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Role of Transcription Factor *Foxb1* in Oligodendrocyte Development

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ABSTRACT

Oligodendrocytes are the myelin-forming cells of the white matter of the central nervous system (CNS). Oligodendrocytes are derived from Oligodendrocyte Progenitor Cells (OPC) or from Neural Stem Cells (NSC) at different development stages. Although numerous factors involved in oligodendrocyte production have been identified, the transcriptional control of oligodendrocytogenesis remains largely unknown. However, the treatment of demyelination diseases like multiple sclerosis (MS) and periventricular leukomalacia (PVL) could greatly benefit from this knowledge.

The forkhead (*Fox*) gene family encodes transcription factors characterized by a DNA binding domain with a variant of the helix-turn-helix configuration. *Foxb1* encodes a forkhead transcription factor expressed in the mouse neural plate and early mesoderm in the primitive streak stage. In midgestation, *Foxb1* is expressed in restricted areas of the neuroepithelium (ventricular zone) as well as the brain parenchyma of midbrain, thalamus, hypothalamus, superior and inferior colliculi, pons, medulla oblongata and spinal cord. Preliminary work in our lab showed that the number of CNS cells belonging to the *Foxb1* lineage (i.e. born from *Foxb1*-expressing ventricular zone) is much larger than the number of CNS cells actually express *Foxb1* in the adult mouse. For my PhD work I wanted to know, first, which CNS cells are generated by *Foxb1*-expressing NSC; second, I wanted to learn about the specific function of *Foxb1* in those cells.

To approach those questions I analyzed the phenotype of heterozygous ($Foxb1^{Cre/+}$) and homozygous ($Foxb1^{Cre/Cre}$) mice of the knock in-knock out *Foxb1-Cre-EGFP* mouse line (generated previously in our lab). I found that the *Foxb1*-expressing neuroepithelium generates large numbers of oligodendrocytes (as well as some astrocytes and neurons); in mice deficient in *Foxb1*, immature oligodendrocytes as well as OPC are abnormally abundant but can differentiate into oligodendrocytes able to produce normal myelin sheaths. I concluded that transcription factor *Foxb1* is a novel player in the regulation of OPC generation, on which it exerts a potent inhibitory function.

-ABSTRACT-

ZUSAMMENFASSUNG

Oligodendrozyten sind die Myelinscheiden-bildenen Zellen der weißen Substanz des zentralen Nervensystems und stammen in Abhängigkeit vom Entwicklungszeitpunkt von oligodendrozytären Vorläuferzellen oder neuralen Stammzellen ab. Obwohl zahlreiche an der Produktion von Oligodendrozyten beteiligte Faktoren identifiziert wurden, sind die molekularen Mechanismen der Oligodendrozytogenese sowie die Herkunft oligodendrozyärer Vorläuferzellen noch weitestgehend unerforscht. Die Multiple Sklerose (MS) und die periventrikuläre Leukomalazie (PVL) bezeichnen zwei wichtige Krankheiten, deren wesentliches pathogenetisches Korrelat die Demyelinisierung darstellt.

Die *forkhead* (FOX)-Genfamilie kodiert für zahlreiche Transkriptionsfaktoren, welche durch eine über die Evolution konservierte DNA-Bindungsdomäne mit einem variablen Helix-Turn-Helix-Motiv charakterisiert sind. *Foxb1* ist ein essentielles Mitglied der forkhead-Genfamilie. Bereits zu einem sehr frühen Zeitpunkt wird es in der Neuralplatte und im frühen Mesoderm exprimiert, zur Mitte der Gestation hin ferner im Mittelhirn, im Thalamus, im Hypothalamus, in den Colliculi superior und inferior, im Pons, in der Medulla oblongata und im Rückenmark. Der Phänotyp von homozygoten *Foxb1*-Knockout-Mutanten zeichnet sich aus durch Störungen des räumlichen Gedächtnisses und der Bewegung der hinteren Gliedmaßen, ebenso ist bei diesen Individuen die Milchproduktion nicht möglich. Vorangegangene Untersuchungen haben gezeigt, dass in homozygoten *Foxb1*-Knockout-Mutanten mehr Zellen der *Foxb1*-Zell-Linie anfärbbar sind, als in heterozygoten *Foxb1*-Mutanten. Ich wollte nun wissen, was es mit den Zellen der *Foxb1*-Zell-Linie auf sich hat und warum gerade die homozygoten Mutanten mehr Zellen der *Foxb1*-Zell-Linie generierten. Sollte es gelingen, diese Zellen zu charaktiersieren, stellt sich weiterhin die Frage, ob die Zellen der homozygoten *Foxb1*-Knockout-Mutanten in ihrer Morphologie und physiologischen Funktion beeinträchtigt oder verändert sind.

In dieser Arbeit habe ich die oben gestellen Fragen beantwortet, indem ich mithilfe des *Cre-loxP*-Rekombinations systems im *Foxb1*-Genlocus transgene Mäuse herstellte und schließlich homozygote Individuen ($Foxb1^{Cre/Cre}$) mit heterozygoten ($Foxb1^{Cre/+}$) verglich. Ich fand heraus, das seine Subpopulation der Oligodendrozyten der Foxb1-exprimierenden Region des Neuroepithels entstammt.

-ABSTRACT-

Foxb1-exprimierende Oligodendrozyten konnten in vitro kultiviert und mittels Entwicklungsstadium-spezifischer Marker angefärbt werden (PDGFRα, NG2, Olig2, GalC, Claudin11 und MBP). Zudem generieren die *Foxb1*-exprimierenden Regionen des Neuroepithels auch einige Neurone und wenige Astroyzten. Die Oligodendrozyten der *Foxb1*-Zell-Linie entwickeln sich zu reifen Oligodendrozyten und bilden Myelinscheiden, welche unter dem Elektronenmikroskop eine normale und pyhsiologische Konfiguration zeigen. Ich habe ferner gezeigt, dass das Ausknocken beiden *Foxb1*-Allele zu einer gesteigerten Proliferation oligodendrozytärer Vorläuferzellen im postnatalen Hirn führt. Das lässt darauf schließen, dass *Foxb1* entweder die Prolifertion der oligodendrozytären Zell-Linie reguliert oder das Schicksal neuroepithelialer Zellen verändert.
Diese Arbeit könnte also dazu beitragen, das demyelinisierende Erkrankungen wie die MS oder die PVL besser verstanden werden und gegebenenfalls neue Therapieansätze entwickelt werden können.

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ABBREVIATIONS

°C	degrees (in Celsius)
4^{th} V	fourth ventricle
AP	alkaline phosphatase
β-gal	beta-galactosidase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BMP	Bone morphogenic protein
bp	Base pairs
BrdU	5'-bromo-2'-deoxyuridine
cDNA	Complementary DNA
Claudin11	Oligodendrocyte specific protein
cm	centimeters
CNS	Central Nervous System
DAPI	4'6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagles's Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alii*. (and other authors)
EtBr	Ethidium bromide
EtOH	Ethanol
Foxb1	Forkhead Box B1
GFP	Green fluorescent protein
GalC	Galactocerebroside
GFAP	Glial fibrillary acidic protein
h	Hour
hPLAP	human Placental alkaline phosphatase
IBA-1	Ionized calcium-binding adapter molecule 1
IRES	Internal ribosomal entry site
ISH	In situ hybridization
L	Liter
MBP	Myelin basic protein
MgCl2	Magnesium chloride

-ABBREVIATIONS-

min	Minute
mM	milli Molar
mRNA	Messenger ribonucleic acid
NBT	Nitro blue tetrazolium chloride
NeuN	Neuronal nuclei
ng	Nano gram
NG2	Neural/glial antigen 2
NTMT	Alkaline phosphatase buffer
OPC	Oligodendrocyte progenitor cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFRα	Platelet derived growth factor receptor, alpha polypeptide
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
V	Volts
VZ	Ventricular zone
WT	Wild Type
µL	micro liter
µM	micro molar
µmol	micro Mol

CHAPTER 1: INTRODUCTION

Oligodendrocytes are the cells that produce and maintain the myelin sheaths around axons of the central nervous system (CNS, including the brain, brainstem and spinal cord). Since the myelin sheath is essential for proper neuronal function, oligodendrocytes are very important cells. Defects in myelination, the process by which oligodendrocytes wrap axons in layers of myelin, cause severe pathological conditions in humans. Since myelination occurs mostly during CNS development, my research has focused on the development of oligodendrocytes.

1.1. Importance of Oligodendrocyte Development

To emphasize the relevance of oligodendrocyte development to the health of human babies, I will briefly introduce here the pathological condition periventricular leukomalacia (PVL), characterized, among other abnormalities, by loss of mature and abnormal abundance of immature oligodendrocytes (Kohelet et al., 2006). PVL is particularly common in *preterm newborns*, that is, babies born before due date (9 months). Specifically, most children born with a weight of 1 kg or less suffer from PVL and show later in life decreased cognitive abilities ranging from mild learning problems to severe mental retardation. Since preterm newborn intensive care has steadily improved for the last 60 years, survival is increasing for preterm neonates and the prevalence of children with cognitive impairments at school age continues to rise (Swaiman, 2012). Up to now, both pathophysiology changes and their relationship to neurodevelopmental outcome remain poorly understood. For this reason, strategies for identifying and preventing causes of disability in PVL patients are important to both physicians and parents (Swaiman, 2012).

1.2. Oligodendrocytes

The adult CNS is composed of three major types of cells; neurons, astrocytes and oligodendrocytes. The histology and morphology of oligodendrocytes was described in great detail by Spanish neuroscientist Pío del Rio Hortega, a disciple of Santiago Ramón y Cajal, in 1921 (Perez-Cerda et al., 2015). The main function of oligodendrocytes is to support and insulate axons in the CNS by wrapping them in

myelin, a specialized structure formed through the accumulation of layers of lipids and specific proteins. One single oligodendrocyte extends its myelinating processes to approximately 50 axons and forms a myelin sheath of about 1 μm around each of those axons, in this way facilitating saltatory conduction of electric impulses generated in the inter-sheath spaces also called Ranvier nodes. In addition, oligodendrocyte can also provide trophic support to axons and promote their viability (Carlson and Birkett, 2013; Emery, 2010; Mitew et al., 2014).

1.2.1. Oligodendrocyte Development

Oligodendrocyte generate from bipolar, migratory oligodendrocyte progenitor cells (OPC) that arise from neural stem cells (NSC) residing in specific zones of the neuroepithelium (ventricular zone) (McTigue and Tripathi, 2008; Tassetto and Gao, 2006; Zhou et al., 2000). OPC are identified by the expression of a series of specific antigens, including NG2 chondroitin sulfate proteoglycan (NG2), platelet-derived growth factor-alpha receptor (PDGFRα), oligodendrocyte transcription factor 2 (Olig2) and sex determining region Y-box 10 (Sox10).

CNS neurogenesis has been the object of intensive analysis. However, the production of glial cells is less well understood. There are different types of OPC, probably heterogeneous in morphology and physiology, and they are generated in specific domains distributed along the germinal neuroepithelium (ventricular zone) (de Castro et al., 2013; Noll and Miller, 1993). Oligodendrocyte for the spinal cord and hindbrain were initially thought to generate only in the ventral side of the neural tube. However, later research showed that these cells originate from multiple regions (Davies and Miller, 2001; Perez Villegas et al., 1999; Vallstedt et al., 2005; Zannino and Appel, 2009). OPC are generated in three waves during development (Richardson et al., 2006). In the mouse telencephalon, the first wave begins in the medial ganglionic eminence (MGE) and associated entopeduncular area (AEP) of the ventral side at about embryonic (E)12.5 (Mitew et al., 2014; Olivier et al., 2001; Tekki-Kessaris et al., 2001). At E18.5, the derived OPC have migrated and spread to most of the developing telencephalon. The second and third OPC waves start in the lateral and caudal ganglionic eminences at E15.5 and, after birth, in the cortex (Kessaris et al., 2006). The process is different in the spinal cord, since the initial wave of OPC

generation begins in the ventral neuroepithelium (at about E12.5), while the second comes from more dorsal progenitor domains at E15.5. This is followed by a third wave after birth. Although most OPC here are from ventral progenitors, the specific origins of the second wave of OPC are still unclear; some of them may be from dorsal progenitors (Mitew et al., 2014; Rowitch and Kriegstein, 2010). Therefore, the generation of oligodendrocyte and OPC follows a double ventral-to-dorsal and caudal-to-rostral gradient (de Castro et al., 2013).

Once at the final destination, oligodendrocyte mature and acquire the expression of specific markers: MBP, PLP, MAG and Claudin11 among others and acquire their typical morphology as well as the ability to myelination (de Castro et al., 2013; Rowitch, 2004).

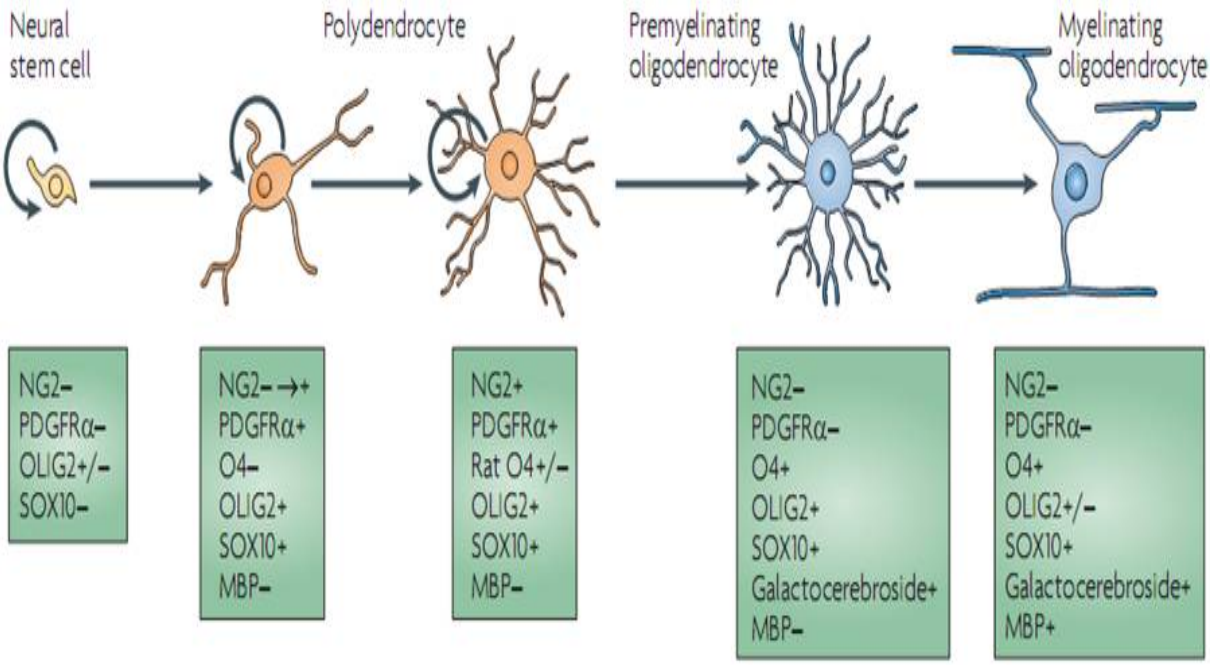


Figure 1. Schematic illustration of oligodendrocyte development.
During oligodendrocyte generation, different stages can be characterized by specific antigens. NG2 and PDGFRα are expressed by proliferating polydendrocytes. Olig2 and Sox10 are expressed throughout the whole development, although it is down-regulated in neural stem cells. Both immature premyelinating oligodendrocytes and mature myelinating oligodendrocytes are identified by Galactocerebroside (GalC) and O4 expression. However, Myelin basic protein (MBP) is only expressed when the oligodendrocyte is able to produce myelin. The morphology is also a good reference to distinguish the different stages of the oligodendrocyte lineage. Neural stem cells and oligodendrocyte progenitor cells are bipolar and show less dendrites. After OPC undergoing differentiation into mature oligodendrocytes, they are able to form myelin sheaths and to develop more processes (Kuhlbrodt et al., 1998; Meijer et al., 2012; Rivers et al., 2008). (Adapted from Nishiyama et al., 2009)

1.2.2 Oligodendrogliogenic Signalling

The migration and differentiation of OPC is precisely regulated by a series of positional and signalling factors (de Castro et al., 2013). For instance, insulin growth factor (IGF)-1 and fibroblast growth factor (FGF)-2 can specify oligodendroglia through activation of the Sonic hedgehog (Shh) pathway. They can also act through phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/ERK2) downstream of mitogen-activated protein kinase (MAPK) signaling respectively (Hsieh et al., 2004; Naruse et al., 2006).

Activation of the Notch pathway can lead to decreased cyclin dependent kinase inhibitor (cdkn1c) activity and increase in OPC differentiation as well as repression of neuronal differentiation (Kim et al., 2008; Mitew et al., 2014; Yuelling et al., 2012).

The formation of ventrally-derived OPCs requires activation of the Shh and bone morphogenic protein (BMPs) pathways and relays on transcription factors such as Pax6, Olig2, Nkx2.2 and Nkx6.1/6.2 (Vallstedt et al., 2005; Yu et al., 2013). The basic helix-loop-helix (bHLH) factor achaete-scute (Asc1/Mash1) plays role in oligodendrocyte specification by restricting the expression of the *distalless* homeobox (Dlx) ½ (Petryniak et al., 2007; Silbereis et al., 2014).

The ventrally originated OPC spread out in a ventro-dorsal and medio-lateral trajectory in spinal cord (Fogarty et al., 2005; Miller and Ono, 1998). By contrast, they show only limited rostro-caudal migration and mostly migrate radially to colonize the rhombomere of origin in quail/chick experiments (Olivier et al., 2001).

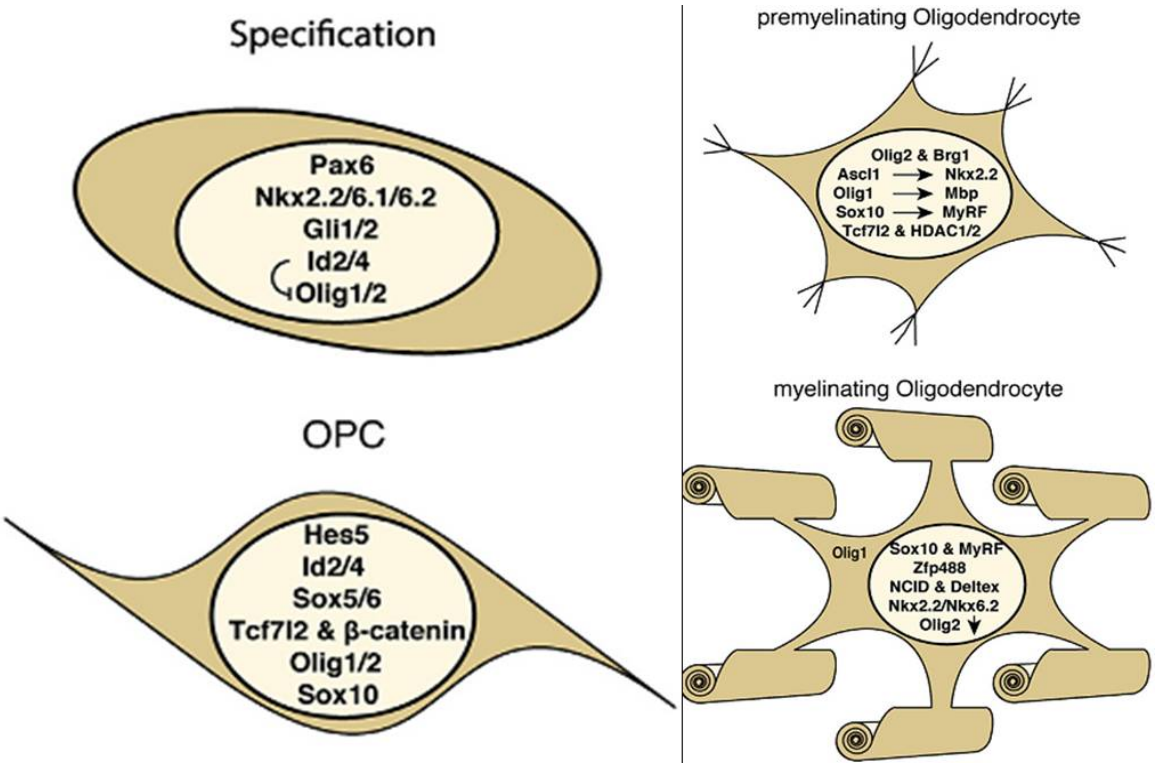


Figure 2. Schematic representation of relevant transcription factors in oligodendrocyte proliferation and differentiation.
During specification and proliferation of oligodendrocyte lineage, several basic helix-loop-helix (bHLH) genes, Olig1, Olig2, Ngn2 and Mash1, together with many transcription factors play fundamental roles in controlling future gene expression and progenitor differentiation. As OPC undergoing terminal differentiation into mature oligodendrocytes, the promyelinating transcription factors are coexpressed with transcriptional repressors to retain myelin homeostasis (Dai et al., 2015; Nieto et al., 2001; Ueno et al., 2012; Zhou et al., 2000). (Adapted from Mitew et al., 2014).

