $\text{medM} > \log_2(1.5)$ and $pM < 10^{-4}$ and $pA < 10^{-2}$. A significant site should have good signal (small pA), and good enrichment (large medM, small pM). Note that $\text{medM} > 0.58$ corresponds to a 1.5 x enrichment in IP-enriched DNA relative to input DNA. The window size (1kb) and cutoffs are somewhat *ad hoc* but arguably reasonable and fairly typical in the ChIP-chip literature. The DNA fragments used in the protocol are 1000bp, and the resolution of these arrays yield ~10 probes per 1000bp window for statistical inference. We do not attempt to adjust the p-values for multiple testing due to the sophisticated dependency between overlapping sliding windows, but instead chose a more stringent p-value cutoff.

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Figure Legends

Figure 1: Overexpression of SoxE factors but not Olig genes is sufficient to drive differentiation toward the oligodendrocyte lineage

(a) Empty virus infected cells do not express O4 in EGF/FGF, while Sox8, Sox9 or Sox10 virus infected cells express O4 at significant levels. In contrast, Olig1 or Olig2 expression does not lead to increased O4 staining in these culture conditions.

(b) The efficiency of O4⁺ cell production after Sox10 expression under non-differentiating conditions (in EGF/FGF containing medium). Results pooled from three separate experiments. Error bars are $\pm$ S.E.M. *** = $P < 0.005$

(c) Cells infected with either Empty or SoxE expressing retroviruses (red) cultured in 2% FBS for 48 hours and stained with O4 antibody (green). Inset shows Sox10 infected cells also stain with CNPase, a marker of terminally differentiating oligodendrocytes.

(d) Cells infected with either Empty or SoxE expressing retroviruses (red) cultured in thyroid hormones for 48 hours and stained with O4 antibody (green). Inset shows Sox10 infected cells also stain with CNPase, a marker of terminally differentiating oligodendrocytes.

(e) The percentage of O4⁺ cells infected with Sox8, 9 or 10 cultured in serum or thyroid hormones for 48 hours. Results are pooled from >3 separate experiments; error bars are $\pm$ S.E.M.

* = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.005$

Figure 2. Suppressor of Fused (Sufu) is a direct target of Sox10

(a) Schematic of the Sufu locus depicting the Sox10 binding site identified in the first intron of the Sufu gene (see **Supplementary Methods**).

(b) Quantitative mRNA profiling using whole mouse transcriptome Affymetrix chips. Each value is shown as a ratio of the experimental value over Empty virus-infected controls. Shown are the relative expression of Sufu mRNA 48 hours after infection with Sox10 virus or 24 hours after infection with Mash1 or Ngn2 retroviruses that drive neurogenic differentiation in EGF/FGF containing medium. Note Mash1 and Ngn2 drive modest increases in Sufu expression, while Sox10 leads to two-fold decrease in expression of Sufu. Normalized intensity values of triplicate chip hybridization experiments are presented. Each sample was prepared from pools of mRNA derived from >3 separate experiments.

(c) Sox10 expression leads to decreased Sufu protein levels in NPCs 24 hours after plating. Note the slightly higher and broader band in the positive control lane was obtained from extracts of HEK-293T cells expressing both human and mouse HA-tagged Sufu. In contrast, NPCs only express endogenous untagged mouse Sufu.

(d) Targeted ChIP for Sox10 bound to the Sufu gene at the first intronic site. Positions of the PCR primers are shown in relationship to the Sufu and Trim8 genes. The PCR reaction yields the expected products for both Sufu and Trim8 after infection with either Empty or Sox10 virus (Input), but only Sox10 infected cells yielded a specific band for Sufu after ChIP (IP: anti-HA). Empty virus-infected cells showed no signal and Sox10 showed no binding to the closely linked Trim8 gene. The arrows indicate the size of the expected Sufu product.

Figure 3. Sufu inhibits Sox10 driven OPC differentiation and endogenous oligodendrocyte differentiation

(a) shRNA-mediated knock down of mouse HA-Sufu protein (solid arrow, top bands) in GP2-293 cells. Note that the endogenous human Sufu protein is unaffected (open arrow, bottom bands).

(b) Percentage of O4⁺ cells co-infected with Sox10 and either Luciferase shRNA, Sufu or Sufu siRNA. Transduced NPCs were cultured in EGF/FGF on laminin-coated plates for 72 hours. Error bars are $\pm$ S.E.M, *** = $P < 0.005$. N=3

(c) Percentage of O4⁺ cells co-infected with Sox10 virus and either Empty or Sufu virus then induced to differentiate by removing EGF/FGF and addition of serum. Error bars are $\pm$ S.E.M, *** = $P < 0.01$. N=3

(d) Percentage of O4⁺ cells infected with Empty or Sufu virus, and cultured in serum on laminin coated plates for 72 hours. Error bars are $\pm$ S.E.M, *** = $P < 0.01$. N=3

Figure 4. Decreased Sufu expression significantly increases the number of oligodendrocyte precursors *in vivo*.

(a) Western blot analysis of equal amounts of protein derived from brain lysates of wildtype (Sufu +/+) mice or mice lacking one copy of Sufu (Sufu +/-).

(b) Olig2 staining in brain sections of Sufu +/+ or Sufu +/- E15.5 brains show an overall increase in Olig2 positive cells in Sufu +/- brains. Higher magnifications in bottom panels.

(c) The number of PDGFR α -positive cells in the forebrain is significantly increased in mice with decreased Sufu expression (Sufu +/-) at embryonic day 15.5 (E15.5) (Error bars are $\pm$ SD, ***=P<0.005. N=3).

(d) The increased number of PDGFR α -positive cells persists in the Sufu +/- postnatal day 2 (P2) forebrain. Error bars are $\pm$ SD, **=P<0.05. N=3

Figure 1.

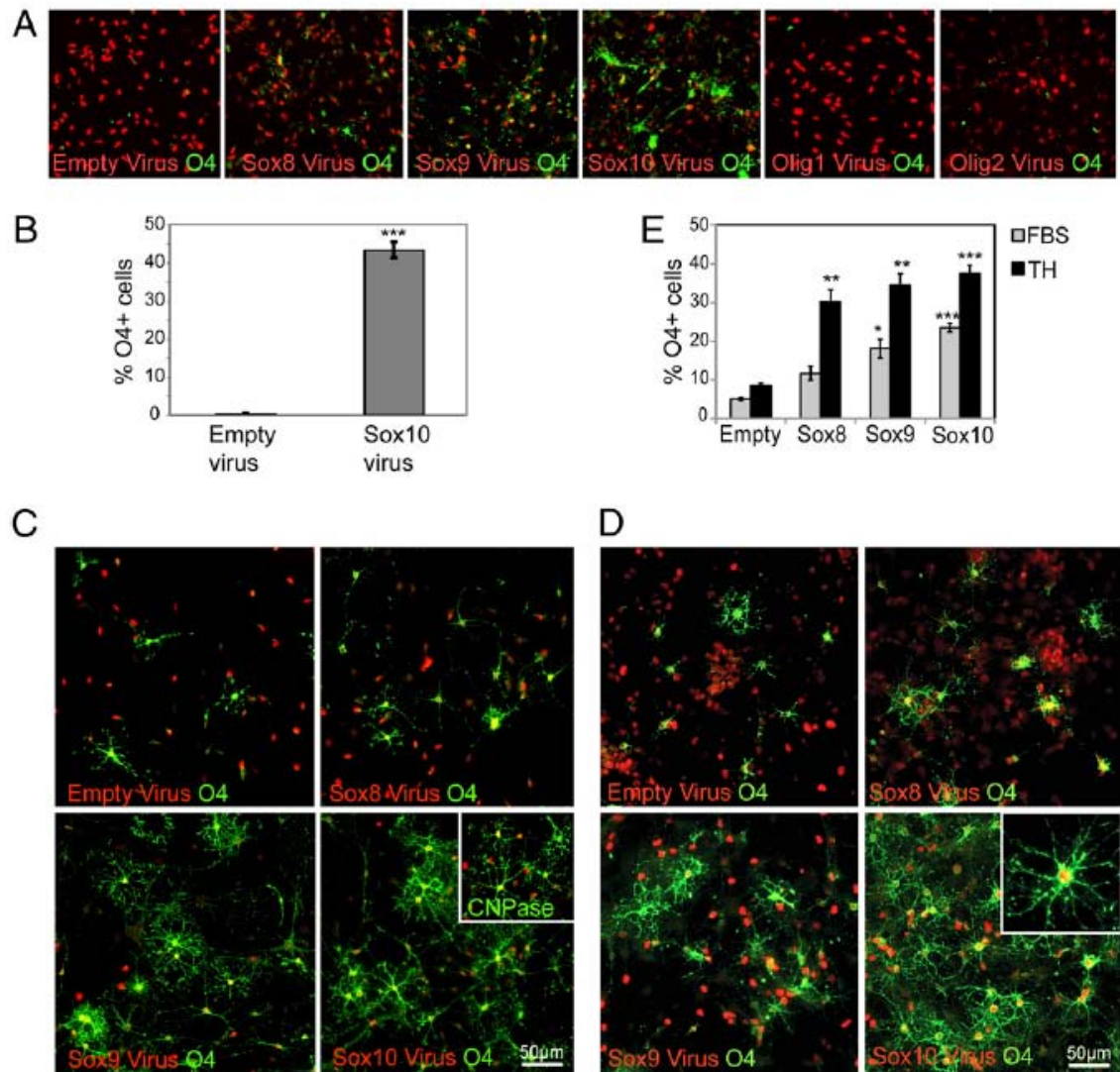


Figure 2.