1.2.3 Demyelination and Remyelination

The early OPC lose their connection to the ventricular side of the neuroepithelium during maturation and migration. At the end of the migration process, they settle and change their morphology from uni or bipolar cell to multiprocessed immature oligodendrocytes. A battery of myelin specific genes, such as MBP and PLP, are then upregulated in order for the oligodendrocyte to be able to form myelin sheaths (Miller and Ono, 1998).

If the myelin sheath is damaged, the axon will lose trophic support and insulation. Consequently, demyelinated axons undergo molecular and physiological changes that result in axonal dysfunction, degeneration and loss of function (including for the patient loss of sensation, movement and/or cognition abilities) (Alizadeh et al., 2015). The two major demyelinating diseases are metachromatic leukodystrophic diseases and myelinoclastic diseases (Felts et al., 1997; Konopaske et al., 2008). Their cause can be either a genetic mutation affecting oligodendrocyte differentiation and generation, or infectious agents which damage oligodendrocytes or impair the integrity of the myelin.

Multiple sclerosis (MS) is mostly caused by inflammation, MS presents demyelination and focal neurological lesions because of the inflammation here are thought to be caused by both genetic and environmental problems. PVL is another important myelin disease, the necrosis and coagulation can be observed near the lateral ventricles. PVL is also a risk of the cerebral palsy; the pathological studies show oligodendroglial loss and ischaemia or infection. Therefore, the affected PVL individuals have motor control problems and delayed development in life (Franklin and Ffrench-Constant, 2008; Olofsson et al., 2015). Precisely describing the cellular and molecular mechanisms of demyelination and remyelination is essential to better understanding the possible therapy for MS and PVL.

Over the past decades, a very large number of regenerative medicine and molecular cell biology papers have been published dealing with possible ways to therapeutically optimize remyelination.

Remyelination is the process of OPCs to form new oligodendrocytes to restoring myelin sheath, to reinstating saltatory conduction and to resolving functional deficits on demyelinated axons (Franklin and Ffrench-Constant, 2008). This process is a highly regulated process, and it is efficient in the healthy white matter.

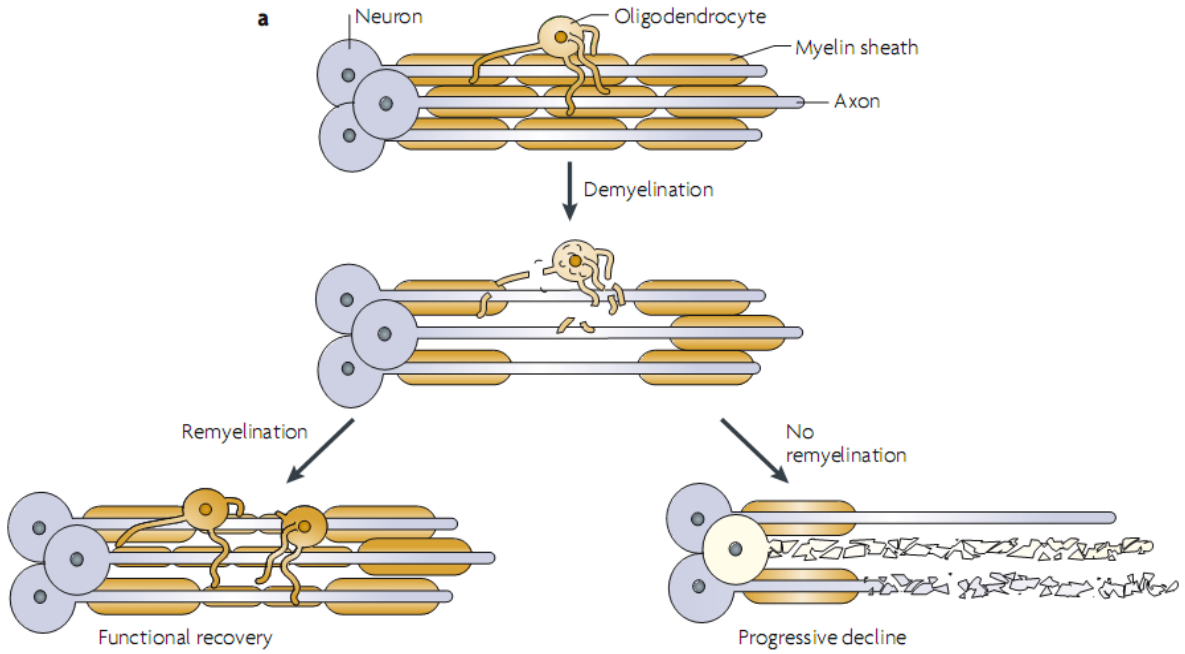


Figure 3. Schematic description of demyelination.
Regardless of which cause, the myelin sheath are damaged by the loss of oligodendrocyte. There are two destinations of an axon after demyelination, 1) the normal reaction is autonomous generation of myelianting oligodendrocytes from OPC to repair myelin sheath, and 2) in some autoimmune or irreversible diseases, mature oligodendrocyte can not be regenerated promptly results in unsuccessful remyelination. The failing myelin sheaths recovery leads to the progressive clinical decline of MS and PVL (Barrette et al., 2013; Kutzelnigg and Lassmann, 2014; Scheller et al., 2015). (Adapted from Franklin and Ffrench-Constant, 2008)

OPC and NPC are responsible to generate oligodendrocyte at different ages. However, the capability of these cells to remyelinate depends on many other factors. For instance, deficiency in certain growth factors can lead to inability to building the highly organized myelin sheath, and to cell death and apoptosis of the newly generated oligodendrocytes (Alizadeh et al., 2015). Although remyelination generates new oligodendrocytes and creates myelin sheath to protect the axons from out of order, we would expect this reconstruction to reach the original myelin sheath thickness and length. However, research has revealed that, during remyelination, a thinner and shorter myelin sheath is built (Franklin and Ffrench-Constant, 2008; Ludwin and Maitland, 1984).

Myelin remodeling in the CNS is independent of cell turnover since it is mainly carried out by mature oligodendrocytes (Yeung et al., 2014). There is still no efficient treatment to prevent demyelination in the chronic stages of demyelinating diseases, although current research is uncovering many possible pathways involved in remyelination.

Remyelination occurs in an environment intrinsically hostile to the oligodendrocyte lineage. This is the cause that, for example, when demyelination is induced by immune response in the MS and in experimental autoimmune encephalomyelitis (EAE), remyelination mechanisms mostly fail (Franklin and Ffrench-Constant, 2008; Lasiene et al., 2008). On the contrary, axons undergoing primary demyelination in experimental and clinical traumatic injury undergo complete remyelination.

Transcription factors may be the most important factor to understand how to treat demyelinating diseases and to better inducing remyelination. Olig1 and Olig2 have been shown to be key factors in developmental myelination. In response to demyelination, local OPC are activated and they regulate several genes that encode the transcription factors Olig2, Myt1, Sox2 and Nkx2.2 (Fancy et al., 2004; Vana et al., 2007). It is believed that microglia and astrocyte are also activated by demyelination, and they induce the rapid proliferative response of OPC to produce more oligdendrocytes for remyelination (Redwine and Armstrong, 1998; Schonrock et al., 1998). This activation is regulated by the cell cycle regulatory protein p27Kip1, FGF and PDGF (Franklin and Ffrench-Constant, 2008; Murtie et al., 2005; Zhou et al., 2006). The Lingo1, Notch-1 and Wnt-b-Catelin are the pathway that involves remyelination. Other factors, such as growth factors IGF-1, Toll-like receptors and

some microRNAs could also play a major role in remyelination (Emery, 2010; Patel and Klein, 2011).

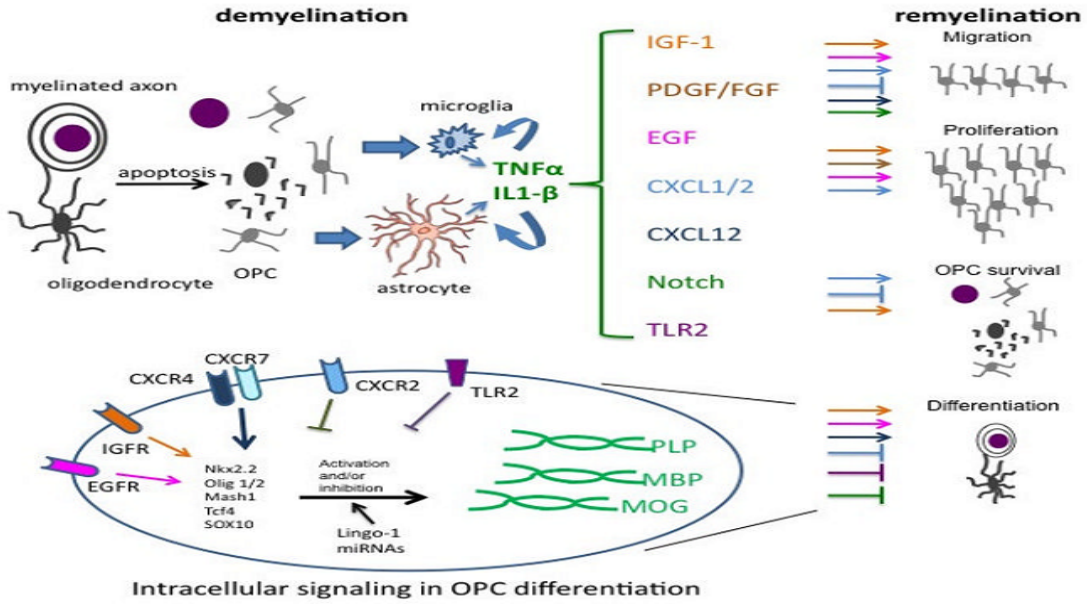
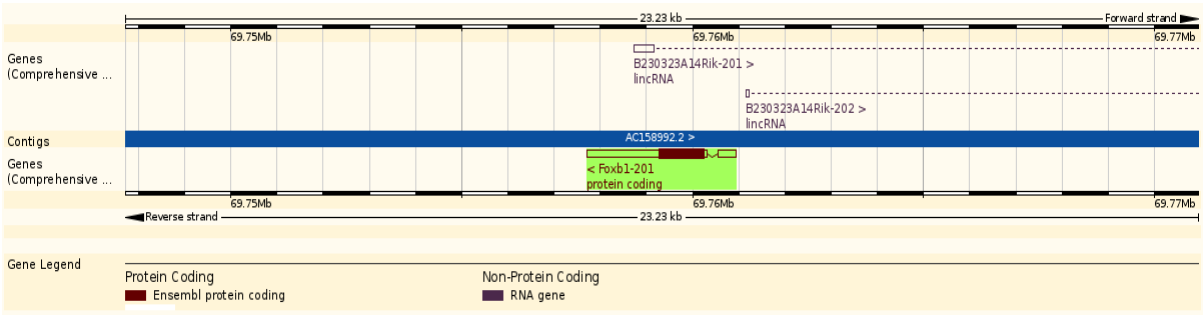


Figure 4. Molecular regulation of remyelination.
After demyelination, oligodendrocyte debris signals activate astrocyte and microglia. The TNFa and IL-1b are stimulated by activation of astrocyte and microglia, and then the oligodendrocyte relevant chemokines and growth factors can be regulated by the secreted cytokines. These alteration are responsible for OPC proliferation, migration and differentiation into myelinating oligodendrocytes. The expression of mature oligodendrocyte antigens, such as PLP, MBP and MOG are regulated by different kinds of chemokines, growth factors, and bHLH transcription factors. On the other hand, TLR2 and Notch1 inhibit oligodendrocyte progenitor survival. This mechanism may account for the remyelination (Aharoni, 2014; Keough and Yong, 2013; Kipp, 2016; Sloane et al., 2010). (Adapted from Patel and Klein, 2011)

1.3 The *Foxb1* Gene

1.3.1 The Structure of the *Foxb1* Gene and the Corresponding Protein

Fork head b1 (*Foxb1*) has also received the names *Fkh5, HFH-e5.1*, *Mf3* and TWH; it is a member of the forkhead gene family, which encodes hundreds of transcription factors identified by its DNA binding domain with winged helix configuration (Alvarez-Bolado et al., 2000a; Carlsson and Mahlapuu, 2002; Weigel and Jackle, 1990; Zhao et al., 2007). The first member of the forkhead family was discovered as a *Drosophila* gene, which promotes differentiation of the terminal, unsegmented ends of the embryo. The structural and functional complexities of the winged helix have been reported and several fork head subfamilies have been detected in different species (Bilella et al., 2014; Kaestner et al., 1993; Kaufmann and Knochel, 1996; Weigel et al., 1989). *Foxb1* is expressed widespread in the early developing neural tube and is later restricted to the ventral and caudal diencephalon. *Foxb1* has been studied in spatial memory, metabolism, aging, tumorgenesis, immunoregulation, thalamic axon guidance and the CNS development (Jonsson and Peng, 2005; Lehmann et al., 2003; Zhao et al., 2007).




```
CLUSTAL O(1.2.1) multiple sequence alignment


Chicken     MPRPGRNTYSDQKPPYSYISLTAMAIQSSPEKMLPLSEIYKFIMDRFPYYRENTQRWQNS
Mouse       MPRPGRNTYSDQKPPYSYISLTAMAIQSSPEKMLPLSEIYKFIMDRFPYYRENTQRWQNS
Human       MPRPGRNTYSDQKPPYSYISLTAMAIQSSPEKMLPLSEIYKFIMDRFPYYRENTQRWQNS
            ************************************************************

Chicken     LRHNLSFNDCFIKIPRRPDQPGKGSFWALHPSCGDMFENGSFLRRRKRFKVLKSEHLAPS
Mouse       LRHNLSFNDCFIKIPRRPDQPGKGSFWALHPSCGDMFENGSFLRRRKRFKVLKSDHLAPS
Human       LRHNLSFNDCFIKIPRRPDQPGKGSFWALHPSCGDMFENGSFLRRRKRFKVLKSDHLAPS
            ************************************************************:*****

Chicken     KPADAAQYLQQQAKLRLSALAATGTHLPQMST--YNL-GVSQPSSFKHPFAIENIIAREY
Mouse       KPADAAQYLQQQAKLRLSALAASGTHLPQMPAAAYNLGGVAQPSGFKHPFAIENIIAREY
Human       KPADAAQYLQQQAKLRLSALAASGTHLPQMPAAAYNLGGVAQPSGFKHPFAIENIIAREY
            ************************:******* :  *** **:***.**************

Chicken     KMPGGLAFSTMQPVPAAYPLPNQLTTVGSSIGTGWPHVYGS-GVIDTATPISMAGGEYGA
Mouse       KMPGGLAFSAMQPVPAAYPLPNQLTTMGSSLGTGWPHVYGSAGMIDSATPISMTSGDYSA
Human       KMPGGLAFSAMQPVPAAYPLPNQLTTMGSSLGTGWPHVYGSAGMIDSATPISMASGDYSA
            *********:****************:***:********** *:**:******:.*:*.*

Chicken     YGVPIKPLCHG-GQTLPAIPVPIKPTPAAVPALPALPAPIPTILSNSPPSLSPTSSQTAT
Mouse       YGVPLKPLCHAAGQTLPAIPVPIKPTPAAVPALPALPAPIPTLLSNSPPSLSPTSSQTAT
Human       YGVPLKPLCHAAGQTLPAIPVPIKPTPAAVPALPALPAPIPTLLSNSPPSLSPTSSQTAT
            ****:*****. ********************************:****************

Chicken     SQSSPATPSETLTSPAPALHSVAVH
Mouse       SQSSPATPSETLTSPASALHSVAVH
Human       SQSSPATPSETLTSPASALHSVAVH
            ***************** ********
```

Figure 5. Schematic structure of *Foxb1* gene.
In mouse, *Foxb1* located in chromosome 9, and this gene has one transcript (splice variant). It is a highly conserved gene between mouse and human.
(Adapted from http://www.ensembl.org/Mus_musculus/Gene, on 19th.Jan, 2016)

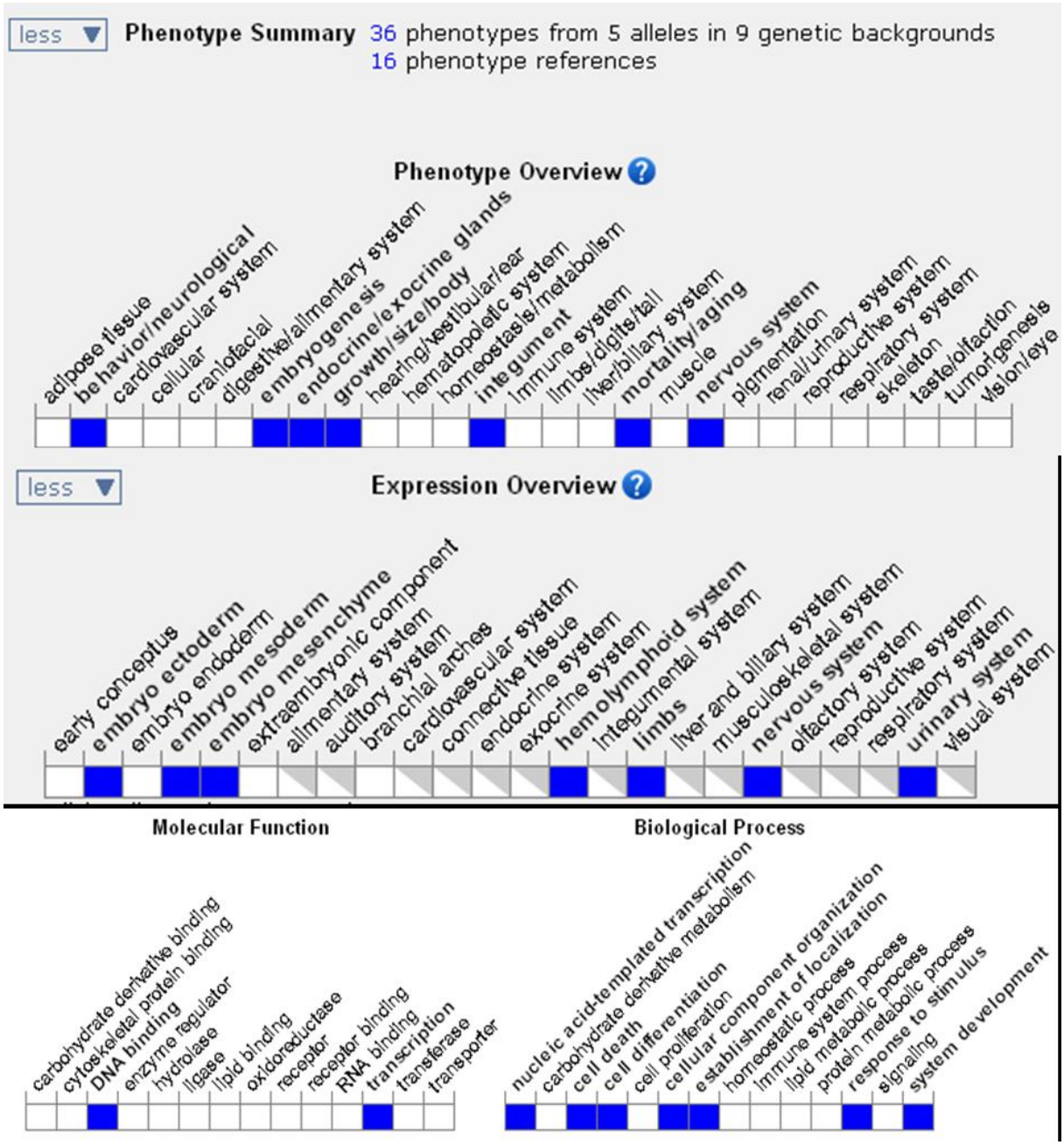


Figure 6. Overview of *Foxb1* phenotype, expression, molecular function and biological process. (Adapted from http://www.informatics.jax.org/marker/MGI:1927549 on19th, Jan, 2016)

1.3.2 Tissue Distribution and Expression

In primitive streak-stage embryos, *Foxb1* labels specific domains in the neural plate and early mesoderm. After this stage, *Foxb1* is expressed in the thalamus and hypothalamus, midbrain, superior and inferior colliculus, pons, medulla oblongata, spinal cord and mammary gland epithelium (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000a; Kaestner et al., 1996; Kloetzli et al., 2001; Zhao et al., 2007). Evidence from *Foxb1* mutant studies suggests roles for *Foxb1* in the development of midbrain, medulla oblongata, hypothalamus and spinal cord, as well as in milk generation or ejection (Alvarez-Bolado et al., 2000b; Dou et al., 1997; Kloetzli et al., 2001; Labosky et al., 1997; Radyushkin et al., 2005). As it is shown above, *Foxb1* is expressed in hindbrain and ventral spinal cord at embryonic age in mice, raising the possibility that the *Foxb1* plays roles in mediating hindbrain differentiation (Alvarez-Bolado et al., 1999; Hardy and Friedrich, 1996). The *Foxb1* expression has been described detailedly at different embryonic ages, making it appealing candidates for regulating neurons and oligodendrocyte generation. Whether *Foxb1* regulates the fate decision of neuroepithelium and how it works on oligodendrocyte lineage production has not been reported.

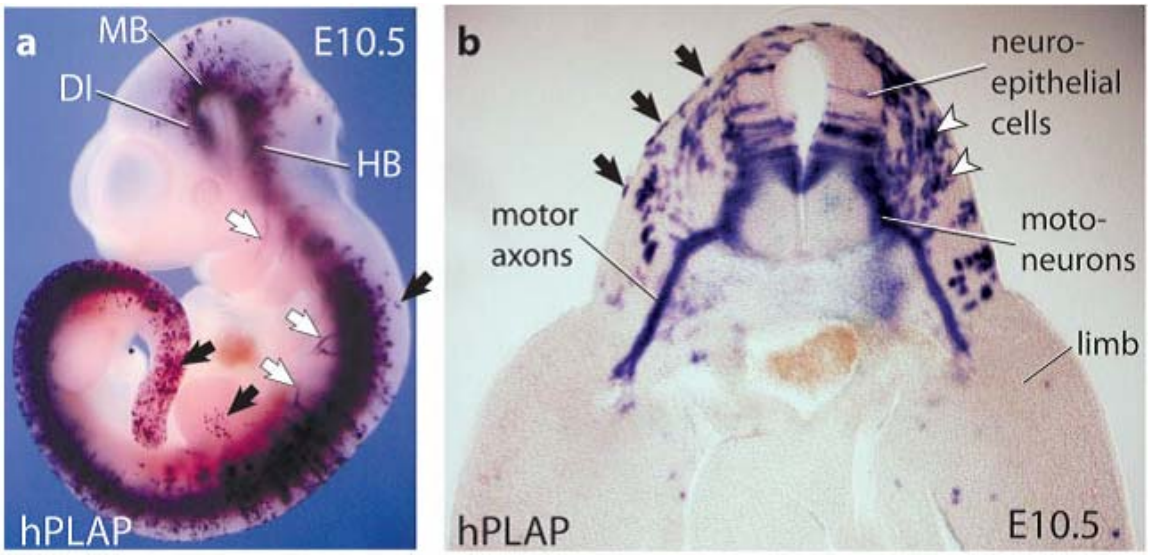
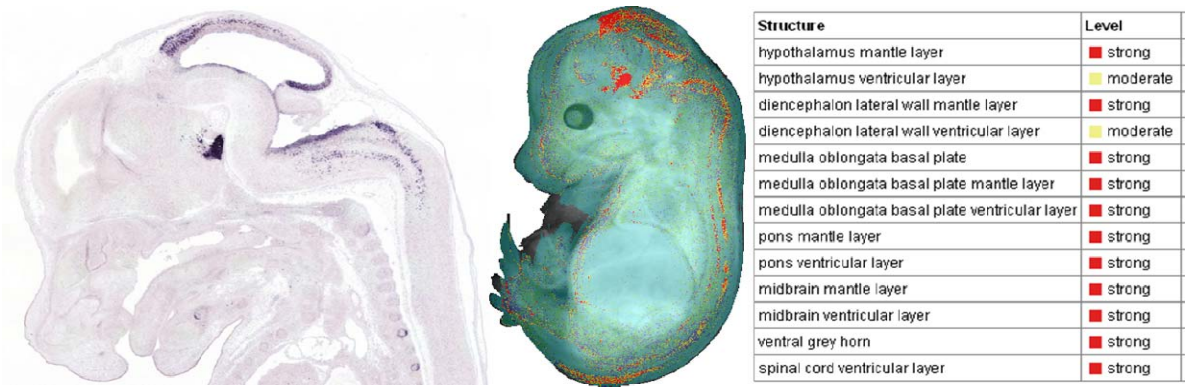


Figure 7. hPLAP reaction on *Foxb1*^{Cre} x *Z/AP* mouse.
At E10.5, the midbrain, hindbrain and spinal cord are labeled by alkaline phosphatase (our lineage reporter). *Foxb1* is expressed by neuroepithelial cells, axonal tracts and motor neurons at this age. (Adapted from Zhao et al., 2007)



Structure	Level
hypothalamus mantle layer	strong
hypothalamus ventricular layer	moderate
diencephalon lateral wall mantle layer	strong
diencephalon lateral wall ventricular layer	moderate
medulla oblongata basal plate	strong
medulla oblongata basal plate mantle layer	strong
medulla oblongata basal plate ventricular layer	strong
pons mantle layer	strong
pons ventricular layer	strong
midbrain mantle layer	strong
midbrain ventricular layer	strong
ventral grey horn	strong
spinal cord ventricular layer	strong

Figure 8. *Foxb1* expression is detected by in situ hybridization at different development stages.
The three dimensional image shows *Foxb1* is expressed strongly in midbrain and hindbrain, but except in forebrain. At E14.5, *Foxb1* is detected in the neuroepithelium which may differentiate into OPC. (Adapted from http://www.emouseatlas.org)

1.4 Hindbrain Anatomy and Function

The hindbrain is a brainstem region including rhombencephalon, pons, medulla oblongata and cerebellum. The hindbrain contains many axon tracts between the spinal cord and higher CNS levels (thalamus and hypothalamus), and it is the destination of many neurons of the reticular formation. In early development, the hindbrain is organized in the rostral-caudal axis by segmentation into rhombomeres. As development proceeds, longitudinal tracts are formed between vestibular networks, the spinal cord and the higher CNS regions (Bosma, 2010). Basically, the hindbrain governs the automated body systems, such as heartbeat, breathing, motor control and sense of equilibrium. The pons controls facial sensation, expressions and movements, eye rotations, breath intensity and frequency, and sense equilibrium. Similarly, the medulla forms the base of the brainstem and it is located between the pons and spinal cord. It regulates cardiac rate and rhythms, breathing rhythms, vasometrics and the reflex actions.

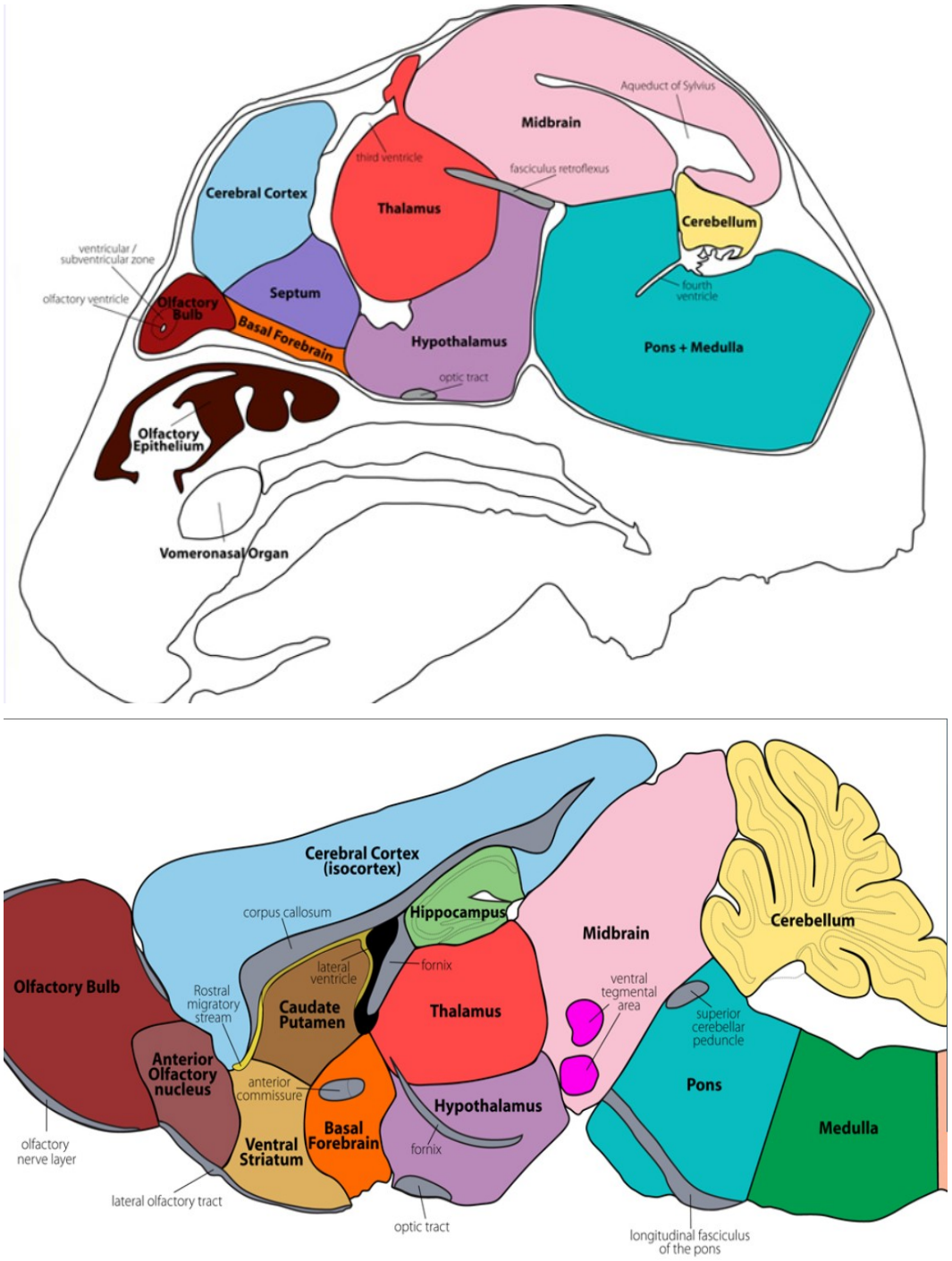


Figure 9. Schematic of sagittal view of E15.5 embryonic brain (upper) and P56 adult brain in mouse (lower). (Adapted from Allen Brain Atlas, http://www.brain-map.org)

1.4.1 The Medulla Oblongata

The medulla oblongata (myelencephalon or simply “medulla”) is the lower part of the hindbrain, posterior to the pons and anterior to the cerebellum. The sensory nuclei, for instance, the solitary nucleus, the trigeminal nerve nuclei, cochlear and vestibular nuclei, the inferior olivary nucleus and the dorsal column nuclei are developed from the alar plate (i.e. the dorsal portion) of the embryonic medulla. In contrast, the motor nuclei, such as the hypoglossal nucleus, nucleus ambiguus, dorsal nucleus of vagus nerve and the inferior salivatory nucleus are produced from the basal plate (i.e. the ventral portion). The medulla is responsible for multiple autonomic functions, e.g. the control of hearbeat and breathing. In human, the medulla oblongata is also called bulbus rachideus, and hence the adjective “bulbar” to refer to this region, especially in clinical usage (Carlson, 2013; Ono et al., 1997; Rovainen, 1985).

1.4.2 The Pons

The pons is located between the midbrain and the medulla oblongata, and it is ventral to the cerebellum. The pons is a bridge to connecting medulla and cerebellum to higher centers (Butler and Hodos, 2005; Ono et al., 1997). The pons is divided into basilar part and pontine tegmentum, and it contains a number of nuclei that deal with primary reflexes. The pons receives sometimes the name of metencephalon, the embryonic brain region that originates it. The V-VIII cranial nerve nuclei are present in the pons, and they play roles in sleep, respiration, swallowing, hearing, taste, eye movement, bladder control and facial sensation.

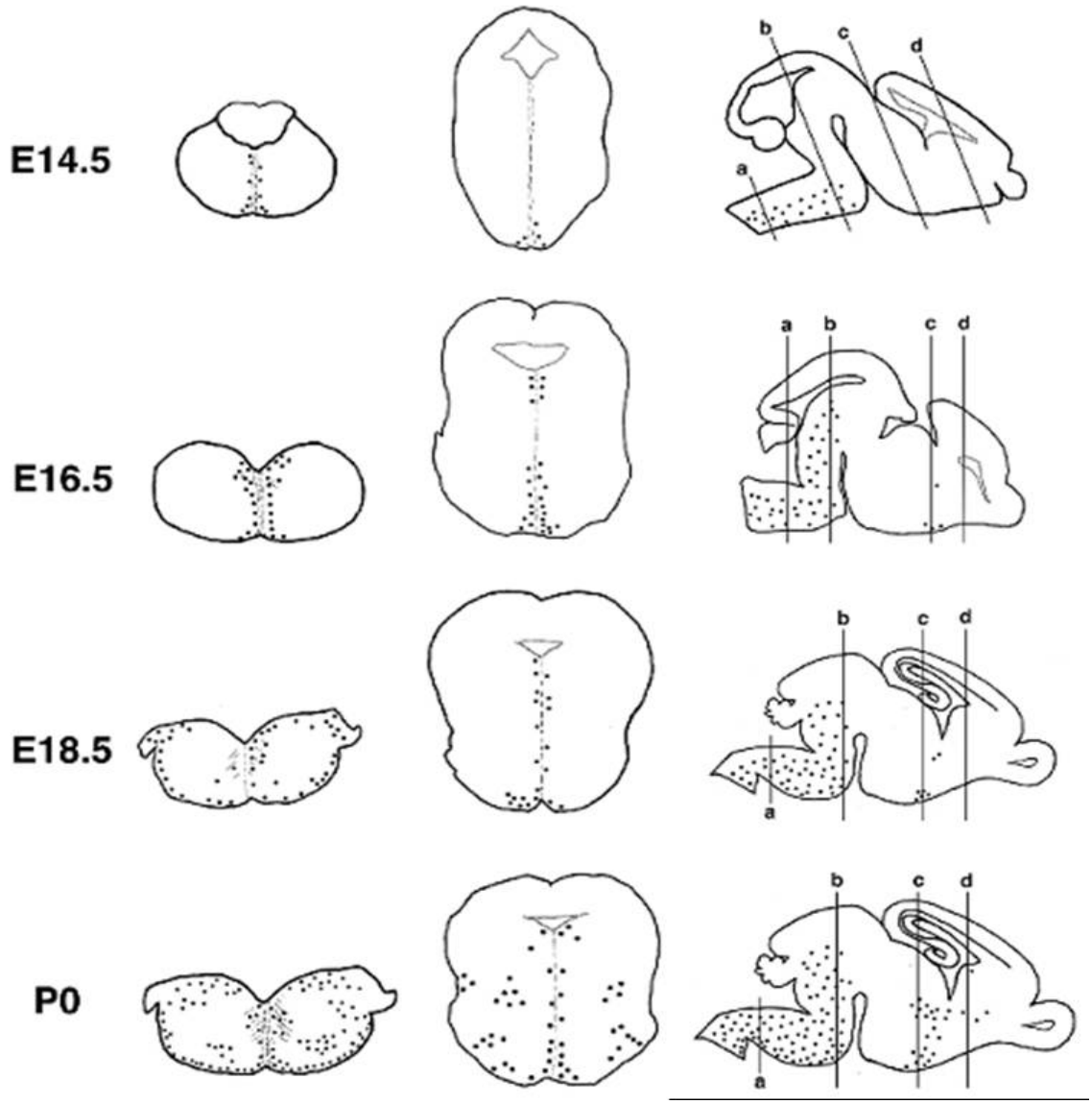


Figure 10. Sketch of the origins of oligodendrocytes in pons and medulla oblongata.
The oligodendrocyte lineage was labeled with specific antibodies during different brain development. OPC are generated from the neuroectodermal cells of the neural tube, and then migrate to the whole pons and medulla oblongata. (Adapted from Hardy and Friedrich, 1996)

1.5 Questions and Aims of the Study

Our group has shown that *Foxb1* is expressed in specific regions of the developing neural tube, for example, diencephalon, midbrain tegmentum, hindbrain and spinal cord. The developmental of oligodendrocyte in the hindbrain has not been studied as thoroughly as in spinal cord. Since, in the spinal cord, oligodendrocytes arise mostly in the ventral ventricular zone adjacent to the floor plate, one could expect them to arise from ventral portions of the brainstem too. Indeed, most hindbrain oligodendrocytes originate ventrally. However, medulla oblongata oligodendrocytes originate from discrete focal clusters around the ventricular or subventricular zones as well (Davies and Miller, 2001; Perez Villegas et al., 1999; Vallstedt et al., 2005; Zannino and Appel, 2009).

We know that *Foxb1* is essential for the survival of specific subpopulations of neurons and for appropriate targeting of some major axonal tracts (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000b; Zhao et al., 2008; Zhao et al., 2007). Since we have detected *Foxb1* expression in portions of the neuroepithelium known to originate OPC, here we ask:
1) Which specific cell types of the CNS are generated by *Foxb1*-expressing ventricular zone (i.e. what is the *Foxb1* cell lineage in the brain)?
2) (since upon answering the first question I found that large numbers of oligodendrocytes belong to the *Foxb1* lineage) What is the role of *Foxb1* in oligodendrocyte development?

To trace the progeny of adult *Foxb1* cells, I crossed *Foxb1-Cre* mouse line, carrying a knockin-knockout *Foxb1* mutation, with the *Z/AP* mouse reporter line, which expresses human placental alkaline phosphatase (*hPLAP*) as *Cre* expression reporter. For the present thesis I approached the above questions through a combination of *Foxb1* lineage *in vivo* and *in vitro* expression analysis and fate mapping, as well as phenotype analysis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

- Acetic acid (Merck)
- Acetic anhydride (Sigma)
- Agarose (Sigma); Alkaline
- Alkaline phosphatase (New England Biolabs)
- DAPI (Roth)
- Distilled water (miliQ water)
- Dimethyl Sulfoxide (DMSO) (Fisher Scientific)
- DMEM Medium, high glucose (Gibco)
- Rnase (Roche)
- dNTPs (Roche)
- EDTA (Calbiochem)
- Ethanol, Pure (Sigma)
- FBS: Fetal Bovine Serum (PAA)
- Glucose (Sigma);
- Glycerol (AppliChem)
- Go Taq DNA Polymerase (Promega)
- Go Taq $MgCl_2$ (Promega)
- PCR buffer (Promega)
- 37%HCl (Merck)
- Isoflurane (Baxter)
- Isopropanol (VWR)
- Kanamycin (Roth)
- KCl (Merck)
- KH_2PO_4 (Roth)
- L-Glutamate (PAA)
- LB powder (Roth)
- LiCl (Fluka)
- Methanol (AppliChem)

- $MgCl_2$ (AppliChem)
- Mowiol (Roth)
- Na_2HPO_4(AppliChem)
- NaCl (AppliChem)
- NaOAc (Merck)
- $NaPO_4$ (Roth)
- NBT (Roche)
- NBT/BCIP (Boehringer Mannheim)
- Normal Goat Serum (Cell Signaling Technologies)
- Normal Horse Serum (AbCam)
- NP40 (Fluka)
- Nuclear fast red (Sigma)
- OCT (A. Hartenstein)
- Paraffin (Langenbrink)
- PBS (Gibco)
- Penicilin/Streptomycin (PAA)
- PFA (Sigma)
- Poly-L-Lysine (Sigma)
- Proteinase K (Roche)
- Skin Antiseptic (Kodan)
- Softasept (Braun)
- Penicillin-Streptomycin (Gibco)
- Sucrose (Sigma)
- Tris-Sodium citrate dihydrate (Merck)
- Trypsin (PAA)
- Tris-Base (Roth)
- Tris-HCl (Roth)
- Triton-X-100 (Sigma)
- Tween-20 (Sigma)
- Xylene (VWR)

2.1.2 Equipments

- Binocular Stereomicroscope (Carl Zeiss Semi 2000-CS)
- Cell Culture Incubator (HERA Cell 150)
- Coated Vicryl Suture (Ethicon)
- Confocal Microscope (Carl Zeiss LSM)
- Cryostat (Leica CM3050S)
- Cryotube Vials (Thermo Scientific)
- Electrophoresis gel chamber (Bio Rad, Sub Cell GT)
- Falcon tubes 15ml, 50ml (Fisher Brand)
- Gel documentation system (Bio Rad)
- Heat block (Eppendorf)
- Heating pad (Beurer TM 20)
- Insulin Syringe 0,5ml (BD Micro-Fine)
- Light Microscope (Leica)
- Microtome (microTec)
- Oxygen flask (medical use)
- Petri dish (Falcon)
- Pipette Pasteur (Fisher Scientific)
- Serological Pippettes 2ml, 5ml, 10ml, 25ml (Cellstar)
- Slides Superfrost plus (Thermo Scientific)
- Small Animal open circuit anesthetic Machine for Isoflurane (Komensaroff)
- Software Zen 2010 (Carl Zeiss)
- Sorvall Centrifuge (Sorvall RC 5B plus)
- T25 flask (Cellstar)
- Table Centrifuge (Eppendorf)
- TECAN rack (TECAN)
- Thermocycler (VWR)
- Vortex (NeoLab)
- Water Bath (Julabo)
- Water purification system (Millipore)

2.1.3 Recipes

10 mM Sodium Citrate Buffer pH=6.0

2.94 g of Tri-odium Citrate (dihydrate)

Adjust volume with dH_2O to 1 L.