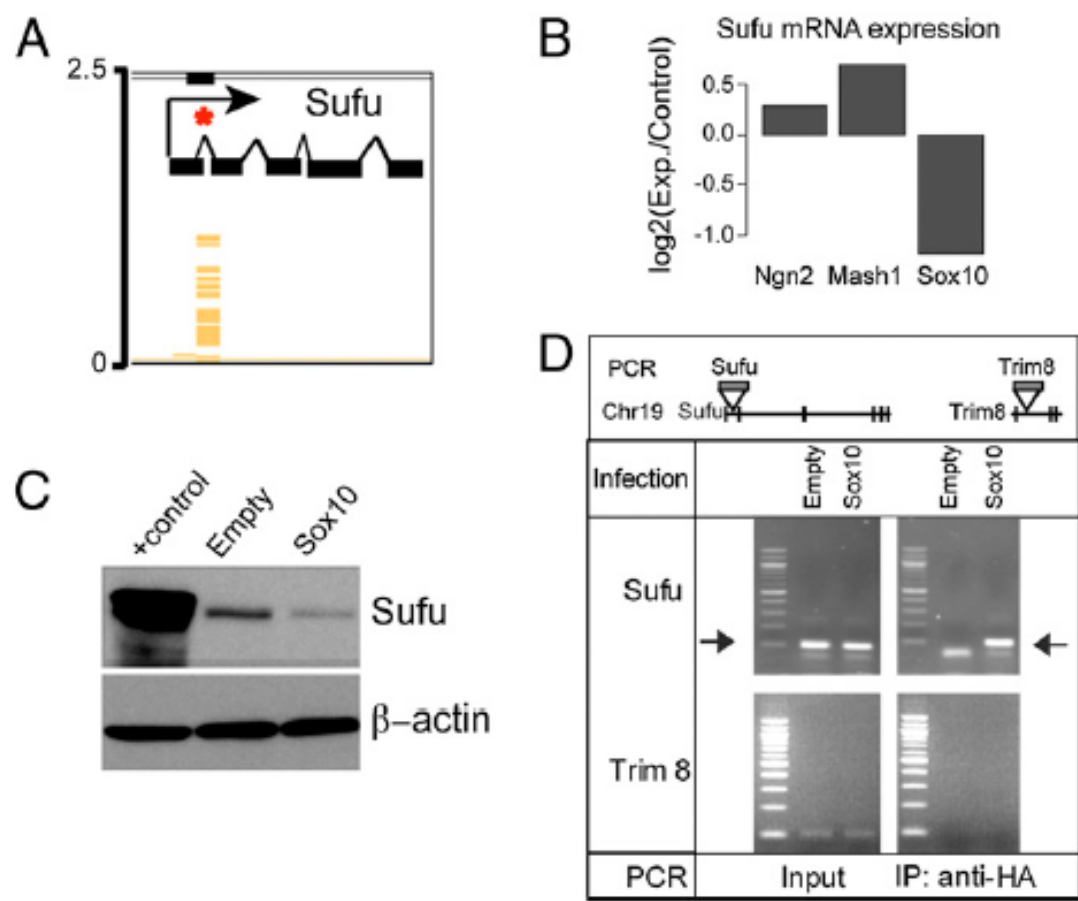


Figure 3.

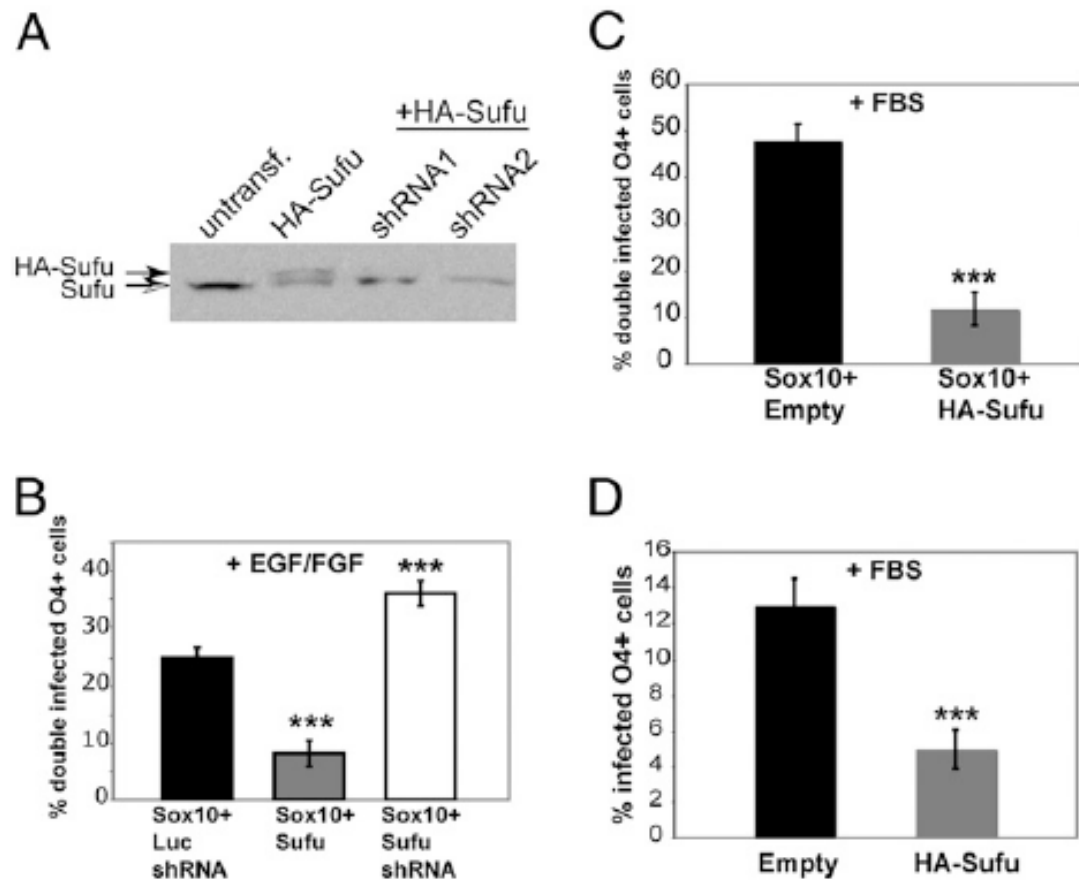
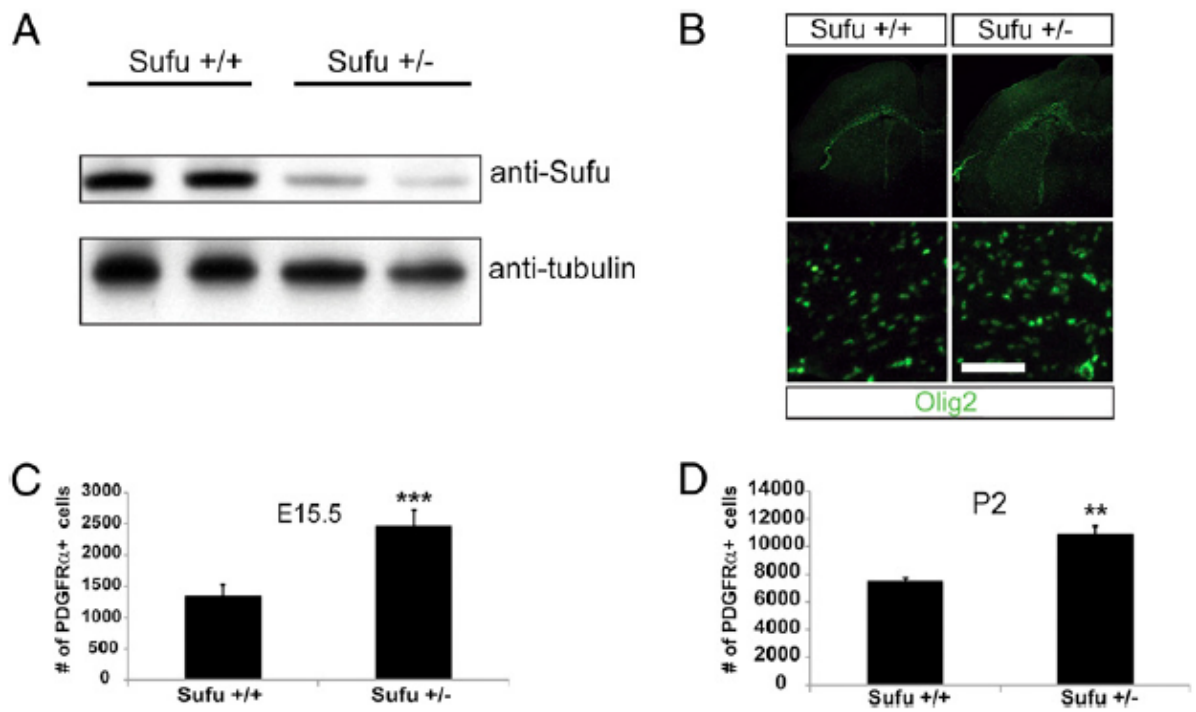


Figure 4.



Chapter 4: Manuscript in Preparation

Suppressor of Fused regulates oligodendrocyte progenitor cell proliferation in development and injury

A.J. Langseth, S.P.J. Fancy, T. Huynh, D.H. Rowitch, and S.J. Pleasure

Abstract

We previously identified Suppressor of Fused (Sufu) as a target of Sox10 regulation. Sox10 inhibits Sufu expression and this increases OPC numbers *in vitro* and in Sufu heterozygous embryos (C. D. Pozniak et al.). Sufu is an inhibitor of the Sonic Hedgehog (L. Ruel and P. P. Therond, 2009) and Wnt (X. Meng et al., 2001) signaling pathways. Sufu heterozygous mice have increased numbers of OPCs in the CNS developmentally, with no changes in the size of progenitor domains, production of OPCs from neural progenitors, OPC apoptosis, or OPC maturation. It is suspected that Sufu specifically regulates the proliferation of OPCs. In these heterozygous mice the increase in OPC numbers is corrected by adult ages. In response to a demyelinating lesion *sufu* heterozygotes have increased numbers of OPCs within the lesion site but no change in the number of mature oligodendrocytes generated to repair the lesion. Mice lacking Sufu in all Olig2-positive cells show no change in the initial production of OPCs but have enhanced proliferation of OPCs and other progenitor cell types that results in large increases in OPC, neuron, and astrocyte numbers and a greatly enlarged CNS at late embryonic ages. Sufu appears to specifically regulate the proliferation of OPCs and other mitotic progenitor cells in the CNS.

Introduction

Oligodendrocyte progenitor cells (OPCs) are generated from multiple progenitor domains in the embryonic forebrain and spinal cord (W. D. Richardson et al., 2006). OPCs migrate and proliferate throughout the CNS (N. Kessaris et al., 2006) before maturing and myelinating axons. A small pool of OPCs is preserved in the adult brain (S. H. Kang et al., ; L. E. Rivers et al., 2008) and is capable of responding to demyelinating injury (R. J. Franklin and C. Ffrench-Constant, 2008). The signals that regulate the proliferation of OPCs in embryonic development and adult injury have not been clearly elucidated.

We previously identified Suppressor of Fused (Sufu) as a target of Sox10 regulation. Sox10 inhibits Sufu expression and this increases OPC numbers *in vitro* and in Sufu heterozygous embryos (C. D. Pozniak et al.). Sufu is an inhibitor of the Sonic Hedgehog pathway and acts by promoting the nuclear export of the Gli transcription factors as well as regulating the processing of Gli's into activator and repressor forms (Y. Chen et al., ; E. W. Humke et al., ; H. Tukachinsky et al., ; C. Wang et al., ; M. H. Chen et al., 2009; J. Jia et al., 2009; L. Ruel and P. P. Therond, 2009). Sufu has also been reported to inhibit canonical Wnt signaling through the nuclear export of beta-catenin (X. Meng et al., 2001). Sufu has been shown to play several roles in the nervous system including patterning during early embryonic development (J. J. Kim et al., ; A. F. Cooper et al., 2005; J. Svard et al., 2006) and maintenance of multipotent progenitor cells (M. A. Cwinn et al.).

The goal of this study was to determine how Sufu regulates OPC numbers in the developing CNS and in response to a demyelinating injury at adult ages. We found that Sufu regulates the proliferation of OPCs without affecting progenitor zone patterning,

production of OPCs from neural progenitors, myelination, or apoptosis. Sufu also regulates other mitotic progenitor cells types in the developing CNS. We are working to determine the signaling pathway regulated by Sufu to have these effects.

Results

Sufu hets have increased numbers of OPCs during development but not at adult ages

The PDGFR α -H2BeGFP mouse line (T. G. Hamilton et al., 2003) which expresses bright nuclear GFP under the PDGFR α promoter was bred into the Sufu mouse line to allow for efficient quantification of OPC numbers. No changes in the number of OPCs in the cervical spinal cord above the level of the forelimb were observed in Sufu hets compared to wt using Olig2 staining at E12.5 (the transgenic GFP was not clearly visible at this early age). There were also no changes in OPC numbers at E18.5 as assessed by PDGFR α -driven GFP expression. Sufu hets have significantly increased numbers of OPCs at P5. There is no difference in OPC numbers between hets and wt's at adult ages (Figure 1).

The increase in OPC numbers is not due to changes in progenitor domain size, production of OPCs from neural progenitors, changes in myelination, or apoptosis of OPCs

We sought to determine the cause of the increased number of OPCs in the developing Sufu het CNS. It is known that mice null for Sufu have severe patterning defects, incomplete neural tube closure, and die at E9.5 (A. F. Cooper et al., 2005; J. Svard et al., 2006). These patterning defects raised the possibility that Sufu hets might

have changes in the size of the pMN or Mash1-positive progenitor domains that give rise to OPCs in the ventral and dorsal spinal cord, respectively. We performed immunohistochemistry with Mash1 on E12.5 Sufu het and wt spinal cord sections and found no significant difference in the size of the Mash1-positive progenitor domain. We also labeled the p2, pMN, and p3 domains with antibodies for Nkx6.1 and Olig2 and found no significant differences in the sizes of these domains (Figure 2). Together these data suggest that changes in the patterning of progenitor domains do not cause the increase in OPC numbers in Sufu het spinal cords.

Changes in the ability of neural progenitors to produce OPCs could account for the change in OPC numbers. We cultured neural progenitors from the brains of Sufu het and wildtype mice at P5 and assessed their ability to produce OPCs upon differentiation with FBS. No significant differences in OPC production were observed (Figure 3).

A block in the maturation of OPCs could cause an increase in OPC numbers. To test this possibility we labeled Sufu het and wt spinal cord sections at E18.5 and P5 with antibodies for MBP and CC1 (APC). No changes in the onset of OPC maturation or the number of mature oligodendrocytes were observed. We also performed Fluoromyelin staining at adult ages and saw no gross differences in the location or extent of myelin (Figure 4). We are currently collaborating with Robin Franklin's lab at Cambridge to assess the myelin of adult Sufu het and wt spinal cords at the ultrastructural level. Together these data indicate that changes in the onset or extent of OPC maturation do not cause the increase in OPC numbers in Sufu het spinal cords.

Differences in apoptosis of OPCs could cause the change in OPC numbers in the Sufu hets. We labeled Sufu het and wt spinal cords with antibodies for active-caspase3 and Olig2 and saw no changes in apoptosis at embryonic or early postnatal ages (data

not shown). This indicates that changes in apoptosis of OPCs are not likely to cause the increase in OPC numbers in *Sufu* het spinal cords at the timepoints analyzed.

We suspect that the increased number of OPCs in *Sufu* hets is due to changes in proliferation of the OPCs. We are currently planning BrdU-labeling experiments to test this possibility.