Adjust pH to 6.0.

Add 500 µL Tween20 (0,05%).

10×PBS (for 1 L solution)

1.37 M NaCl

0.027 M KCl

0.1 M Na_2HPO_4

0.02 M KH_2PO_4

Adjust pH to 7.4.

Add volume with dH_2O to 1 L and autoclave.

10×TN (for 1L solution)

1 M Tris

1.5 M NaCl

Adjust pH to 7.5.

Adjust volume with dH_2O to 1 L and autoclave.

PBND buffer (Lysis buffer)

50 mM KCl

10 mM Tris; pH=8.3

(5 ml of 1 M Tris-HCl stock)

2.5 mM $MgCl_2$ (1.25 ml 1 M of $MgCl_2$ stock)

0.45% v/v NP-40

0.45% v/v Tween-20

Adjust volume with dH_2O to 500 ml and autoclave. Store at 4°C

TE buffer pH=8.0

1 M Tris-HCl; pH8.0

0.5 M EDTA; pH8.0

Tris-EDTA Buffer pH=9.0

Prepare 1 L of 100 mM Tris-Base pH=9.0 autoclave

Prepare 1 L of 10mM EDTA pH=8.0 autoclave

For 1 L Tris-EDTA buffer: add 100 mL Tris stock

100 mL EDTA stock and fill with miliQ water until 1 L. Check pH (to be 9.0).

Add 500 µl Tween20 (0.05%)

AP buffer

100 mM Tris-HCl, pH=9.5,

100 mM NaCl,

10 mM $MgCl_2$

NBT/BCIP Staining Buffer

100 mM Tris-HCl, pH=9.5

100 mM NaCl,

50 mM $MgCl_2$,

0.01% sodium deoxycholate

0.02% NP-40,

337 µg/ml NBT and 175 µg/ml BCIP.

2.2 Methods

2.2.1 Mutant Mouse Lines

All mouse lines were housed and fed according to the German animal welfare regulations (TierSchG) and the European Communities Council Directive in the Interfacultary Biomedical Faculty (IBF), University of Heidelberg. The authorization of collecting mice brain and handling animal samples were approved by the Regierungspraesidium Karlsruhe, Baden-wuerttemberg. Most of the experiments were carried out in the Neuroanatomy Department, University of Heidelberg.

To track *Foxb1* cell lineage during development, mutant mouse lines were generated or obtained from different sources. The details of the mutant sources are summarized in table. The *Foxb1-Cre* mouse was generated by our group (Zhao et al., 2007). In order to create *Foxb1* mutant allele, iCre-IRES-EGFP in the *Foxb1* locus was knocked in between exon1 and coding region of exon2. Then we deleted the positive selection cassette PGK-Neo by crossing the mutant allele mouse with the FLPeR deleter mouse. Therefore, the *Foxb1* coding region was replaced by the Cre recombinase cDNA. Cre is then expressed under the control of the regulatory sequences of *Foxb1*. Cre recombinase activity can be examined under UV light; the *Foxb1* expression regions show EGFP fluorescence. In previous studies, our group has shown *Foxb1* expression pattern by in situ hybridization or by observing the EGFP signal (Alvarez-Bolado et al., 1999; Zhao et al., 2008; Zhao et al., 2007). The *Z/AP* was explained detailedly as it expresses human placental alkaline phosphatise (hPLAP) (Lobe et al., 1999). hPLAP is a GPI-linked cell surface marker and is useful for labelling the full extension of axonal processes. After crossing between $Foxb1^{Cre}$ and *Z/AP* mouse lines, hPLAP will be a lineage marker of *Foxb1* expression. In $Foxb1^{Cre/+}$ *x Z/AP* mouse, the *Foxb1* expressing cell itself and all the progeny cells of *Foxb1* lineage are detected by hPLAP permanently. In this way, we can detected *Foxb1* lineages by doing hPLAP reaction or by labelled the cells with anti-hPLAP antibody (Lobe et al., 1999).

When dealing with antibodies which detect proteins present in the cell nucleus (for instance, transcription factors, like *Olig2*), the use of a lineage reporter also expressed

in the cell nucleus makes it easier to ascertain cellular colocalization of both marker and reporter. *ROSA26R* (a gift from Prof. P. Soriano) is such a reporter mouse line (i.e. with nuclear expression). In this mouse, β-galactosidase is the reporter gene and it is inserted in the ROSA locus downstream of a floxed stop codon. After Cre recombination, the stop codon is deleted and β-galactosidase expression is activated; detection of this reporter molecule by an anti-β-galactosidase antibody produces a characteristic punctate pattern in the cell nucleus (Labosky et al., 1997; Soriano, 1999). Therefore, we crossed the *Foxb1*^{Cre} and *ROSA26R* mouse lines and determined that the presence of β-galactosidase in the nucleus of Cre-expressing cells is detected initially at E8.5 (Ang et al., 1993; Zhao et al., 2008; Zhao et al., 2007). Thus, *Foxb1*^{Cre} x *ROSA26R* mouse can be applied to identify *Foxb1* cell lineage by detecting β-galactosidase expression.

Table 1. Mutant mouse lines

Name	Description	Origin
Foxb1-Cre	The Cre recombinase cDNA was inserted in the *Foxb1* coding region between exon1 and exon2	Gonzalo Alvarez-Bolado (Zhao et al., 2007)
Z/AP	hPLAP was inserted into the downstream of the *loxP-flanked βgeo/3xpA* and as a reporter of *Foxb1* lineage after *Foxb1*^{Cre}*:Z/AP* Cre recombination	Corrinne Lobe (Lobe et al., 1999)
ROSA26R	β-galactosidase is the cell lineage marker of *Foxb1*^{Cre}*:ROSA*$^{loxP\text{-}STOP\text{-}loxP\text{-}LacZ}$ after Cre recombination	Philippe Soriano (Soriano, 1999)

2.2.2 Genotyping

2.2.2.1 Lysis Tails

The adult mouse tails were clipped (about 5 mm length) by the animal caretakers of the mouse facility (IBF). I clipped the embryonic tails (2 mm) by my self. For tail lysis, 300 µl genomic DNA lysis buffer and 60 µg Proteinase K were added to each tube. The tails were then incubated at 55°C and mixed for 550 rpm overnight in a heat block. The proteinase K was deactivated at 85°C for 1 hour in the next morning. The tails were centrifuged shortly and were kept at 4°C.

2.2.2.2 Genotyping PCR

The DNA amplification was performed with the polymerase chain reaction (PCR) method. The following primers were used (5'-3'):

Foxb1
Forward: CAC TGG GAT GGC GGG CAA CGT CTG
Reverse: CAT CGC TAG GGA GTA CAA GAT GGC
EGFP: CTC GGC ATG GAC GAG CTG TAC AAG

hPLAP:
Forward: TTT AAC CAG TGC AAC ACG ACA CGC
Reverse: CTG TAG TCA TCT GGG TAC TCA GGG

β-galactosidase
Forward: CGT CAC ACT ACG TCT GAA CGT CG
Reverse: CAG ACG ATT CAT TGG CAC CAT GC

The PCR master mix was prepared and the programs were used as follows:

PCR master mix	Volume (µl)
5 x PCR buffer	5
25 mM $MgCl_2$	2
10 mM dNTPs	0.5
100 mM forward primer	0.125
100 mM forward primer	0.125
5 U/µl Taq DNA polymerases	0.125
Genomic DNA	1
RNase-free water	to 20 µl

PCR conditions:

	Initial denaturation	94 °C	5 min
35 cycles	Denaturation	94 °C	20 sec
	Annealing	64 °C	30 sec
	Extension	72 °C	40 sec
	Final extention	72 °C	10 min
	Cool down	8 °C	Hold

2.2.2.3 DNA Fragments Checking

After amplification, the samples were run in a 1.5% agarose gel in 1x TAE buffer at 100V. The gel was then stained in ethidium bromide (EB) solution for 15 min and washed in water for 15 min. The specific bands were then observed and recorded under UV-light.

2.2.3 Collection of Tissue Samples

The adult mice were anesthetized with isofluorane or by cervical dislocation. Then I cut the mouse head and open the skull to expose brain. The dissected brain was processed according to the certain purpose. Different methods for tissue handling will be described later. With respect to special antibodies, for instance, NG2 antibody and PDGFRa antibody, the mouse should be perfused with 4% PFA. Postnatal mouse was anesthetized with isofluorane quickly and was intracardiac perfusion with 4% PFA in 37°C PBS buffer which contained 20U heparin per 100g body weight. The brain was removed and dissected same as before. For embryos, pregnant mice were sacrificed by cervical dislocation. E12.5, E15.5 or E18.5 pups were taken out from the uterus and the brains were dissected in cold PBS. The hPLAP can only be detected after E9.5 age (Zhao et al., 2007), so I chose E12.5 brains to characterize neuroepithelium and *Foxb1* lineage. All the procedures were performed according to the Animal Welfare Act (Tierschutzgesetz).

2.2.3.1 Paraffin Sections

1. The brains were fixed in 4% PFA overnight and then were washed in PBS sufficiently for 5 hours.

2. The tissue was then dehydrated in different concentration of ethanol solutions serially. The exact protocols are listed as follows:

Table 2. Processing of paraffin blocks

Reagents	E9.5-E15.5	E15.5-E18.5	P0-Adult
50% Ethanol	0.5 hour	1 hour	2 hours
70% Ethanol	0.5 hour	1 hour	4 hours
80% Ethanol	0.5 hour	1 hour	2 hours
90% Ethanol	0.5 hour	1 hour	2 hours
100% Ethanol	1 hour	1 hour	2 hours
100% Ethanol	1 hour	2 hour	2 hours
Xylene1	0.5 hour	1 hour	2 hours
Xylene2	1 hour	2 hour	4 hours
Paraffin1	4 hours	4 hours	overnight
Paraffin2	overnight	overnight	overnight

3. Turn on the embedding machine and hot plate for 4 hours and clean all the mold and tools with 70% ethanol.
4. Fill the bottom of metal cassette with paraffin liquid firstly, and then orientate brains at the right position.
5. Fill more paraffin liquid until the whole brain was embedded.
6. Place the mold on cooling plate to accelerate solidification.
7. After the paraffin was solid thoroughly, turn on the microtome and fill sterile dd H_2O to water bath, keep the temperature of water bath to 42°C.
8. Adjust the block and trim tissue, and then use the manual control mode to cut the section thickness to 10 μm serially.
9. Put the sections in water bath and wait until full extension, pick up the sections to a SuperFrost slide.
10. Place the slides horizontally at 42°C overnight.
11. Store slides at room temperature and keep them from light.

.

2.2.3.2 Fixed Frozen Sections

1. Fix brains in 4% PFA overnight at 4°C; the fixative volume was 30-50 times greater than the size of brain.
2. Wash samples in PBS for 4 hours at 4°C.
3. Protect tissues by immersing in 10% and 30% sucrose in PBS until brains sink to bottom.
4. Mount brains in OCT embedding compound on dry ice and freeze them at -80°C.

5. Transfer block at -20°C overnight before cutting.
6. Section the frozen tissue to 20 µm thickness using cryotome.
7. Pick up section onto SuperFrost slides and dry the sections overnight at room temperature.
8. Store sections in a sealed box at -20°C for immunohistochemistry.

2.2.3.3 Fresh Frozen Sections

1. After dissection, directly mount brains in OCT embedding compound on dry ice and freeze blocks at -80°C.
2. Transfer block at -20°C overnight before cutting.
3. Section the frozen tissue to 20 µm thickness using crytome.
4. Pick up section onto SuperFrost slides and dry the sections overnight at room temperature.
5. Store sections in a sealed box at -80°C for immunohistochemistry.

2.2.4 hPLAP Reaction

Human placental alkaline phosphatise (hPLAP) is resistant to heat and many chemical inhibitors that can inactivate other endogenous alkaline phosphatases in mouse. hPLAP has been used widely as lineage marker to label membranes and to define the outer surface of transduced cells, as well as neuron processes and axonal bundles. To get a primary impression of the *Foxb1* lineage, I did phosphatase/NBT staining firstly (Gierut et al., 2014; Lobe et al., 1999).

2.2.4.1 Preparation of Tissues and Embryos for hPLAP Staining

1. Dissect brains or embryos in PBS buffer on ice.
2. Fix in 4% PFA solution includes 0.02% NP-40 and 0.01% sodium deoxycholate at 4°C for 30 min.
3. Wash samples in PBS three times for 30min at 4°C.
4. Inactivate endogenous alkaline phosphatise in PBS at 72°C for 30 min.
5. Rinse in PBS three times for 10 min at room temperature.
6. Wash in AP buffer two times for 10 min
7. Stain tissues with 100 mg/ml NBT and 50 mg/ml BCIP in AP buffer at 4°C until the optimal results appear.

8. Wash samples in PBS extensively to reduce the background.
9. Mount slides in Mowiol mounting medium and observe results under light microscope.

2.2.4.2 Preparation of Sectioned Brains and Tissues

1. Fix frozen sections in 4% PFA and 0.02% NP-40 in PBS for 1 hour at 4°C.
2. Rinse sections in PBS three times for 10 min and wash slides in AP buffer for 10 min at 4 °C.
3. Transfer slides to 72 °C PBS for 30 min to inactivate endogenous alkaline phosphatase.
3. Stain sections with 100 mg/ml NBT and 50 mg/ml BCIP in AP buffer at 4°C until the optimal results appear.
4. Rinse slides in PBS three times for 5 min and in AP buffer for 10 min at room temperature.
5. Mount slides in Mowiol mounting medium and sotre slides at 4 °C.

2.2.5 Immunohistochemistry (IHC)

PLAP staining can observe the general expression of *Foxb1* lineage, however, the cell membranes and processes are not identified well. In addition, some endogenous alkaline phosphatase is still activated to influence the hPLAP signal. Therefore, to label *Foxb1* lineage more clearly, monoclonal antibody specific to PLAP were purchased (Sigma, Saint Louis, USA) and were used for immunohistochemistry (De Groote et al., 1983; Zoellner and Hunter, 1989).
hPLAP is expressed in cytoplasm or cell membranes, in contrast, some antigens are expressed in nucleus (e.g. Olig2), I used another mouse line *Foxb1-Cre x ROSA26R* to label *Foxb1* lineage by β-galactosidase. Frequently, β-galactosidase is expressed in nucleus or as a punctate expression as well. It is possible to use this mouse line to colocalize nucleus antigens and *Foxb1* lineage.

2.2.5.1 Protocol for Paraffin-embedded Brain Sections

1. Prepare solutions and make them available before IHC.
2. Put slides in two containers of xylene for 10 min each.
3. Place slides in two containers of 100% ethanol for 5 min each.

4. Place slides in 95%, 90%, 80%, 70% and 50% ethanol gradually for 5 min each.
5. Rinse slides in dH_2O two times for 5min each to rehydrate sections.
6. Remove cross-linked antigens by antigen retrieval in 10 mM sodium citrate buffer.
    a. Place slides in 10mM sodium citrate buffer container.
    b. Put container in a pressure cooker with 500 ml dH_2O and heat the water to boiling.
    c. Turn down the power to avoid vigorous boil and heat the slides for 10 min more.
    d. Take out slides from cooker and cool for 30 min at room temperature.
7. Rinse sections in dH_2O two times for 1 min each.
8. Put slides in 3% H_2O_2 for 15 min at temperature to remove endogenous peroxidise.
9. Rinse sections in dH_2O two times for 5 min each.
10. Wash sections in PBS three times for 5 min each.
11. Draw a circle around the tissue by creating a hydrophobic boundary to avoid solutions going out.
12. Block sections in 10% horse serum in PBS for 1 hour at room temperature.
13. Remove blocking solution and add 100 µl primary antibody in recommended dilution per section. Incubate slides in a humidified chamber at 4 °C overnight.
14. Wash slides in PBS three times for 10 min each.
15. Cover secondary antibody to slides at temperature for 2 hours.
16. Wash slides in PBS three times for 10 min each.
17. Control staining with 1:10000 DAPI solution in PBS.
18. Wash slides in PBS two times for 5 min each.
19. Rinse slides in dH_2O shortly.
20. Mount sections with Mowiol medium and store slides at 4 °C.

2.2.5.2 Protocol for Frozen Sections

2.2.5.2.1 Fixed frozen sections

1. Equilibrate the sealed box to room temperature, and then take out the desired sections.

2. Fix slides in 4% PFA or cold acetone for 15 min at room temperature.
3. Wash slides in PBS three times for 5 min each.
4. Permeate cell membrane in 0.1%-0.5% Triton X-100 in PBS for 15 min (if available).
5. Wash slides in PBS three times for 5 min each.
6. Same as step 10 to step 20 in paraffin part.

2.2.5.2.2 Fresh frozen sections

1. Open sealed box quickly in cooling room, and then take out the desired sections.
2. Fix slides in 4% PFA or cold acetone for 20 min at room temperature.
3. Wash slides in PBS three times for 5 min each.
4. Permeate cell membrane in 0.1%-0.5% Triton X-100 in PBS for 15 min (if available).
5. Wash slides in PBS three times for 5 min each.
6. Same as step 10 to step 20 in paraffin part.

Table 3. Antibody list

Source	Abbreviation Name	Description	Dilution	Working Conditions
Sigma (A2951)	hPLAP	Monoclonal anti- human placental alkaline phosphatase antibody	1:2000	Paraffin, Fixed and Frozen sections.
Abcam (ab9361)	β-gal	Chicken polyclonal to beta Galactosidase	1:200	Fixed frozen
Abcam (ab53041)	Claudin11	Rabbit polyclonal to oligodendrocyte specific protein	1:500	Fixed frozen
Chemicon (AB142)	GalC	Rabbit polyclonal to galactocerebroside	1:100	Perfused tissue
Sigma (M3821)	MBP	Rabbit polyclonal to myelin basic protein	1:100	Fixed frozen
BD Pharmingen (558774)	PDGFRα	Rat monoclonal anti-mouse CD140a	1:300	Fresh frozen
Chemicon (AB5320)	NG2	Rabbit polyclonal to NG2 chondroitin sulphate proteoglycan	1:100	Perfused tissue
Chemicon (AB9610)	Olig2	Rabbit polyclonal to oligodendrocyte transcription factor 2	1:200	Fixed frozen
Chemicon (AB5804)	GFAP	Rabbit polyclonal anti-glial fibrillary acidic protein	1:300	Fixed frozen
Abcam (ab18207)	β-Tubulin III	Rabbit polyclonal to beta III Tubulin	1:600	Fixed frozen
Abcam (ab177487)	NeuN	Rabbit monoclonal to NeuN neuronal marker	1:500	Fixed frozen
Abcam (ab105389)	Nestin	Rabbit monoclonal to nestin	1:200	Fixed frozen
Chemicon (AB1782)	Glast	Guinea pig polyclonal to glutamate transporter glial	1:100	Fixed frozen
Abcam (ab16508)	NeuroD1	Rabbit polyclonal to NeuroD1	1:100	Fixed frozen
Abcam (ab92547)	Vimentin	Rabbit monoclonal to	1:200	Fixed frozen
Chemicon (ABN14)	BLBP	Rabbit polyclonal to brain lipid binding protein	1:300	Fixed frozen
Chemicon (MAB3222)	BrdU	Mouse monoclonal to Bromodeoxyuridine	1:100	Fixed frozen
Invitrogen (A11012)	Rabbit A-594	Goat polyclonal anti-rabbit IgG (H+L) secondary antibody, AlexaFluor@594 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A11034)	Rabbit A-488	Goat polyclonal anti-rabbit IgG (H+L) secondary antibody, AlexaFluor@594 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A10667)	Mouse A-488	Goat polyclonal anti-mouse IgG / IgA / IgM (H+L) secondary antibody, AlexaFluor@488 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A11032)	Mouse A-594	Goat polyclonal anti-mouse IgG (H+L) secondary antibody, AlexaFluor@594 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A11006)	Rat A-488	Goat polyclonal anti-rat IgG (H+L) secondary antibody, AlexaFluor@488 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A11039)	Chicken A-488	Goat polyclonal anti-chicken IgY (H+L) secondary antibody, AlexaFluor@488 conjugate	1:500	Paraffin, Fixed and Frozen sections.
Invitrogen (A11076)	Guinea pig A-594	Goat polyclonal anti-guinea pig IgG (H+L) secondary antibody, AlexaFluor@594 conjugate	1:500	Paraffin, Fixed and Frozen sections.

2.2.6 Proliferation Assay

For proliferation studies, 50 mg/kg of BrdU in dH_2O were injected into P10 mice intraperitoneally three times, at four hour intervals (Bu et al., 2004). Mice were sacrificed 2 hours after the last injection. The brains were dissected and fixed in 4% PFA overnight at 4 °C. In the next morning, brains were washed in PBS buffer for 4-6 hours and then were cryoprotected in 0.1 M PBS containing 15% sucrose and 30% sucrose gradullay until the tissue are sinked. The brains were then embedded in OCT compound on dry ice. Tissues were strored at -80 °C. To do immunohistochemistry of BrdU and Olig2, fixed frozen blocks were transferred to -20 °C overnight. Sections of 20 µm thickness were cut. Sections were then fixed in cold acetone for 15 min at room temperature. Slides were washed for three times 10 min each. The sections were incubated in 1 M HCl for 10 min on ice and this is followed by 2 M HCl for 30 min at 37 °C. After HCl incubation, sections were neutralized by putting the samples in 0.1 M sodium borate buffer three times each 5 min at room temperature. The unspecific antigens were blocked in 10% horse serum in 0.3% Triton X-100 PBS for 1hour at room temperature. BrdU immunohistochemistry was done with standard staining procedure as described in above protocols.

2.2.7 Cell Culture and Immunocytochemistry (ICC)

Tissues from newborn pups have been used to cultivate primary neurons and glia from different regions of CNS (Kaech and Banker, 2006). All the instruments and materials were sterilized. The detailed protocols are following:

2.2.7.1 Primary Cell Culture of Hindbrain

1. Prepare culture dish and coverslips with 0.001% poly-l-lysine in dH_2O and incubate in 5% CO_2 at 37 °C cell culture incubator for 1 hour.
2. Rinse petra dish and 24-well plate with dH_2O after coating. Put coverslips in 24-well plate and dry them under clean bench.
3. Newborn *Foxb1-Cre x Z/AP* mice were anesthetized by Isoflurane prior to decapitate animals. Hindbrain was isolated and dissected under sterile conditions, washed in PBS without Ca^{2+} and Mg^{2+}.

4. The meninges were removed carefully to exclude fibroblast contamination. The brain tissue was minced into small pieces and pipetted for 30-50 times up and down in DMEM.
5. Digest the cell cluster to single cell in 0.05% trypsin and 0.02% EDTA solution and incubate in 5% CO_2 at 37 °C cell culture incubator for 30 min.
6. Stop digestion by adding 10% FBS in DMEM. Centrifuge at 1500 rpm for 5 min, and then discard supernatant.
7. Suspend cells in 10% FBS, 1x penicillin-streptomycin in high glucose-DMEM at desity of 5 x 10^6 cells/ml, and seed cells in petri dish and 224-well plate.
8. Refresh half medium at time points of 72 hours and 144 hours. Stop cell culture at day 9.

2.2.7.2 Immunocytochemistry (ICC)

1. Wash coverslips in 37 °C PBS for three times 5 min each.
2. Fix cells in 4% PFA for 30 min at room temperature
3. Wash cells in PBS for three times 5 min each.
4. Penetrate cell membrane by adding 0.1% Triton X-100 in PBS for 15 min at room temperature.
5. Block cells in 10% horse serum in PBS for 1 hour at room teperature
6. Incubate cells in diluted primary antibodies overnight at 4 °C.
7. Wash coverslips in PBS for three times 10 min each.
8. Add secondary antibodies on coverslips and incubate for 1 hour at room temperature.
9. Wash coverslips in PBS for three times 10 min each.
10. Add 1:10000 DAPI to stain nuclei for 5 min.
11. Wash in PBS for two times 5 min each.
12. Rinse in dH_2O shortly and mount coverslips to slides with Mowiol mounting medium.

2.2.8 Confocal Microscope and Electron Microscope

2.2.8.1 Confocal Microscope

The results were observed and analyzed under fluorescent microscope (Zeiss Axiover 200M) and confocal laser-scanning microscope (Zeiss LSM 510). The software what I used is ZEN 2010.

2.2.8.2 Electron Microscope

To observe whether or not myelination is normal in *Foxb1*$^{Cre/Cre}$ mouse, the transmission electron microscope was used. The samples were processed according to the general guidelines of electron microscope.

1. Dissect *Foxb1*$^{Cre/Cre}$ hindbrain and cut tissue to about 1mm^3 size.
2. Fix small sample in 4% PFA overnight at 4 °C and Immerse samples in 2.5% glutaraldehydefor 4 hours at room temperature.
3. Wash sample in 0.1 M cacodylate buffer three times (each 5 min duration).
4. Postfix sample in 1% osmium tetroxide in cacodylate buffer for 1 hour at room temperature. Wash in cacodylate buffer for 3 times 5 min each.
5. Dehydrate tissue in 50% ethanol, 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol, 100% ethanol and propylene oxide for 15 min each solution gradully. Place sample in 1:1 propylene oxide/Epon resin overnight.
6. Immerse sample with Epon for 4 hours and Embed in fresh Epon. Place mold containing sample at 60 °C for 24 hours.
7. Take ultra thin sections and place them on grids and stain grids in uranyl acetate for 2 hours and in lead citrate for 5 min.
8. Observe ultra sections under electron microscope.
9. Analyze images and calculate G-ratio.

The G-ratio is the relationship between axon diameter and myelin sheath thickness. It is expressed by the diameter of the axon divided by the diameter of the myelin sheath (Liu and Schumann, 2014; Paus and Toro, 2009). Therefore, G-ratio is defined as the inner axonal diameter divided by the total outer fiber diameter (axon plus myelin). The optimal range of the G-ratio is studied from 60% to 75% (Chomiak and Hu, 2009; Goldman and Albus, 1968; Paus and Toro, 2009). An increase in G-ratio suggests a reduction in the thickness of the myelin sheath.

2.2.9 Statistical Analysis

Three mice of each genotype were chosen for cell counting. Thickness of section was 20 µm. Sagittal sections were cut in four series (A, B, C and D) from an area spanning the middle (rostro-caudally) of the hindbrain. Eight sections were counted per mouse. The counting bin for marker-labeled cells was 600 µm x 600 µm in medulla oblongata. The counting bin for Olig2 plus BrdU was 0.1 mm^2 in medulla oblongata. The localization of the bins were chosen randomly.
Statistical assessment was performed with Prism 5 software (GraphPad Software, San Diego, CA, USA). To compare the difference between two groups, the Mann-Whitney test was used. This is a nonparametric test to compare the hypothesis that two groups from the same population against the alternative hypothesis, particularly if a population tends to have larger values than the other. A *p*-value ≤ 0.05 is considered statistically significant.

CHAPTER 3: RESULTS

3.1 The *Foxb1* Lineage in the CNS Produces Abundant Oligodendrocytes

Since *Foxb1* is expressed in extensive regions of the neuroepithelium (ventricular zone) (Alvarez-Bolado et al., 1999), first of all I asked which cell types of the CNS are generated in *Foxb1*-expressing neuroepithelium, and in which regions these cells resided; that is, my first question was: what is the cell lineage of *Foxb1* in the brain. I used a mouse with lineage-labeling capabilities in order to learn the cell generation capabilities of *Foxb1*-expressing neural stem cells of the neuroepithelium as well as their distribution in vivo. For this purpose I examined the brains of mice heterozygous for *Foxb1-Cre-EGFP x Z/AP*. The *Foxb1*-heterozygotes specifically lack a neuronal nucleus called the mammillary body of the hypothalamus, and they lack as well certain motoneurons corresponding to hindleg muscles (Alvarez-Bolado et al., 2000a; Alvarez-Bolado et al., 2000b; Dou et al., 1997). Otherwise they are normal and will be used here as proxies for the wild type. Careful analysis of a developmental series of brain sections from these mice stained with antibody against hPLAP (lineage marker for *Foxb1*), showed, as expected, labeling of cells in the early neuroepithelium (Fig. 1 A). Intriguingly, however, the antibody labelled extensive areas of the diencephalon and brainstem as well (Fig. 1 B to I). As postnatal development proceeded, these areas gradually resolved into large but discrete groups of cells with a “fluffy” appearance (Fig. 1 A-I).

Next, I wanted to identify these cells, which under the microscope appeared to be the most abundant cellular component of the *Foxb1* lineage in the brain. The “fluffy” morphology of most hPLAP-labeled cells suggested that these cells could be oligodendrocytes. In order to confirm this impression, I stained sections of adult *Foxb1*$^{Cre/+}$ brains with antibodies against hPLAP as well as with antibodies against one of the best known markers of mature oligodendrocytes, myelin basic protein or MBP (Barratt et al., 2016; Lourenco et al., 2016; Martenson et al., 1969; Poduslo and Braun, 1975). The abundance of MBP in adult brain tissue, however, as well as the abundance of hPLAP-labeled cells, produced results that were interesting but difficult to interpret accurately (Fig. 2).

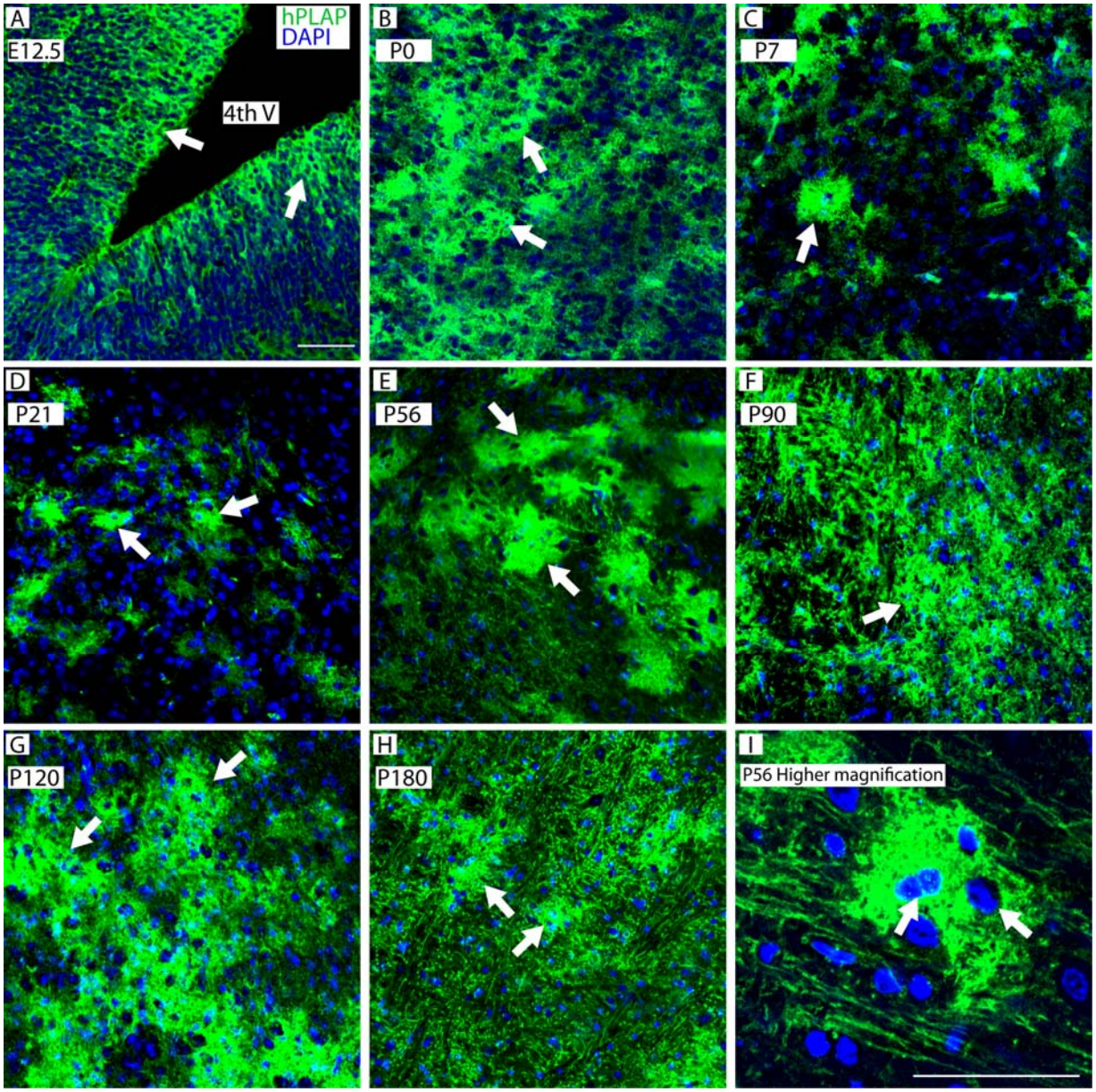


Figure 1. "Fluffy cells" of the *Foxb1* lineage labeled with anti-hPLAP antibody during development in *Foxb1*$^{Cre/+}$ x *Z/AP* mouse.
(A) E12.5 coronal section through the 4th ventricular zone showing radial glial like cells of *Foxb1* lineage as visualized by immunofluorescence.
(B-H) Representative images of *Foxb1* lineage cells at different ages after birth.
The *Foxb1* cells are clustering after birth as arrows pointing to cell bodies at P0 (B), P7 (C) or P21 (D). The hPLAP+ cells show larger size and "fluffy" morphology in adult mice at different ages from P56 to P180 (E-H).
(I) Higher magnification views of P60 medulla showing hPLAP+ cells morphology could be oligodendrocyte lineage.
(A-H) Same magnification. Scale bars=50 μm.

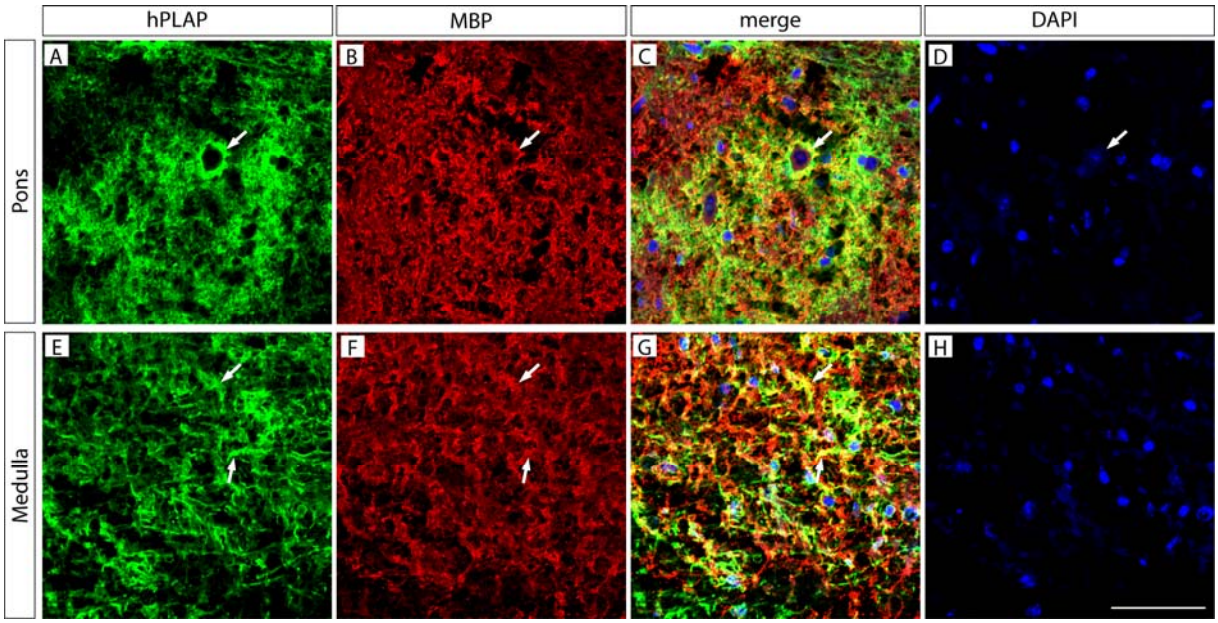


Figure 2. Mature myelin membrane marker is colocalized with *Foxb1* lineage reporter.
(A-H) Immunofluorescence on *Foxb1*$^{Cre/+}$ x *Z/AP* mouse medulla sections showing that *Foxb1* lineage cells (green, A and E) colocalizes the myelin membrane and cytoplasmic side of myelin marker MBP (red, B and F). White arrows indicate colocalization of myelin and *Foxb1* lineage reporter in merged image (yellow color in C and G). The nuclei are stained by DAPI (D and H). Scale bars=50 μm.

Another known marker of mature oligodendrocytes is Claudin 11 (Anitei et al., 2006; Bronstein et al., 2000; Bronstein et al., 1997; Bronstein et al., 1996; Morita et al., 1999). Many of the cells labeled by the anti-hPLAP antibody were also labeled by an anti-Claudin 11 antibody on sections (Fig. 3 A, B). Claudin 11-positive (Claudin 11+) structures were long and resembled as expected myelin sheaths along axons (Fig. 3 A, B). On these structures, Claudin 11 and hPLAP labeling often overlapped (arrows in Fig. 3 B). However, in the same tissue regions, extensive hPLAP+ structures did not overlap with Claudin 11 labeling (arrowheads in Fig. 3 B). While it was possible that both markers labeled different subcellular compartments in one single cells, it could also be that the markers were expressed by different but closely apposed cells. Therefore, for final proof of the expression of hPLAP by oligodendrocytes, I labeled primary cultures of *Foxb1*-heterozygous brainstem cells with anti-Claudin 11. The results showed numerous double-labeled cells with definite mature oligodendrocyte morphology, including their myelinating processes, with characteristic wide shape (Ness et al., 2005; von Budingen et al., 2015) (arrowheads in Fig. 3 C).

Finally, I colocalized hPLAP with a marker of mature and immature oligodendrocytes, GalC (Miller, 2002; Nishiyama et al., 2009; Raff et al., 1978; Ranscht et al., 1982; Rapport et al., 1964; Steiner et al., 2014; Woodruff et al., 2001). The results (Fig. 4) showed numerous GalC+ cell bodies overlapping areas of hPLAP expression (arrows in Fig. 4 A). Under higher magnification, AP and GalC seemed to find themselves in different subcellular compartments. While the cell nucleus occupied the center of the round or oval gaps which are characteristic of the hPLAP-expressing tissue in these mice (arrowheads in Fig. 4 B), the cell body was also part of it (arrows in Fig. 4 B), while hPLAP expression seemed confined to a region beyond the cell body and presumably corresponding to a region of very dense and thin processes (Fig. 4 B). On primary cultures of *Foxb1*$^{Cre/+}$ brainstem cells I found abundant unequivocal images of double-labeled oligodendrocytes (Fig. 4 C). I concluded that oligodendrocytes are a very abundant cellular component of the *Foxb1* lineage.