Sufu hets have increased numbers of OPCs in a demyelinating lesion but no changes in remyelination

Sufu het mice have increased numbers of OPCs during development but no discernable changes in OPC numbers or myelin at adult ages. This apparent return to wildtype numbers of OPCs and normal myelin at adult ages allowed us to test the response of *Sufu* het mice to a demyelinating injury to determine whether the increased capacity for OPC production observed in development would be recapitulated in the adult CNS in response to injury.

We induced demyelination of the dorsal white matter tract of the adult spinal cord by lysolecithin injection using previously described methods (S. P. Fancy et al., 2009). Maximal OPC recruitment to the area of demyelination occurs at 5 days post lesion (dpl). We observed significantly increased numbers of OPCs within the lesion in *Sufu* hets compared to wt's by counting either Olig2 immunohistochemistry or PDGFR α -GFP expression (Figure 5).

We next sought to determine whether this increased number of OPCs within the lesion site resulted in increased oligodendrocyte numbers. OPCs begin to mature into oligodendrocytes at 10dpl and the new oligodendrocytes begin to wrap myelin around axons within the lesion at 14 dpl. We performed in situ's for PLP and observed no changes in the number of oligodendrocytes within the lesion at 5, 10, or 14dpl (Figure 6).

Together these data indicate that adult *Sufu* hets have increased numbers of OPCs within a demyelinating lesion but no changes in OPC maturation.

Loss of Sufu enhances proliferation of multiple progenitor types

We sought to determine the effect of loss of *Sufu* in the progenitors that generate OPCs and in the OPCs themselves. We used a *sufu* conditional mouse line (M. H. Chen et al., 2009) crossed to *Olig2-cre* mice (U. Schuller et al., 2008) to selectively remove *Sufu* from *Olig2*-positive progenitors and the oligodendrocyte lineage. *Olig2-cre;Sufu-fl/fl* (mutant) mice show no significant changes in OPC numbers at E14.5 compared to *Olig2-cre;Sufu-fl/+* (wildtype) littermate controls as assessed by *Olig2* staining, but they have a large increase in OPCs at E18.5. The size of the central nervous system is greatly increased at E18.5 and there is incomplete bone covering of the dorsal spinal cord at this age (Figure 7). These mice do not survive the birth process.

These mice also have increased numbers of mitotic progenitors at E14.5 and 18.5 (Figure 8). They have increased numbers of astrocytes and neurons but not oligodendrocytes at E18.5 (Figure 9).

Together these results suggest that the selective loss of *Sufu* in *Olig2*-positive progenitors and the oligodendrocyte lineage results in enhanced proliferation of OPCs as well as other mitotic cell types.

Discussion

Sufu is known to be an inhibitor of the Sonic Hedgehog signaling pathway (Y. Chen et al., ; E. W. Humke et al., ; H. Tukachinsky et al., ; C. Wang et al., ; M. H. Chen et al., 2009; J. Jia et al., 2009; L. Ruel and P. P. Therond, 2009) and may also inhibit the

canonical Wnt signaling pathway (X. Meng et al., 2001). It has been shown to control some patterning events during development of the CNS (J. J. Kim et al., ; A. F. Cooper et al., 2005; J. Svard et al., 2006) and regulate neural progenitor cells in the retina (M. A. Cwinn et al.). We have identified a novel role for *sufu* as a regulator of the number of OPCs in the CNS during embryonic development and in response to a demyelinating lesion.

Mice heterozygous for *Sufu* appear to have normal patterning of spinal cord progenitor domains and normal production of OPCs early (E12.5-18.5). By postnatal ages they have increased numbers of OPCs with a normal onset and extent of myelination. By adult ages this increase in OPC numbers has undergone compensation by an unknown mechanism and returned to wildtype levels. It is suspected that *Sufu* regulates the proliferation of OPCs because no changes in OPC production from progenitors, apoptosis, or maturation were observed. In support of this idea, mice lacking *sufu* in all *Olig2*-positive cells (a pool of neural progenitors plus all cells of the oligodendrocyte lineage) show no changes in OPC numbers at E14.5 when OPC production from progenitors has just begun, but greatly expanded numbers of OPCs at E18.5 when OPCs are migrating and proliferating to cover the CNS before the onset of myelination.

The increased ability of *Sufu* het mice to generate OPCs during development appears to be paralleled in adult ages in response to a demyelinating injury, as has been observed with other signaling pathways and molecules that regulate the oligodendrocyte lineage (S. P. Fancy et al.). Increased numbers of OPCs are observed in a region of demyelination but this does not result in increased numbers of mature oligodendrocytes.

These results are consistent with the idea that Sufu regulates OPC numbers without influencing the production of OPCs from neural progenitors or the maturation of OPCs into oligodendrocytes. The identification of a signal that specifically controls the proliferation stage of the oligodendrocyte lineage is very exciting. We plan to specifically test this hypothesis by removing Sufu from OPCs using the CNP-cre mice (C. Lappe-Siefke et al., 2003) and observing the effects on proliferation and maturation of OPCs. We will also test Sufu's role in the proliferation of other mitotic progenitors using mouse lines such as Nestin-cre. Regulation of OPC proliferation may be useful for promoting regeneration in response to demyelinating injury or disease by providing a way to rapidly expand the pool of OPCs available in the CNS.

Our finding that Olig2-cre;Sufu-fl/fl mice have enlarged nervous systems with increased numbers of mitotic progenitors persisting both along the spinal cord ventricle and away from the ventricle and large increases in numbers of neurons and astrocytes by late embryonic ages indicates that Sufu may be involved in the maintenance of the pool of mitotic neural progenitor cells. Sufu may regulate the proliferation and cell cycle exit of these progenitors to control neuron and astrocyte numbers. Thus Sufu may not exclusively regulate the proliferation of OPCs but may also regulate the proliferation of less restricted progenitors within the nervous system.

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Figure Legends

Figure 1. Sufu heterozygous mice have increased OPC numbers during development.

Olig2 staining revealed no significant difference in OPC numbers between Sufu hets and wildtypes at E12.5 (a-b). GFP expression driven by the PDGFR α promoter revealed no significant difference in OPC numbers between Sufu hets and wildtypes at E18.5 (c-d) but there was a significant difference at P5 (e-f). This difference was not present at adult (8-12 week) ages (g-h). These results are quantified in (i).

Figure 2. Progenitor domain sizes are not changed in Sufu heterozygous mice.

Olig2 and Nkx6.1 staining were used to determine the size of the p2, pMN, and p3 domains of Sufu het and wildtype spinal cords at E12.5 (a-b). No significant difference was found in the sizes of any of these domains and these results are quantified in (e). Mash1 staining was used to define the dorsal OPC-producing progenitor domain (c-d). No significant difference was found in the size of this domain and these results are quantified in (f).

Figure 3. OPC production from neural progenitors is not changed in Sufu heterozygous mice.

Neural progenitor cell cultures were taken from the lateral wall of the lateral ventricle of P5 Sufu het and wildtype mice. These cultures were passaged in EGF/FGF 2 times and then plated as a monolayer and induced to differentiate for 2 days *in vitro* with FBS. No significant difference was observed in the ability of sufu het cultures to produce OPCs.

Figure 4. Onset and extent of myelination is not changed in Sufu heterozygous mice.

MBP and CC1 (not shown) were used to label oligodendrocytes in E18.5 and P5 Sufu het and wildtype spinal cord sections (a-d). No significant differences were observed in the number of oligodendrocytes (g). FluoroMyelin stain was used to visualize the white matter in adult (8-12 week) cords. No differences were observed in the gross white matter morphology between Sufu hets and wildtypes (e-f).

Figure 5. Sufu hets have increased numbers of OPCs in a demyelinating lesion.

A lesion in the dorsal white matter tract of adult Sufu het and wildtype mice was induced by lysolecithin injection. At 5 days post lesion, when OPC recruitment to the lesion is maximal, an increased number of OPCs is observed within the area of the lesion as assessed by Olig2 or PDGFR α -GFP staining (a-b). These results are quantified in (c).

Figure 6. Sufu hets have no change in oligodendrocyte numbers in a demyelinating lesion.

PLP in situ was used to quantify the number of mature oligodendrocytes within the lesion area in lysolecithin-lesioned cords. At 5 dpl, no PLP-positive cells were observed within the lesion (a-d). At 10 dpl, a time of maximal OPC maturation, no significant difference in PLP-positive cells was observed between Sufu hets and wildtypes (e-h). At 14 dpl, a time when the new oligodendrocytes begin to form new myelin wraps, no significant difference in PLP-positive cells was observed between Sufu hets and wildtypes (i-l). These results are quantified in (m).

Figure 7. Olig2-cre;Sufu-fl/fl mice have increased OPC numbers and increased CNS size.