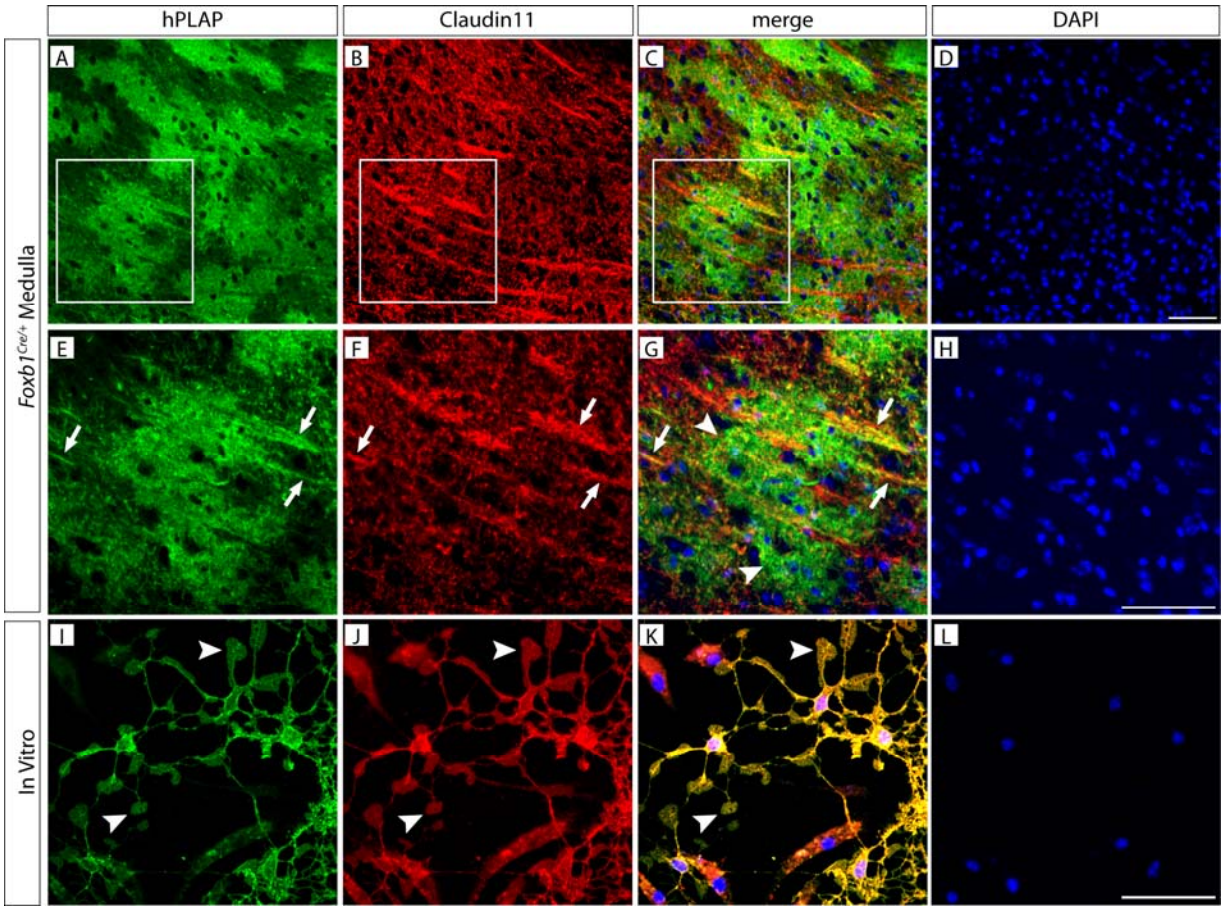


Figure 3. Mature oligodendrocyte specific protein is colocalized with *Foxb1* lineage reporter.
(A-H) Confocal images showing hPLAP+ expression (green, A and E) are also labeled by mature oligodendrocyte marker Claudin11 (red, B and F) on P56 *Foxb1*$^{Cre/+}$ x *Z/AP* medulla. Boxed areas in (A-C) are shown at higher magnification in (E-H). White arrows indicate the coexpression between Claudin11 and hPLAP (yellow, G). Arrowheads indicate not all hPLAP+ positive cells are *Foxb1* lineage (green+red-, E-H). Nuclei are illustrated by DAPI (blue, D and H).
(I-L) Immunocytochemistry on adherent monolayer primary cell cultures of newborn *Foxb1*$^{Cre/+}$ x *Z/AP* mice brain using antibodies against mature oligodendrocyte Claudin11 (red, J) and *Foxb1* lineage marker hPLAP (green, I). Arrowheads indicate the lamella extension of mature oligodendrocyte, which allows oligodendrocyte to myelinate multiple axons (J). The merged image shows coexpression of Claudin11 and hPALP (yellow, K).
Nucleus marker is DAPI (blue, L). Scale bars=50 μm.

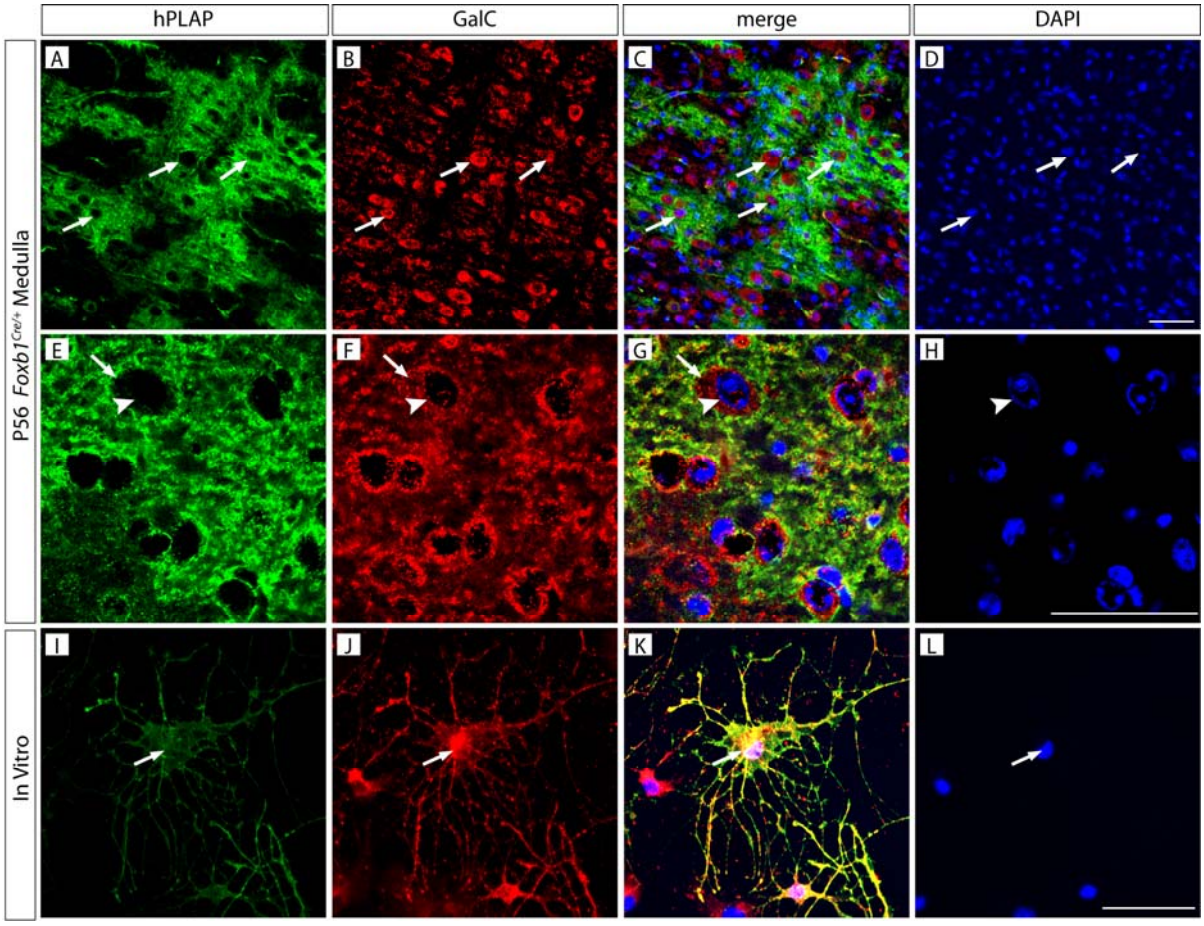


Figure 4. Colocalization of hPLAP and GalC are observed in both immature and mature oligodendrocytes.
(A-H) Confocal images showing immature oligodendrocyte and mature oligodendrocyte marker GalC (red, B and F) are immunoreactive to hPLAP on P56 *Foxb1*$^{Cre/+}$ x *Z/AP* medulla sections (green, A and E). The details of immuno reaction are shown at higher magnification in (E-H). GalC is expressed in cytoplasm and cell membrane (F), but hPALP is expressed majorly outside of cell body (E). The merged images show hPLAP and GalC immunoreactivity in the same cell as arrow indicated (C and G). (I-L) Immunocytochemistry on primary cell culture of newborn *Foxb1*$^{Cre/+}$ x *Z/AP* mouse hindbrain. Arrows indicate a typical morphology of oligodendrocyte which is labeled by GalC (red, J and K). The *Foxb1* lineage maker is also stained after immuno reaction (green, I and K). The merged image shows colocalization of oligodendrocyte marker and hPLAP in vitro (yellow, K).
Nuclei are labeled by DAPI (blue, D, H and L). Scale bars=50 μm.

3.2 The *Foxb1* Lineage in the CNS Includes Neurons and Astrocytes

In order to identify if other cell types belong to the *Foxb1* lineage, I colocalized cell type-specific markers with the lineage marker hPLAP at P56, when the cells appeared most differentiated and could be expected to show their most distinctive characteristics. First, I colocalized neuron-specific markers β-tubulin-III and NeuN (Alexander et al., 1991; Caccamo et al., 1989; Eriksson et al., 1998; Ferreira and Caceres, 1992; Fortino et al., 2014; Ikeda and Ikeda, 2015; Magavi et al., 2000; Mullen et al., 1992; Sarnat et al., 1998; Vanella et al., 2015; Wolf et al., 1996) with hPLAP (Fig. 5 A-C, D-F). Both antibodies labeled cell bodies inside the typical gaps or openings in the hPLAP pattern (arrows in Fig. 5 A-C, D-F) present in the AP+ regions (arrowheads in Fig. 5 A-C, D-F), making it difficult to ascertain if one single cell was labeled in two different compartments or if on the contrary the image corresponded to two different cells in tight contact with each other. Using anti-hPLAP and anti- β -tubulin-III antibodies on primary cultures of postnatal day zero (P0) brainstem cells (Fig. 5 G-I) I could confirm that, although most neurons are not hPLAP+, a few of them certainly are (arrow in Fig. 5 G-I).

Next, I asked if the *Foxb1* lineage includes astrocytes. Colocalizing astrocyte-specific protein glial fibrillary acidic protein (GFAP) (Borit and McIntosh, 1981; Cobb et al., 2016; Cohen et al., 1979; Duffy, 1982; Jessen and Mirsky, 1980; Lolait et al., 1983) with hPLAP also showed a small number of *Foxb1*-lineage astrocytes on sections (Fig. 6 A-D) as well as on primary cultures of *Foxb1*$^{Cre/+}$ brainstem cells (Fig. 6 E-H). As expected, no anti-hPLAP-labeled cells colocalized with microglial marker IBA1, since microglia originates in very early embryonic hematopoietic sites outside the brain (Fig. 6 I-L) (Ginhoux et al., 2013; Ito et al., 2001; Kadowaki et al., 2007; Meyer et al., 2015; Shapiro et al., 2009).

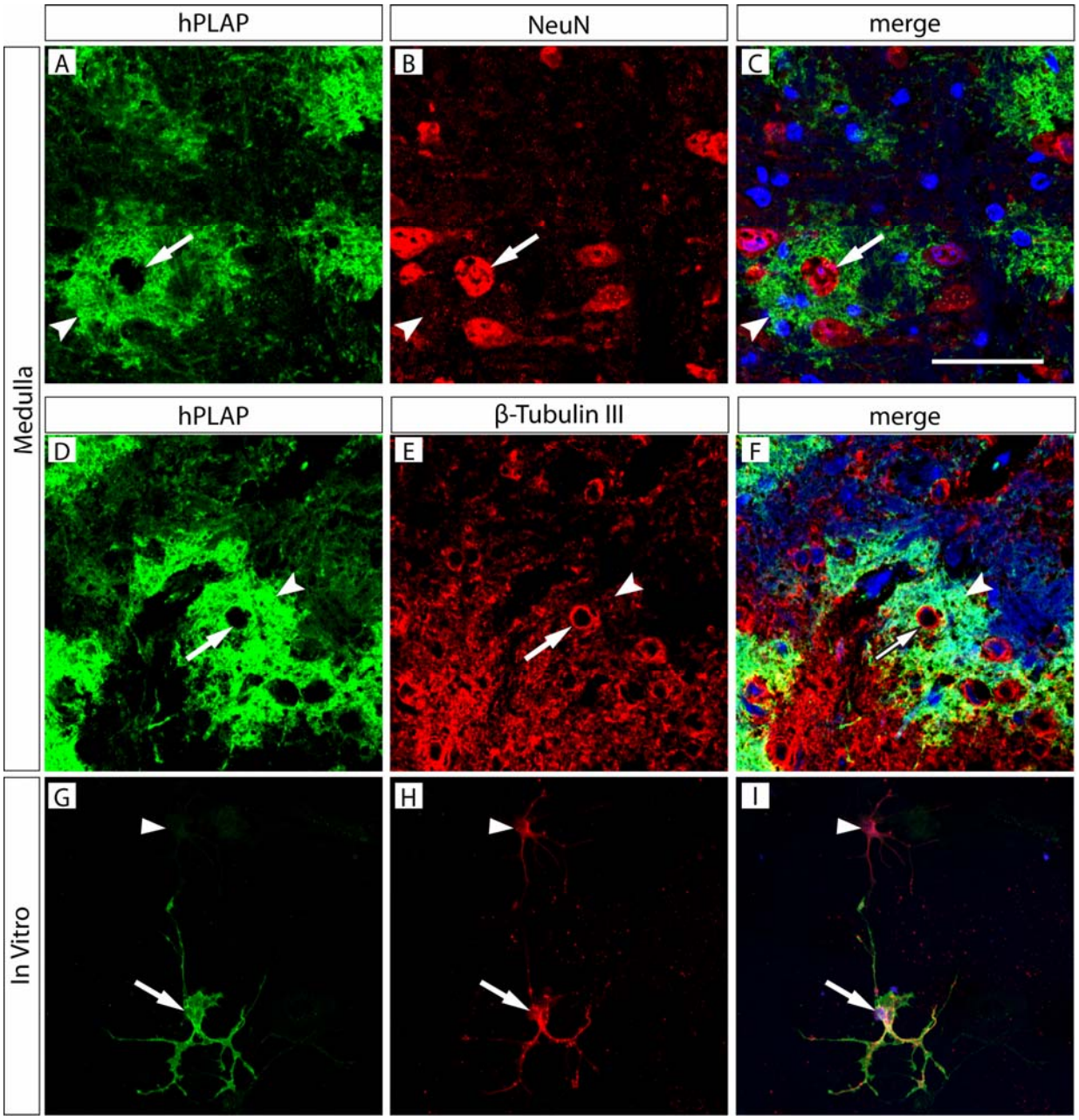


Figure 5. Some neuronal markers positive cells and hPLAP are coexpressed in P56 *Foxb1*$^{Cre/+}$ x *Z/AP* mouse.
(A-G) Colocalization of neuronal markers (NeuN and β–Tubulin III) with hPLAP.
Images of hPLAP+ cells (green, A and C) are immunoreactive to NeuN (red, B and C). Arrows indicate hPLAP and NeuN are coexpressed in a same cell (A-C). Another neuronal marker β–Tubulin III is expressed in cytoplasm and cell membrane (red, E and F). Arrows indicate the β–Tubulin III+ cells belong to *Foxb1* lineage (D-F). Arrowheads in (D-F) show cell boundary of *Foxb1* lineage.
(G-I) Immunocytochemistry on primary cell culture of newborn *Foxb1*$^{Cre/+}$ x *Z/AP* mouse brain show some neuron is *Foxb1* lineage (arrows in G-I). However, some neurons are still not coexpressed with *Foxb1* lineage marker (arrowheads in G-I).
Nuclei are labeled by DAPI. Scale bars=50 μm.

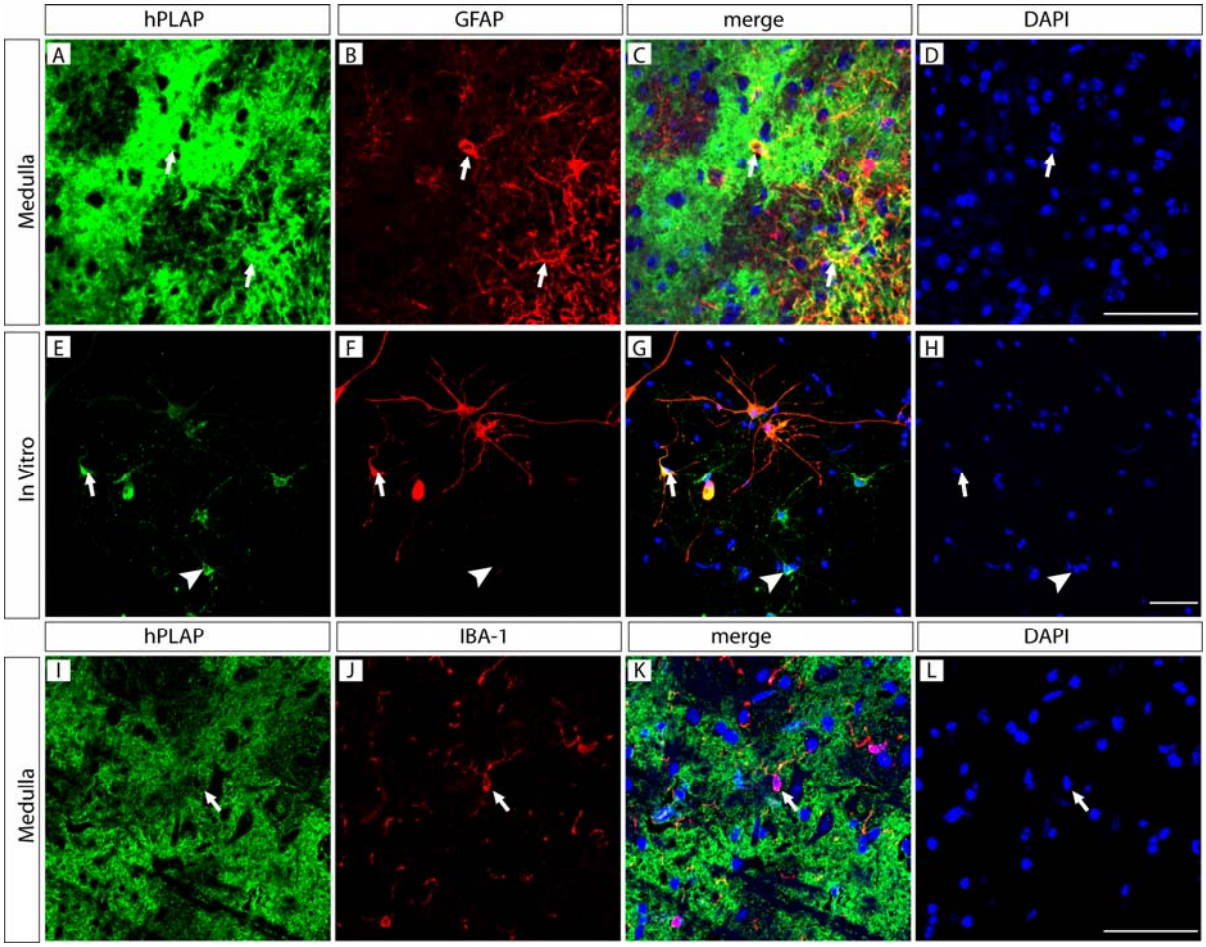


Figure 6. Few astrocytes are *Foxb1* lineage, but no microglia is coexpressed with *Foxb1* lineage marker in hindbrain.
(A-C) Colocalization of astrocyte marker GFAP and *Foxb1* lineage marker hPLAP.
Arrows indicate coexpressed astrocytes (red, B and C) and hPLAP (green, A and C) in P56 *Foxb1*$^{Cre/+}$ x *Z/AP* mouse medulla (yellow, C).
(E-F) Immunocytochemistry on primary cell culture of newborn *Foxb1*$^{Cre/+}$ x *Z/AP* mouse brain show few GFAP+ cells is colocalized with hPLAP (arrows in E-H). Arrowheads indicate not all *Foxb1* lineage cells are labeled by GFAP (green, E and G).
(I-K) Confocal images show there is no microglia (green, I and K) labeled by hPLAP (red, J and K).
Arrows indicate the hPLAP negative microglia (I-L).
Nuclei are labeled by DAPI (blue, D, H and L). Scale bars=50 μm.

Finally, since I based this analysis on the detection of the enzyme hPLAP, an alkaline phosphatase, it was in principle possible that I was detecting different kinds of cells expressing an endogenous form of alkaline phosphatase unrelated to the *Foxb1* lineage. Fortunately, the *Z/AP* mouse line (Lobe et al., 1999; Nagy et al., 1993) has a built-in control for specificity of labeling. This consists of a floxed β-*galactosidase* gene followed by three STOP cassettes inserted upstream the alkaline phosphatase reporter gene. In that way, cells expressing the *Cre* recombinase (i.e., in our case, cells expressing *Foxb1*) will delete the control insertion (β-*galactosidase* plus STOP cassettes) and express alkaline phosphatase reporter. Contrariwise, cells no expressing *Cre* (i.e. not expressing *Foxb1*), will not only not express the alkaline phosphatase reporter, but they will indeed express β-*galactosidase*. That is, the cells of our mutant mice will express either hPLAP or β-*galactosidase*. Performing both staining on sections of heterozygote brains showed as expected no overlap between areas expressing β-*galactosidase* and areas expressing hPLAP (Fig. 7). Additionally, I never detected any staining by the hPLAP antibody in brain regions where *Foxb1* is normally not expressed, like the telencephalon (not shown).