OPCs were quantified in E14.5 Olig2-cre;Sufu-fl/+ (control) and Olig2-cre;Sufu-fl/fl (mutant) spinal cords with Olig2 staining. No changes in OPC numbers were observed. At E18.5, OPC numbers were dramatically increased in mutant mice. In addition, the size of the spinal cord and brain were greatly increased.

Figure 8. Olig2-cre;Sufu-fl/fl mice have increased numbers of proliferative cells.

Ki-67 positive cells were quantified in E14.5 and E18.5 Olig2-cre;Sufu-fl/+ (control) and Olig2-cre;Sufu-fl/fl (mutant) spinal cords. At E14.5 increased numbers of mitotic cells were observed along the spinal cord ventricle of mutant mice. At E18.5 a pool of mitotic progenitors along the spinal cord persists in the mutants where none is seen in the

wildtype controls and increased numbers of mitotic cells are also observed away from the ventricle.

Figure 9. Olig2-cre;Sufu-fl/fl mice have increased numbers of astrocytes and neurons but not mature oligodendrocytes.

BLBP and GFAP staining showed greater numbers of astrocytes throughout the spinal cords of E18.5 Olig2-cre;Sufu-fl/fl (mutant) mice than Olig2-cre;Sufu-fl/+ (control). NeuN staining revealed that mutant mice also have more neurons than control mice and that some neurons were located ectopically in the white matter tracts. MBP staining showed no increase in the number of mature oligodendrocytes in mutant mice.

Figure 1.

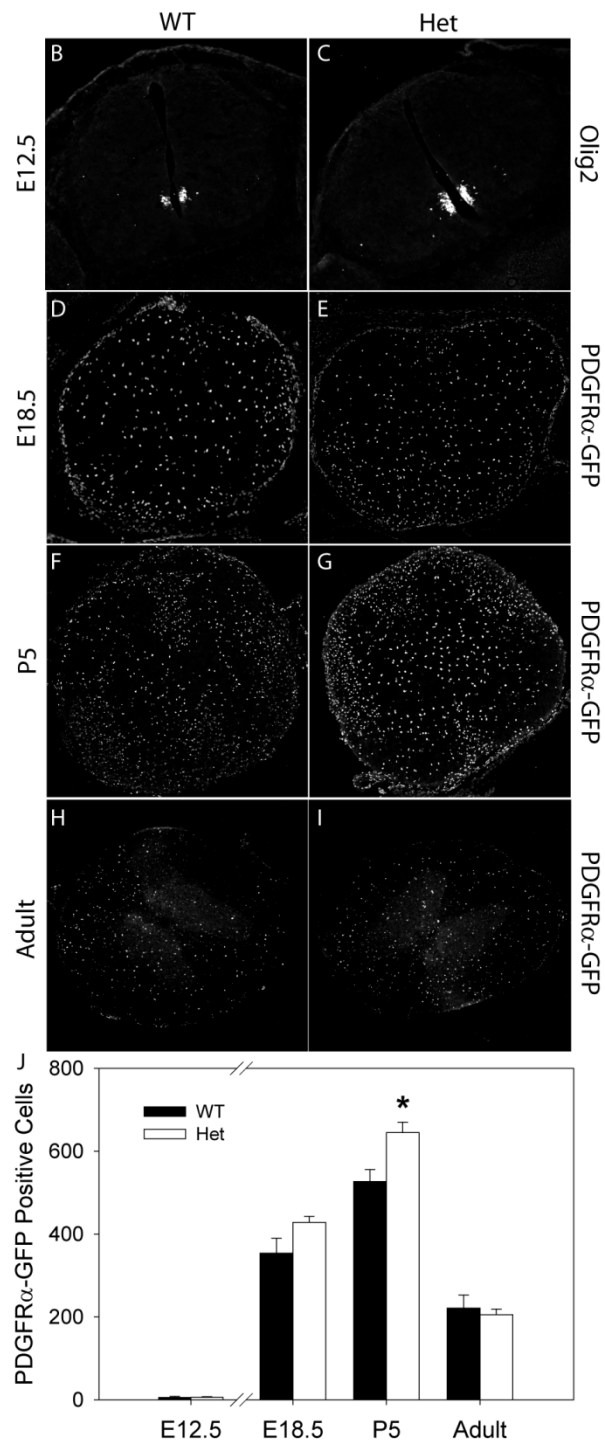


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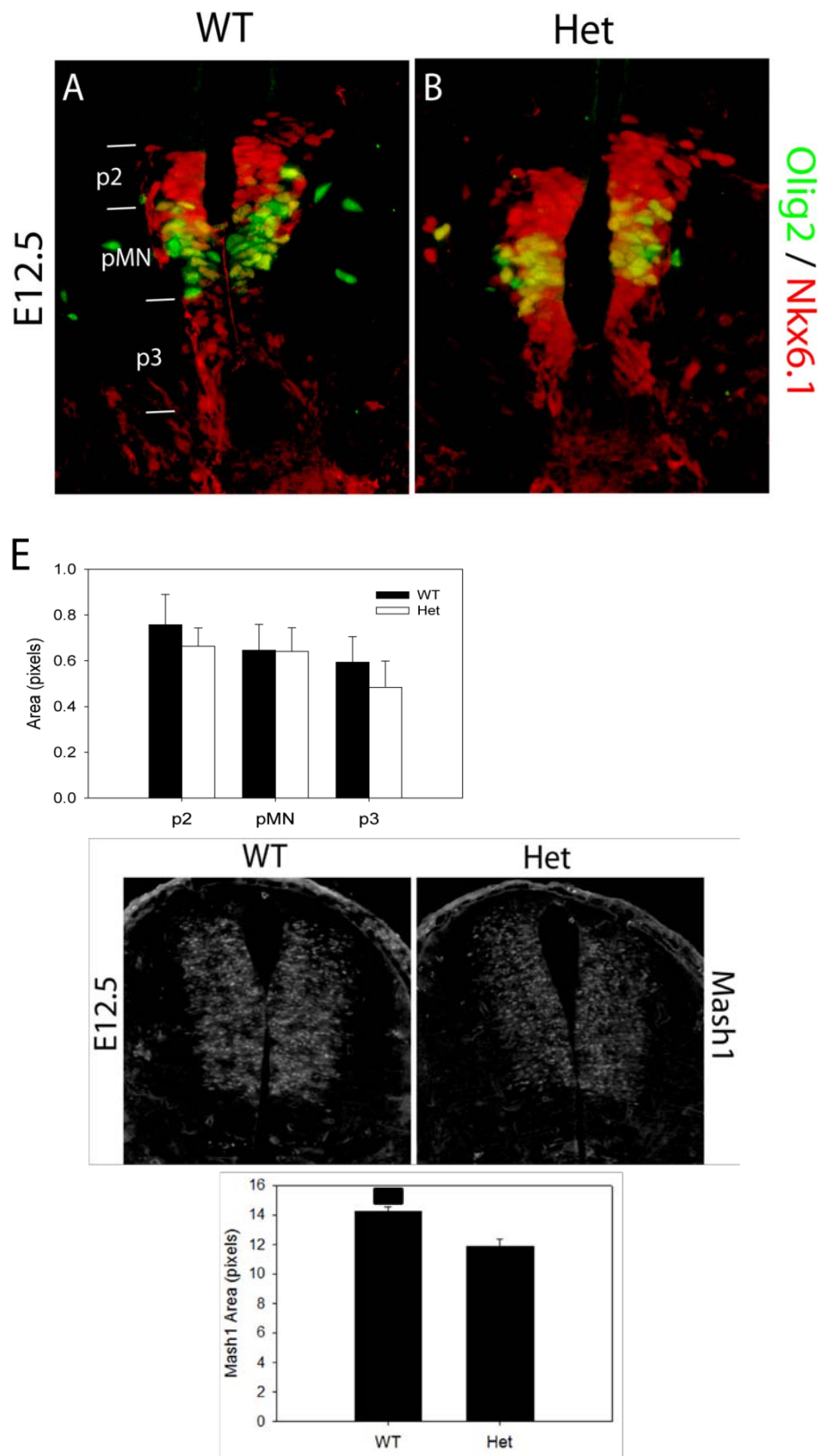


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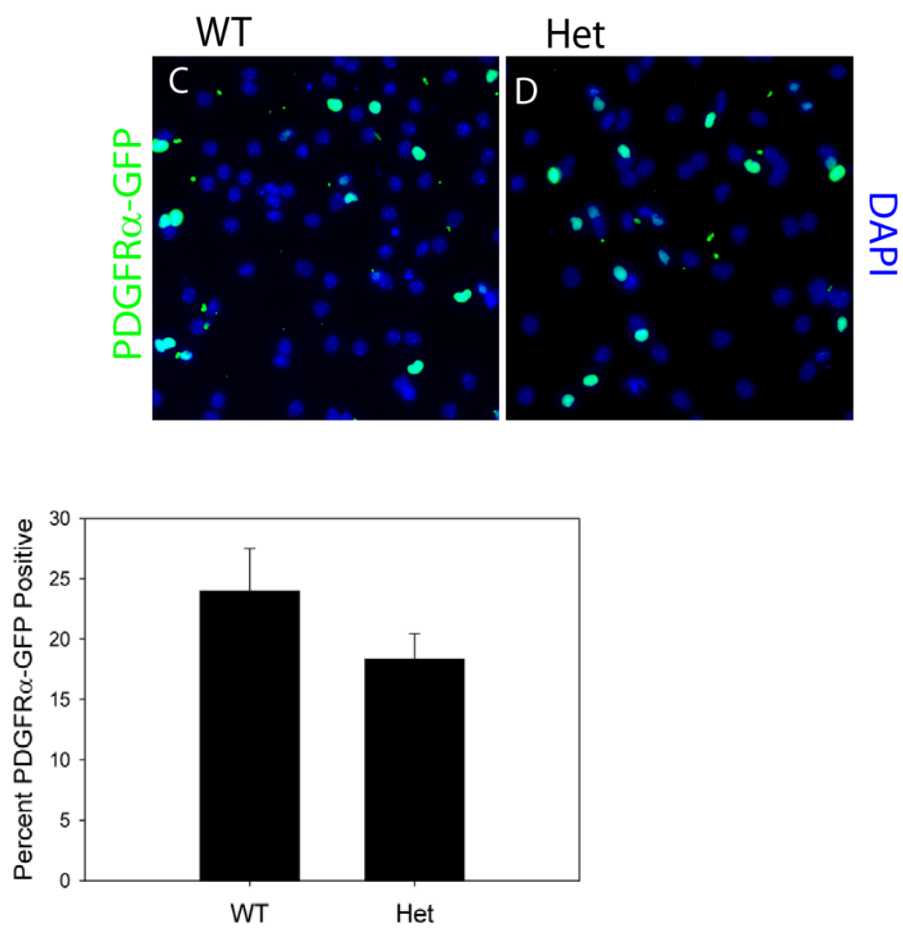


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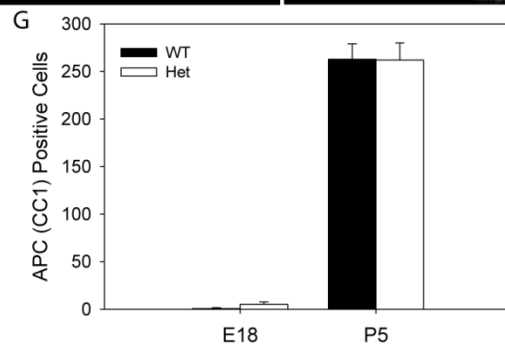
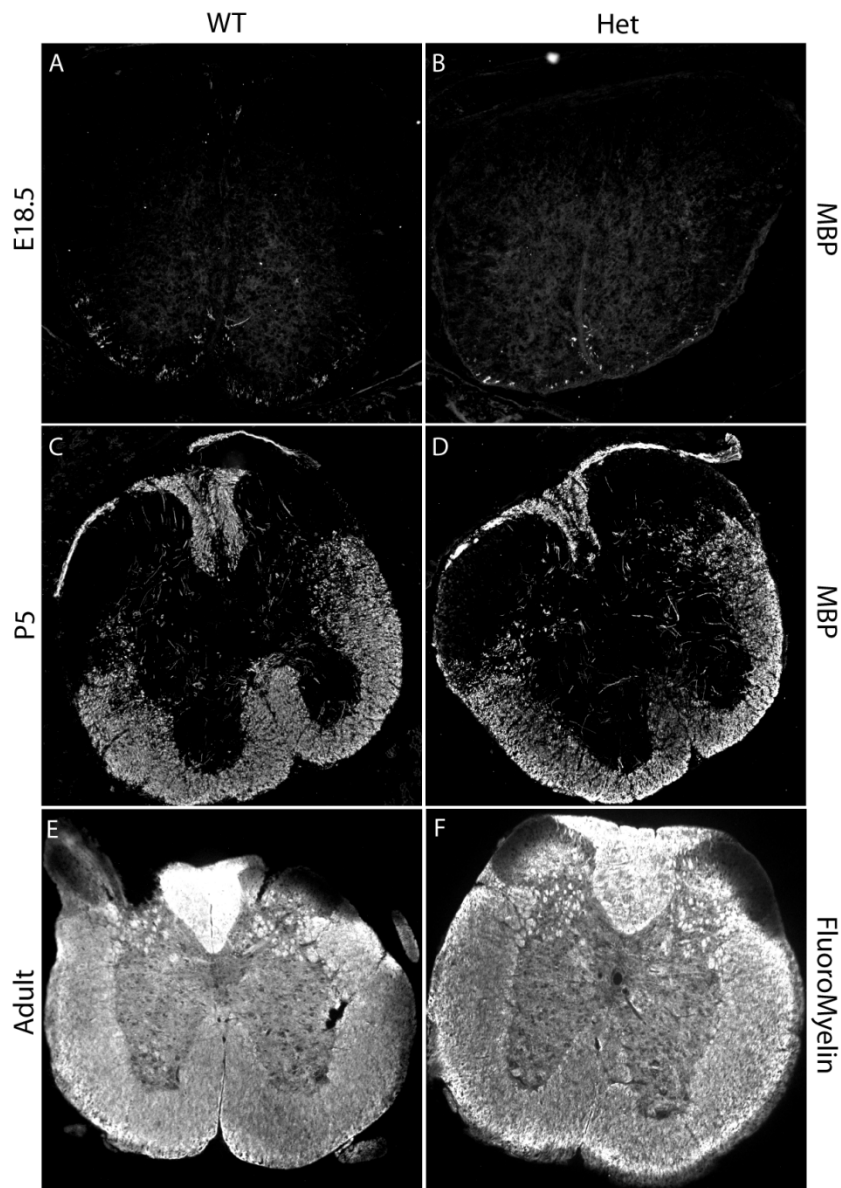


Figure 5.