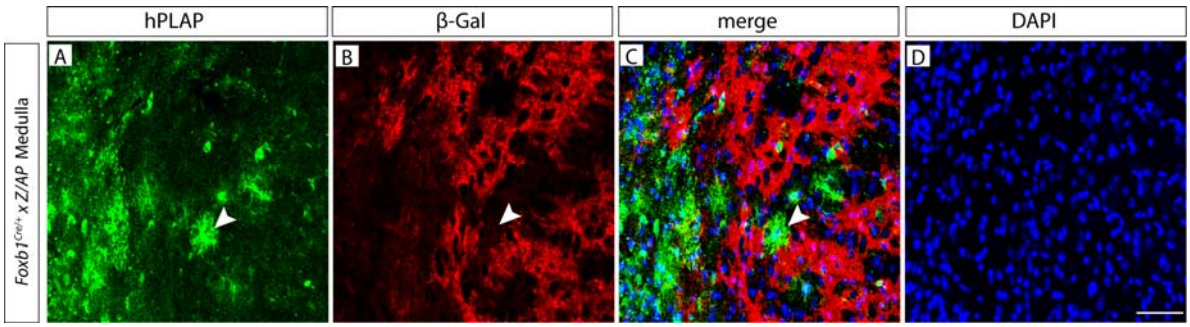


Figure 7. Verification of experimental animals.
(A-D) Specificity of *Foxb1*$^{Cre/+}$ x *Z/AP* mouse. Cre-lox recombination is a site-specific recombinase technology, the cells are only labeled by β-gal (red, B and C) if Cre recombinase is inactive. After Cre recombination, Loxp sites are excised that leads to hPLAP expression. hPLAP (green, A and C)and β-gal (red, B and C) are exclusively expressed in hindbrain.
Nuclei are labeled by DAPI (blue, C and D). Scale bars=50 μm.

3.3 Some Oligodendrocyte Progenitor Cells (OPC) Belong to the *Foxb1* Lineage

Since I identified numerous *Foxb1*-lineage oligodendrocytes, I reasoned that I should be able to find OPC expressing the AP reporter in our heterozygotes. To identify them I colocalized hPLAP and OPC-specific markers on brain sections of postnatal heterozygous mice of the *Foxb1-Cre* line. One such marker is the α subunit of the platelet-derived-growth-factor receptor (PDGFRα) (Boulanger and Messier, 2014; Hart et al., 1989; Hill and Nishiyama, 2014; Hill et al., 2014; Motomura et al., 2012; Nishiyama et al., 2009; Pringle et al., 1989; Pringle et al., 1992; Rivers et al., 2008; Wilson et al., 2006). The "patches" of brain tissue expressing the hPLAP reporter contained cells labeled also by anti-PDGFRα antibody (Fig. 8 A-D). Another key marker of OPC is NG2 (Baracskay et al., 2007; Bu et al., 2004; Dimou and Gallo, 2015; Hill and Nishiyama, 2014; Kucharova and Stallcup, 2010; Nishiyama, 2007; Nishiyama et al., 2009; Nishiyama et al., 2002; Sakry and Trotter, 2015). However, NG2 is also expressed by endothelial cells of the capillaries found in the brain (Chekenya et al., 2002; Grako and Stallcup, 1995; Guichet et al., 2015; Pouly et al., 2001). Therefore, first I developed a protocol to detect NG2 in OPC efficiently. To test it, I colocalized NG2 with PDGFRα (Fig. 8 E-H); this first experiment demonstrated that my protocol reliably identifies NG2-expressing OPC on brain sections. Then I colocalized NG2 with hPLAP on similar brain sections (Fig. 8 I-L). These results demonstrated, as expected, that a number of OPC belong to the *Foxb1* lineage. Finally, I confirmed this result on primary cultures of *Foxb1*$^{Cre/+}$ hindbrain cells (Fig. 8 M-P).

Olig2, a transcription factor protein specific of OPC (Douvaras and Fossati, 2015; Emery and Lu, 2015; Kuspert and Wegner, 2015; Li and Richardson, 2015; Ligon et al., 2006; Miller, 2005; Motomura et al., 2012; Nishiyama et al., 2009; Parras et al., 2007; Ross et al., 2003; Takebayashi et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2000), was also present in the nucleus of some hPLAP-expressing cells. Since hPLAP, however, seems to label a cytoplasmic compartment, while Olig2 is localized in the nucleus, I wanted to identify our *Foxb1*-lineage cells by a different reporter (not hPLAP) expressed also in the nucleus. To this effect, I generated crossed our *Foxb1-Cre* mice with the *ROSA26R* mouse line, generating *Foxb1-Cre-ROSA26R* mice. In these mice, the reporter is LacZ (β-*galactosidase*), which is expressed as a

punctate nuclear pattern. By treating sections of *Foxb1-Cre-ROSA26R* brains with anti-Olig2 and anti-β-galactosidase antibodies, I detected colocalization of both markers in brain cell nuclei (Fig. 8 O-T).

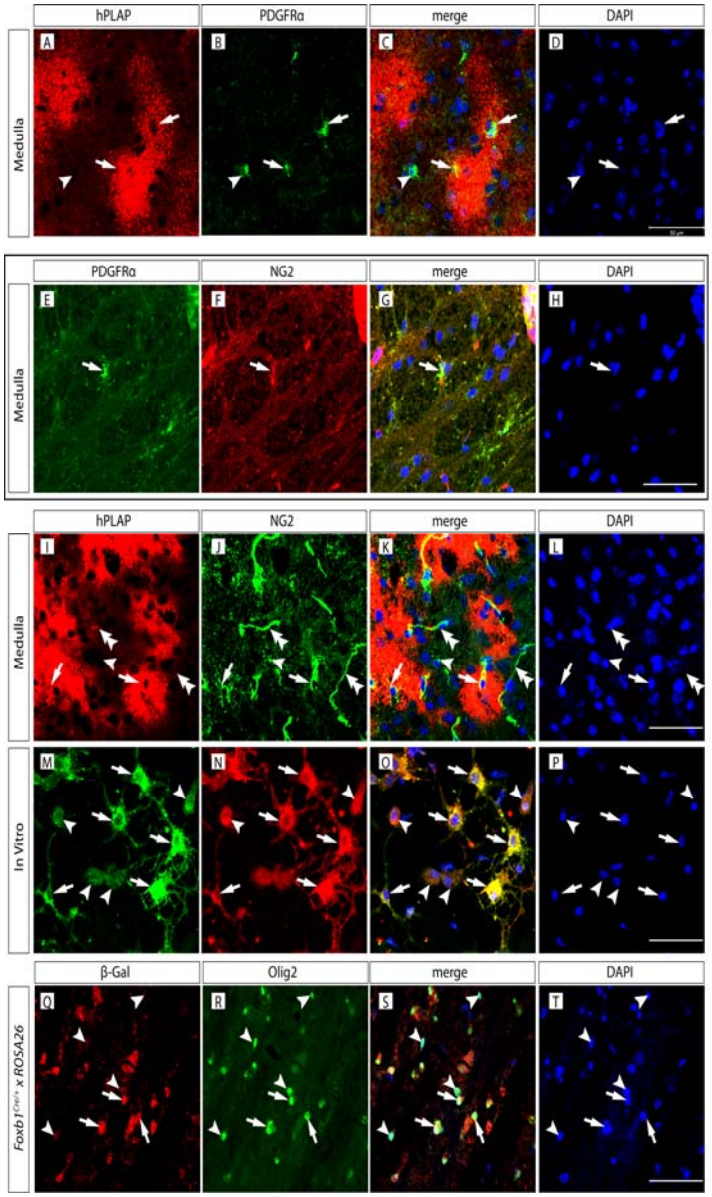


Figure 8. Some oligodendrocyte progenitor cells generated from *Foxb1* lineage.
(A-D) Colocalization of OPC specific marker PDGFRα and hPLAP. OPCs are labeled with PDGFRα antibody in *Foxb1*$^{Cre/+}$ x *Z/AP* medulla oblongata (green, B and C). The merged images show colocalization of PDGFRa and hPLAP (arrows indicate in C). Arrowhead indicates that not all PDGFRα cells are *Foxb1* lineage (PDGFRα+hPLAP-, A-C).
(E-F) The intracellular proteins NG2, a chondroitin sulfate proteoglycan is specific marker to identify OPC and pericyte in brain. Nevertheless, PDGFRa is a well known marker to characterize OPC in mouse brain. To test the specificity of NG2 staining in OPC, PDGFRα (green, E and G) and NG2 (red, F and G) are co-stained for identifying OPC. The merged image shows colocalization between the two antibodies which is indicated with arrows (G).
(I-L) Immunofluorescence microscopy of *Foxb1*$^{Cre/+}$ x *Z/AP* medulla oblongata, staining for hPLAP (red, I and K) and NG2 (green, J and K). Arrows show colocalized cells with both antibodies. Arrowheads indicate some NG2+ OPC are not *Foxb1* lineage. Double arrowheads indicate the endothelial cells are labeled only with NG2, but not with hPLAP (I-K).
(M-P) Immunocytochemistry of primary cell culture from newborn *Foxb1*$^{Cre/+}$ x *Z/AP* medulla oblongata. Arrows indicate the *Foxb1* lineage OPCs (M-O); arrowheads show OPC are not of *Foxb1* lineage (O).
(Q-T) Verification of *Foxb1* lineage with the other mouse line *Foxb1*$^{Cre/+}$ x *ROSA26* mouse. In order to colocalize *Foxb1* lineage cells with the nuclei staining antibody, β-gal is the lineage marker in *Foxb1*$^{Cre/+}$ x *ROSA26* mouse line. Arrows indicate many cells of coexpression between Olig2 (green, R and S) and β-gal (red, Q and S) antibodies. However, some Olig2+ cells don't belong to *Foxb1* lineage which are indicated with arrowheads (Q-S).
Nuclei are counterstained with DAPI (D, H, L, P and T). Scale bars=50 μm.

3.4 *Foxb1*-lineage Neuroepithelium

The presence of *Foxb1*-lineage oligodendrocytes as well as OPC indicated that there must be stem cells in the neuropithelium (ventricular zone) belonging to this lineage as well. In order to confirm this hypothesis I first labeled sections of embryonic heads by means of a histochemical reaction which detects alkaline phosphatase activity. The results showed a restricted pattern of alkaline phosphatase activity in a paired region of the ventral side of the neuroepithelium of the fourth ventricle (Fig. 9 A-C). I proceeded to investigate this region by colocalizing several general ventricular zone markers with hPLAP by means of antibodies (Fig. 9 D-L). The three markers (vimentin, Fig. 9 D-F; nestin, Fig. 9 G-I; NeuroD1, Fig. 9 J-L) showed abundant double-labeled cells. This indicated that, as expected, abundant neuroepithelial cells in a specific region of the neural tube belong to the *Foxb1* lineage.
Radial glial cells are essential components of the ventricular zone (neuroepithelium) since they divide to generate most neurons and glia (Dimou and Gotz, 2014; Dromard et al., 2007; Hartfuss et al., 2001; Jakovcevski et al., 2009; Kessaris et al., 2008; Malatesta et al., 2003; Mo and Zecevic, 2009). Therefore, I colocalized hPLAP with several specific markers for radial glial cells (GLAST, GFAP and BLBP) in the ventral neuroepithelium of the fourth ventricle (Fig. 10). Again, the three markers showed abundant radial glial cells belonging to the *Foxb1*-lineage (Fig. 10).

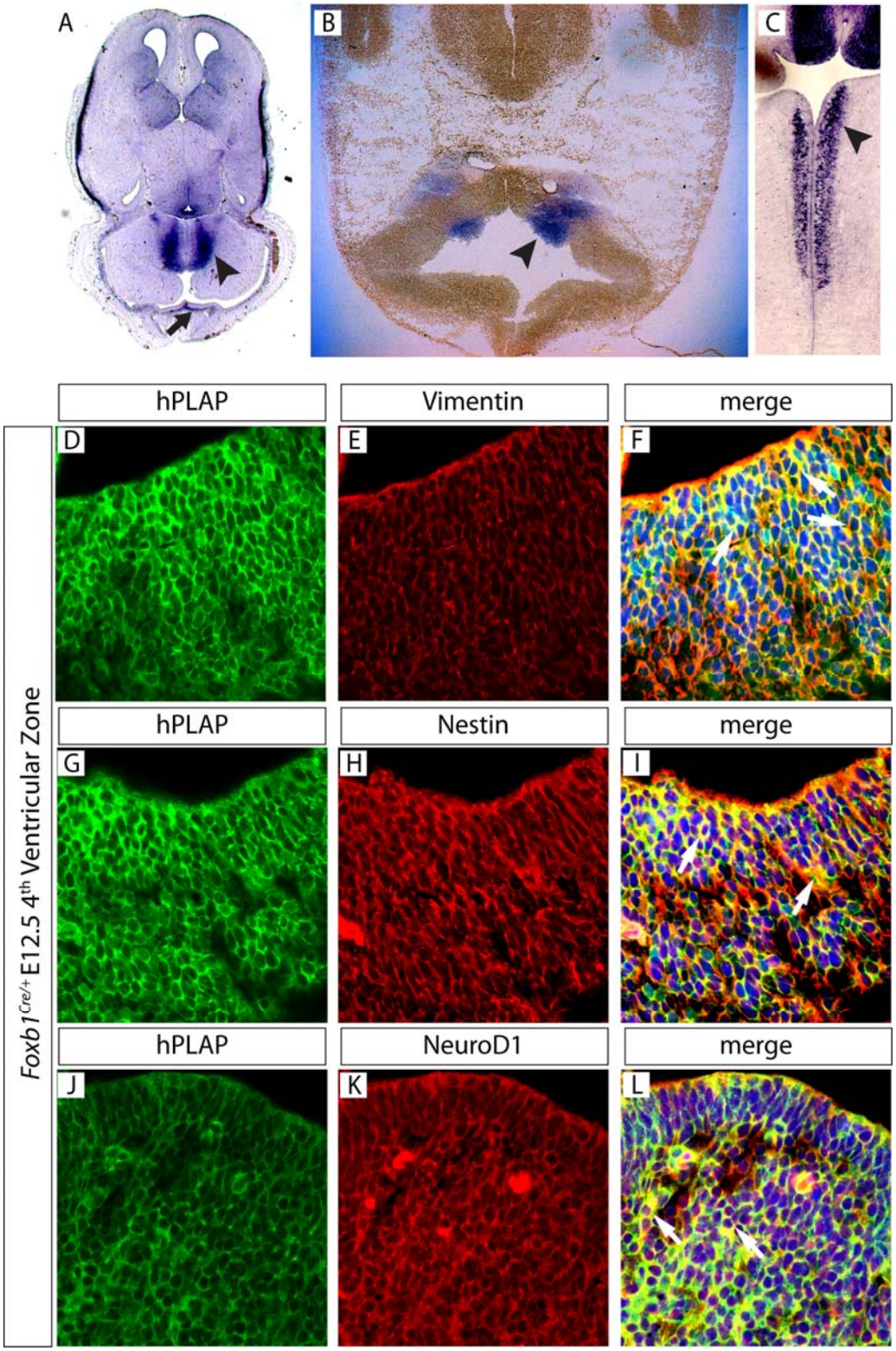


Figure 9. Different orientation of E12.5 *Foxb1*$^{Cre/+}$ mice after hPLAP staining. Colocalization of neuroepithelial markers with hPLAP in the 4th ventricular zone.
(A-C) Different orientation of hPLAP staining on E12.5 *Foxb1*$^{Cre/+}$ x *Z/AP* mouse brain. Black arrows indicate *Foxb1* lineage cells are stained in purple color after hPLAP reaction (A-C). The positively reacted region will develop into hindbrain. The metencephalon are shown in horizontal (A) and coronal (B) orientation. The longitudinal brain sections reveal the pre-medulla are stained by hPLAP (C).
(D-L) Colocalization of neuroepithelial markers (Vimentin, Nestin and NeuroD1) with hPALP in the ventricular zone of E12.5 *Foxb1*$^{Cre/+}$ x *Z/AP* mouse 4th ventricle. White arrows indicate this group of neuroepithelium are *Foxb1* lineage.
Nuclei are counterstained with DAPI (F, I and L). Scale bars=50 μm.

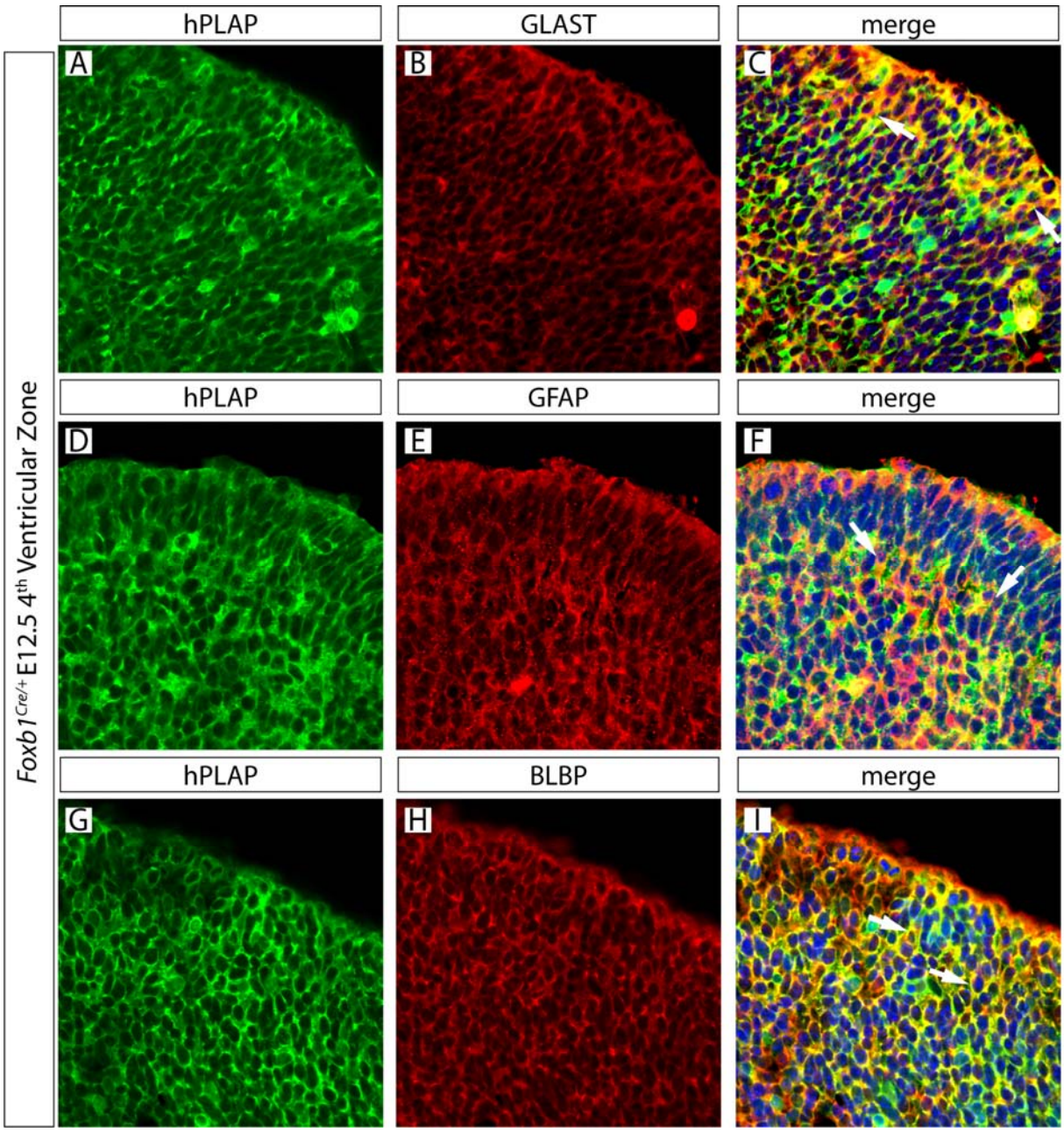


Figure 10. Colocalization of radial glial markers with *Foxb1* lineage marker at E12.5 ventricular zone.
(A-I) Immunohistochemistry of radial glial markers (Glast (red, B and C), GFAP (red, E and F), BLBP (red, H and I)) with hPLAP (green, A, B and G) on *Foxb1*$^{Cre/+}$ x *Z/AP* brain sections. The *Foxb1* lineage neuroepithelium are indicated with white arrows (yellow, C, F and I).
Nuclei are counterstained with DAPI (C, F and I). Scale bars=50 μm.