Lyso 5dpl Analysis

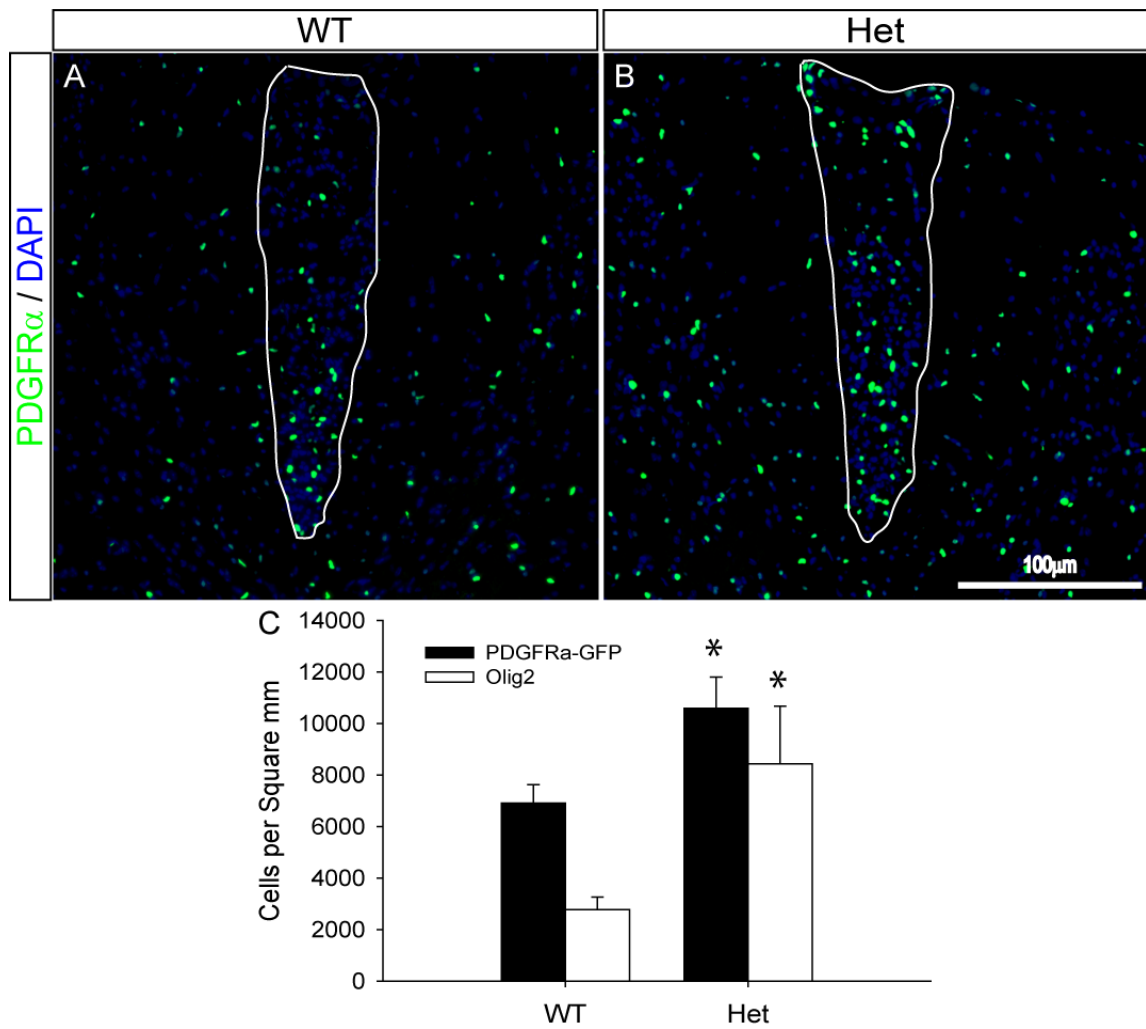


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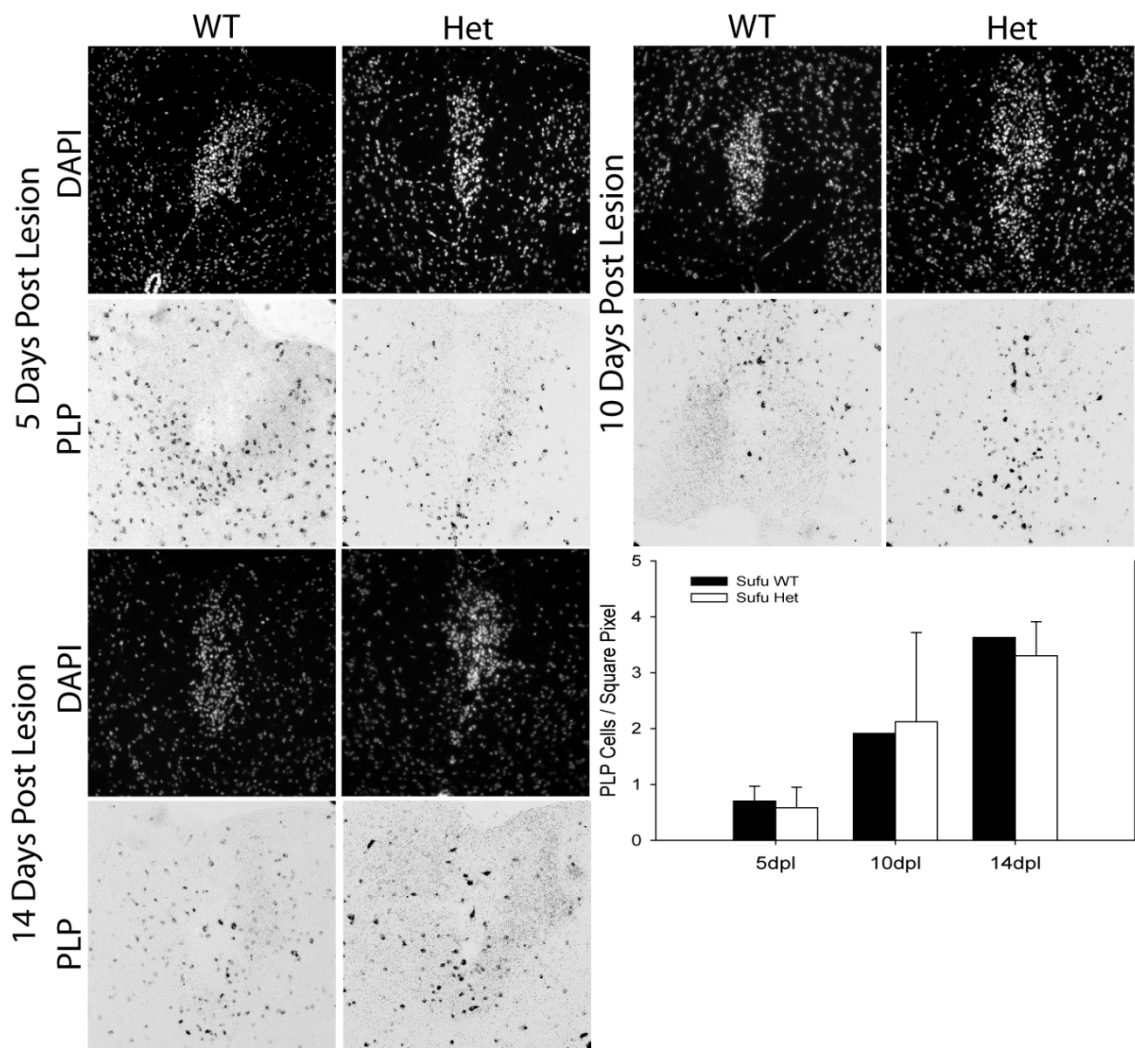


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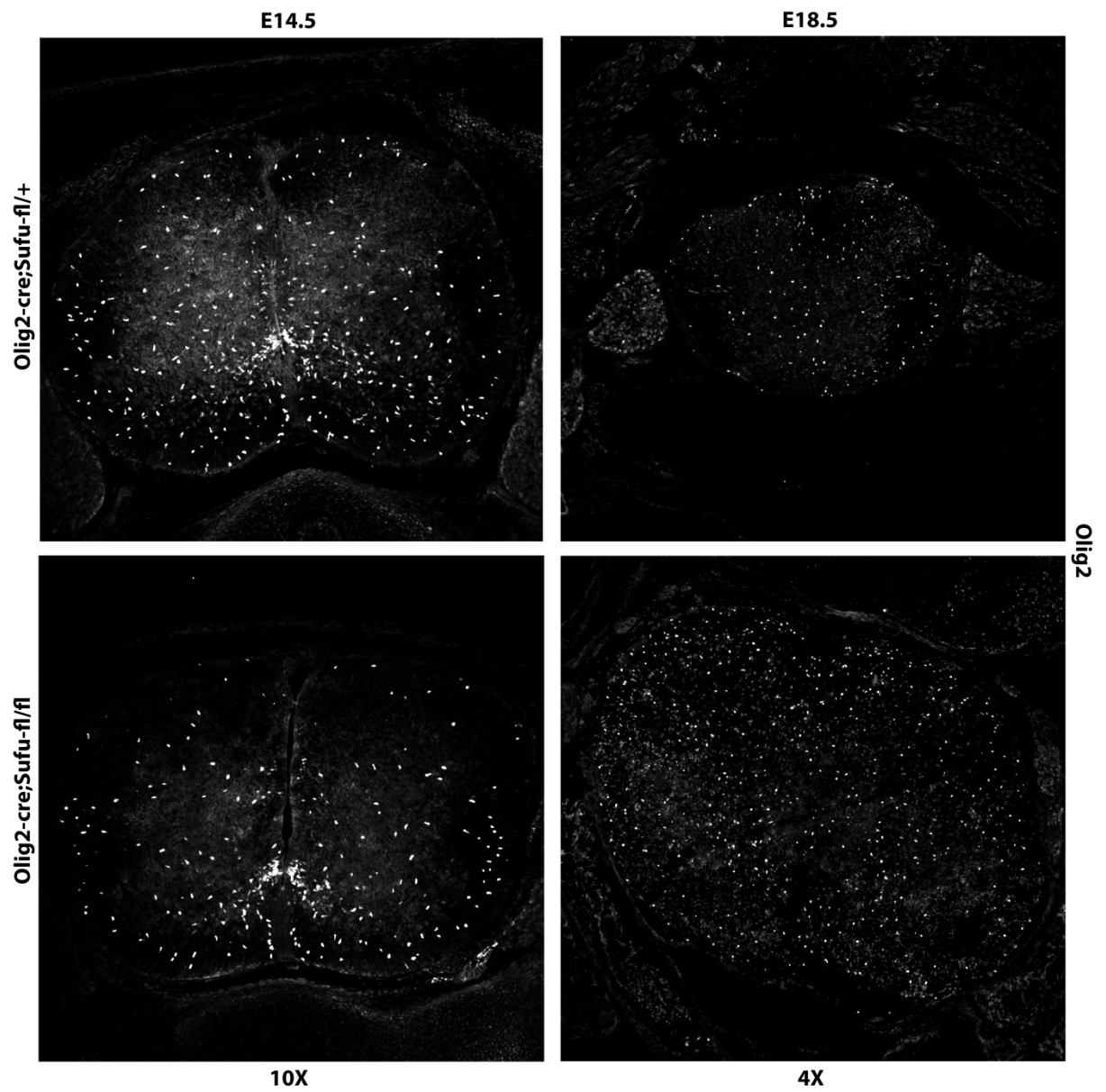


Figure 8.

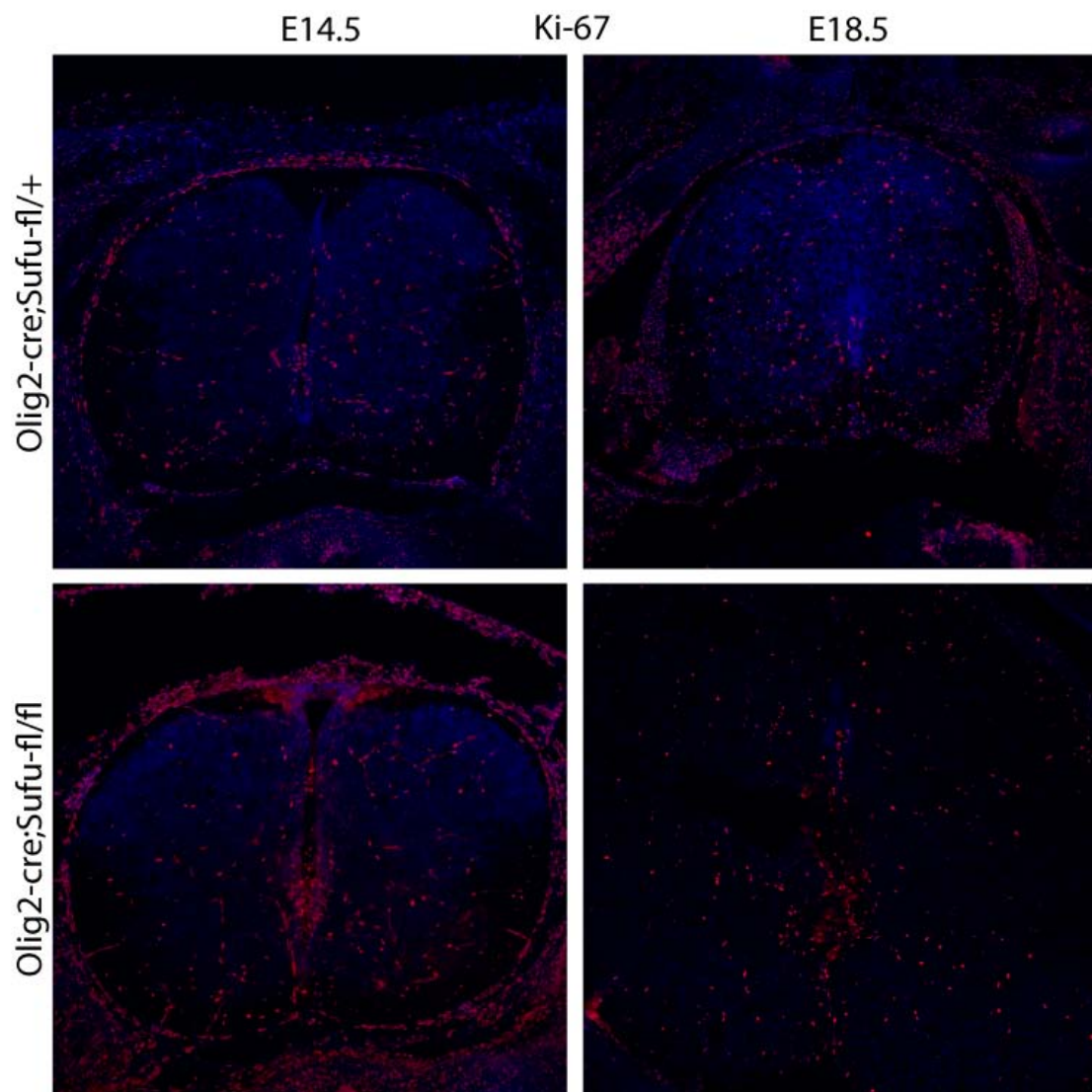
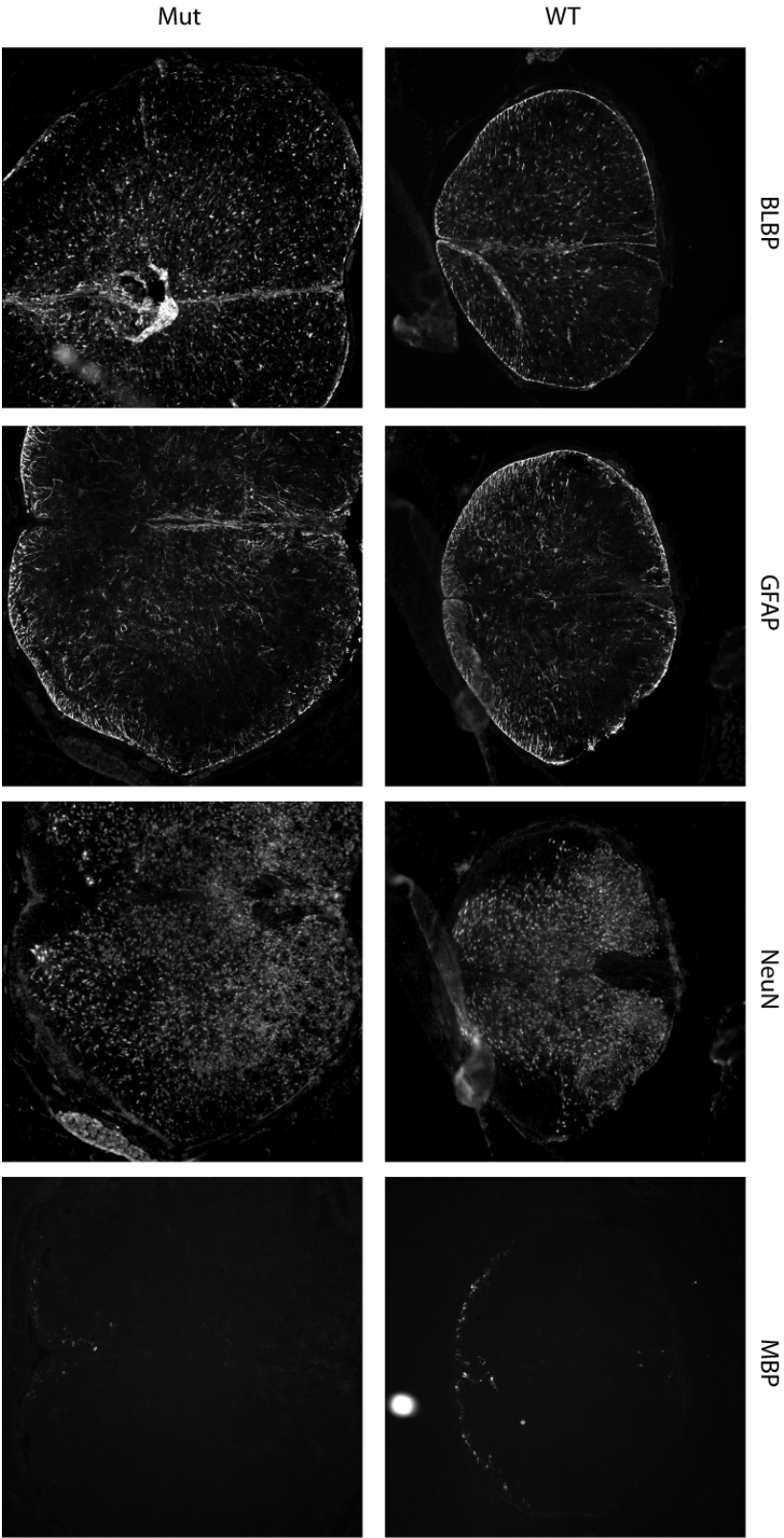


Figure 9.



Publishing Agreement

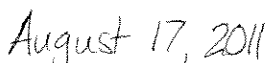
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