3.5 *Foxb1*-lineage OPC are More Abundant in Null Mutants

In order to learn about the possible function of *Foxb1* in the oligodendrocyte lineage, I used hPLAP labeling on the hindbrain of $Foxb1^{Cre/Cre}$ mutants. Simple examination of the sections under the microscope showed that the labeled regions were much more extensive than in of $Foxb1^{Cre/+}$ brains (Fig. 11, compare A, B to C, D and E, F to G, H). Next, I used antibodies to colocalize *Foxb1*-lineage reporter hPLAP with the same markers used previously for the analysis of heterozygotes (Figs. 3, 4, 5, 6, 8). Cell countings and statistical analysis (Figs. 12a and 12b, summarized in Fig. 13) showed clear differences between the heterozygous and the homozygous brains. The OPC triplicate their number in the $Foxb1^{Cre/Cre}$ mutants (Fig. 12a G-H and Fig. 12b A-C), as do the immature oligodendrocytes (i.e. those cells expressing GalC but not Claudin11) (compare Fig. 12a A to D). The increase is not only in absolute number of cells per counting bin, but also in the proportion of *Foxb1*-lineage cells that are OPC or immature oligodendrocytes (Fig. 13). Mature oligodendrocytes, on the contrary, are increased in absolute number (Fig. 12a A) but reduced in percent of total hPLAP+ cells (Fig. 12a C and Fig. 13). The absolute number of astroglia are also increased in *Foxb1* homozygous hindbrain (Fig. 12b G, I and Fig. 13). Finally, in homozygotes, neurons are reduced in absolute numbers as well as in percent of all hPLAP+ cells (Fig. 12b D, F). A last class of cells, belonging to the *Foxb1* lineage but not expressing any of the markers I used (black portions of the columns in Fig. 13), increased also in proportion in $Foxb1^{Cre/Cre}$ mutants.

These results indicate that, in mice deficient in transcription factor *Foxb1*, the *Foxb1*-expressing proliferating cells of the nervous system are strongly biased towards oligodendrocyte production.

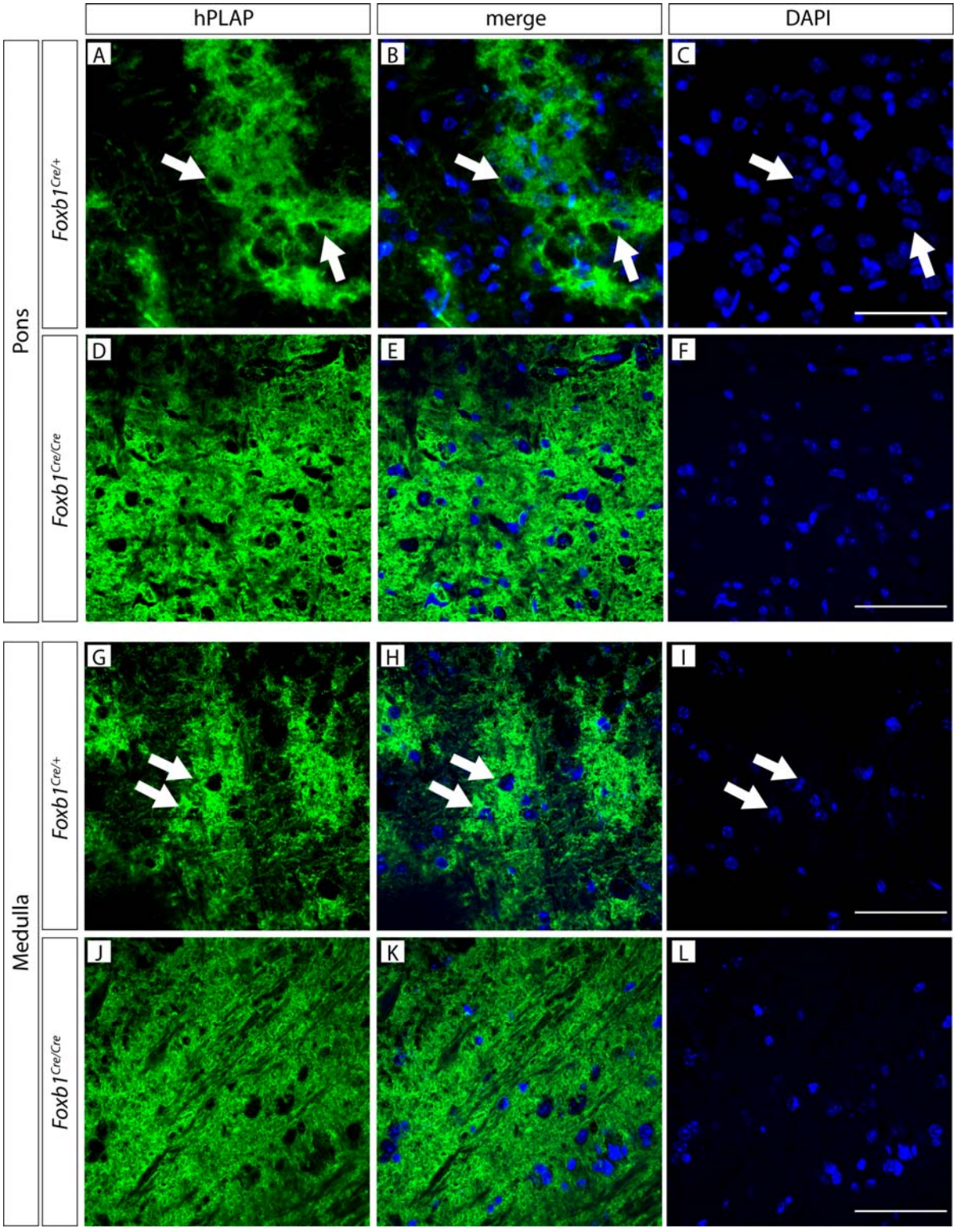


Figure 11. More cells are labeled by *Foxb1* lineage marker in *Foxb1*$^{Cre/Cre}$ mice.
White arrows indicate the *Foxb1* lineage cells in pons (A-C) and medulla oblogata (G-I) of adult *Foxb1*$^{Cre/+}$ x *Z/AP* mouse. There are more hPLAP+ cells in adult *Foxb1*$^{Cre/Cre}$ x *Z/AP* pons (D-F) and medulla (J-L) than in adult *Foxb1*$^{Cre/+}$ x *Z/AP* mouse.
Nuclei are stained with DAPI (C, F, I and L). Scale bars=50 μm.

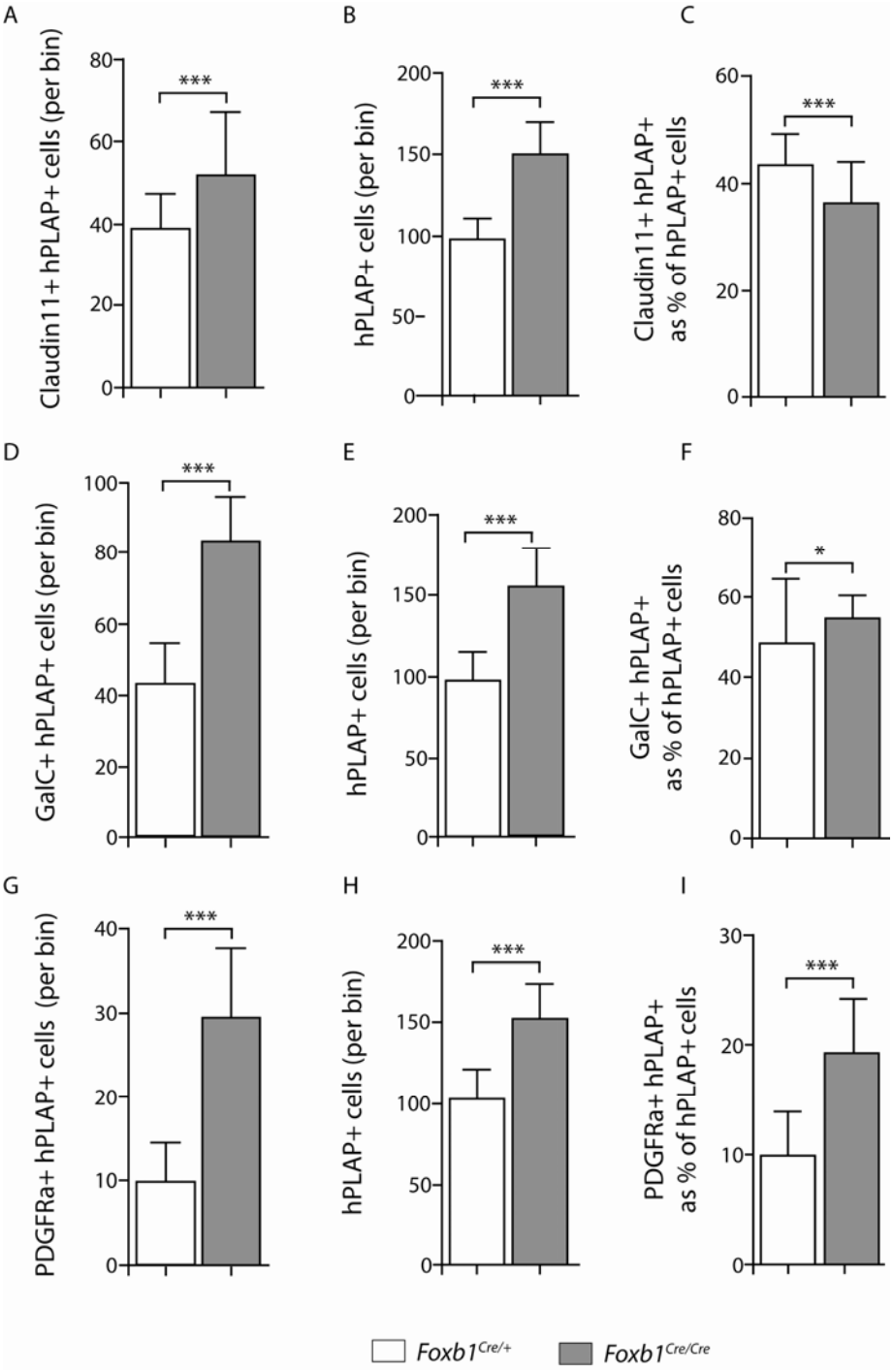


Figure 12a. Increased oligodendrocyte numbers in the pons and medulla of *Foxb1* mutant mice.
Quantification of the number of cells expressing different markers in hindbrain.
(A) There are more Claudin11+hPLAP+ mature oligdodendrocytes in *Foxb1* homozygotes than in *Foxb1* heterozygotes. However, the proportion of mature oligodendrocytes in *Foxb1* lienage is less in *Foxb1* homozygotes (C).
(D) *Foxb1* homozygous hindbrain has more GalC+hPLAP+ immature and mature oligodendrocytes which belong to *Foxb1* lineage. The proportion of GalC+ in *Foxb1* lineage are also increased in homozygotes (F).
(G-I) PDGFRa+hPLAP+ OPC has more absolute number in *Foxb1* homozygous hindbrain. The proportion of *Foxb1*-lineage OPC are also more in homozygotes than in heterozygotes (I).
The absolute hPLAP+ cell numbers are shown in (B, E and H). It reveals there are significantly more *Foxb1* lineage cells in homozygotes hindbrain.
(For all quantifications, 10 sections per animal and 3 mice each group were counted. All sections were cut sagitally and serially. Mean ± SD; *p<0.05, **p<0.01, ***p<0.005; Nonparametric Mann-Whitney test).

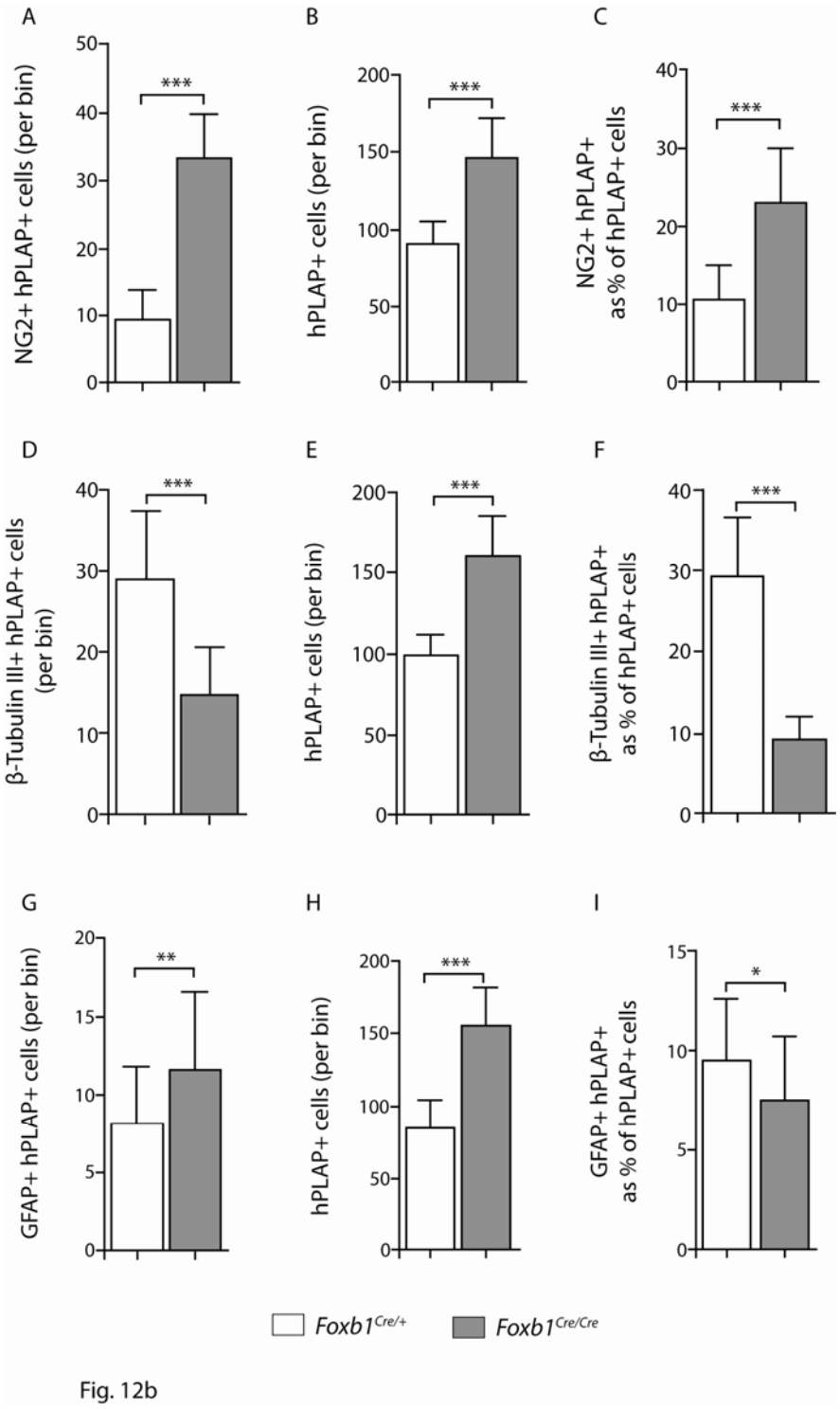


Figure 12b. Increased OPC numbers in the pons and medulla of *Foxb1* mutant mice
(A-I) Quantification of the number of cells expressing different markers (NG2+ (A-C), β-Tubulin III+ (D-F) and GFAP+ (G-I)) in hindbrain.
(A) There are moreNG2+hPLAP+ OPC in *Foxb1* homozygotes than in *Foxb1* heterozygotes. The proportion of mature oligodendrocytes in *Foxb1* lienage is more in *Foxb1* homozygotes (C).
(D) *Foxb1* homozygous hindbrain has less β-Tubulin III+hPLAP+ neurons. The proportion of β-Tubulin III+ in *Foxb1* lineage also declines in homozygotes (F).
(G-I) GFAP + hPLAP+ astrocyte has more absolute number in *Foxb1* homozygous hindbrain. However, the proportion of *Foxb1*-lineage astrocytes is less in homozygotes than in heterozygotes (I).
The absolute hPLAP+ cell numbers are shown in (B, E and H). It reveals there are significantly more *Foxb1* lineage cells in homozygotes hindbrain.
(For all quantifications, 3 mice each group were counted. All sections were cut sagitally and serially. Mean ± SD; *p<0.05, **p<0.01, ***p<0.005; Nonparametric Mann-Whitney test).

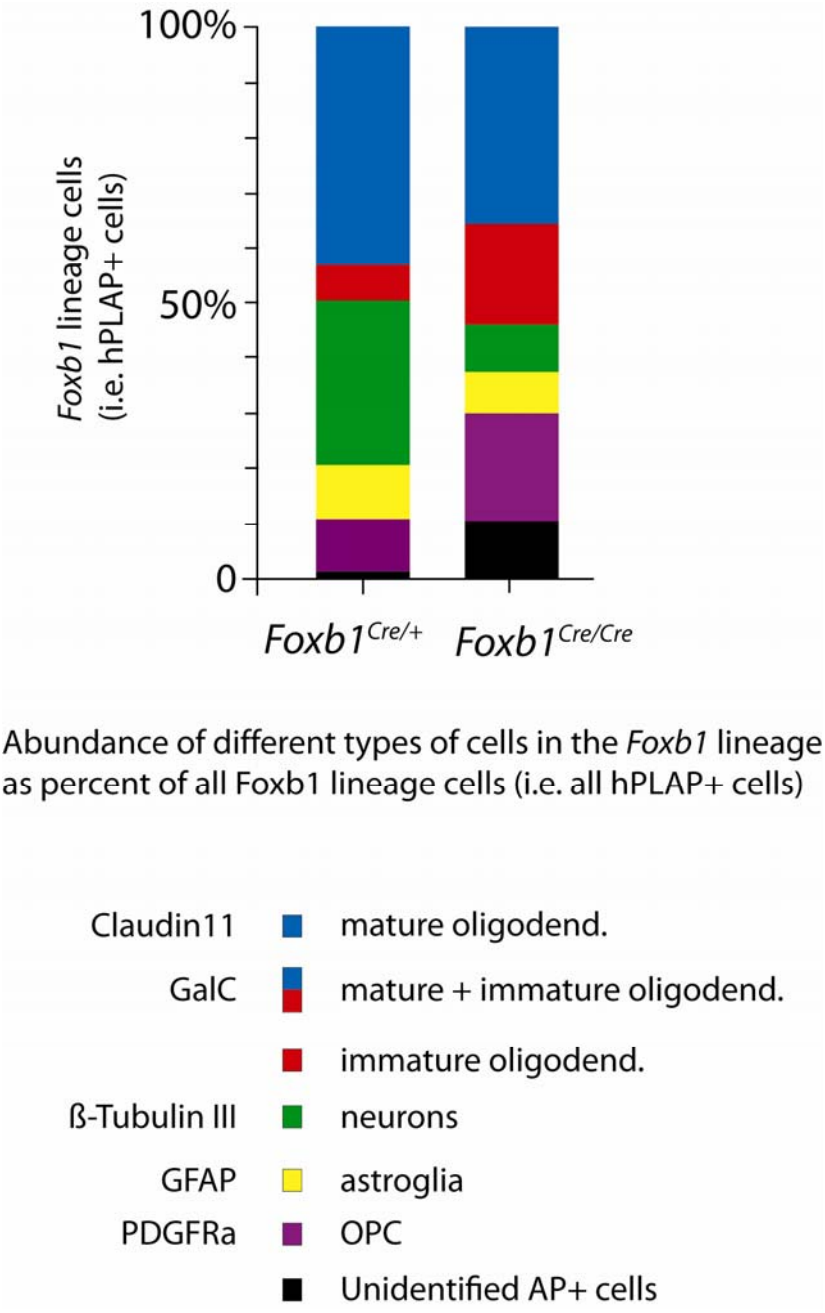


Figure 13. Deficiency of *Foxb1* in hindbrain expands the oligodendrocyte lineage.
Graphical illustration of the proportion of *Foxb1* cell lineage in heterozygous and homozygous pons and medulla oblongata.
Five colors represent populations of cells that belong to mature oligodendrocyte (blue), mature and immature oligodendrocyte (blue and red), neuron (green), astrocyte (yellow) and OPC (purple). Unidentified cells are represented in black color.
It shows more immature oligodendrocytes in *Foxb1* homozygotes. The proportion of OPC is also increased in homozygous mice. In contrast to oligodendrocyte lineage, neurons (green) percentage of hPALP+ cells is less in *Foxb1* homozygous hindbrain.

3.6 *Foxb1*-lineage OPC Proliferate More in Null Mutants

I reasoned that the increase in OPC and in oligodendrocytes could be due to an increase in proliferation by OPC. To test this hypothesis, I performed intraperitoneal injections of proliferation marker bromo-deoxy-uridine (BrdU) into P10 *Foxb1*$^{Cre/+}$ and *Foxb1*$^{Cre/Cre}$ mutants (see Material and Methods for details). Then I used antibodies to colocalize BrdU and Olig2 on sections of medulla oblongata of these mice (Fig. 14 A-F) and counted the double labeled cells (see Material and Methods for details). Our results show an increase in the number of proliferating OPC in homozygotes (Fig. 14 G-I).

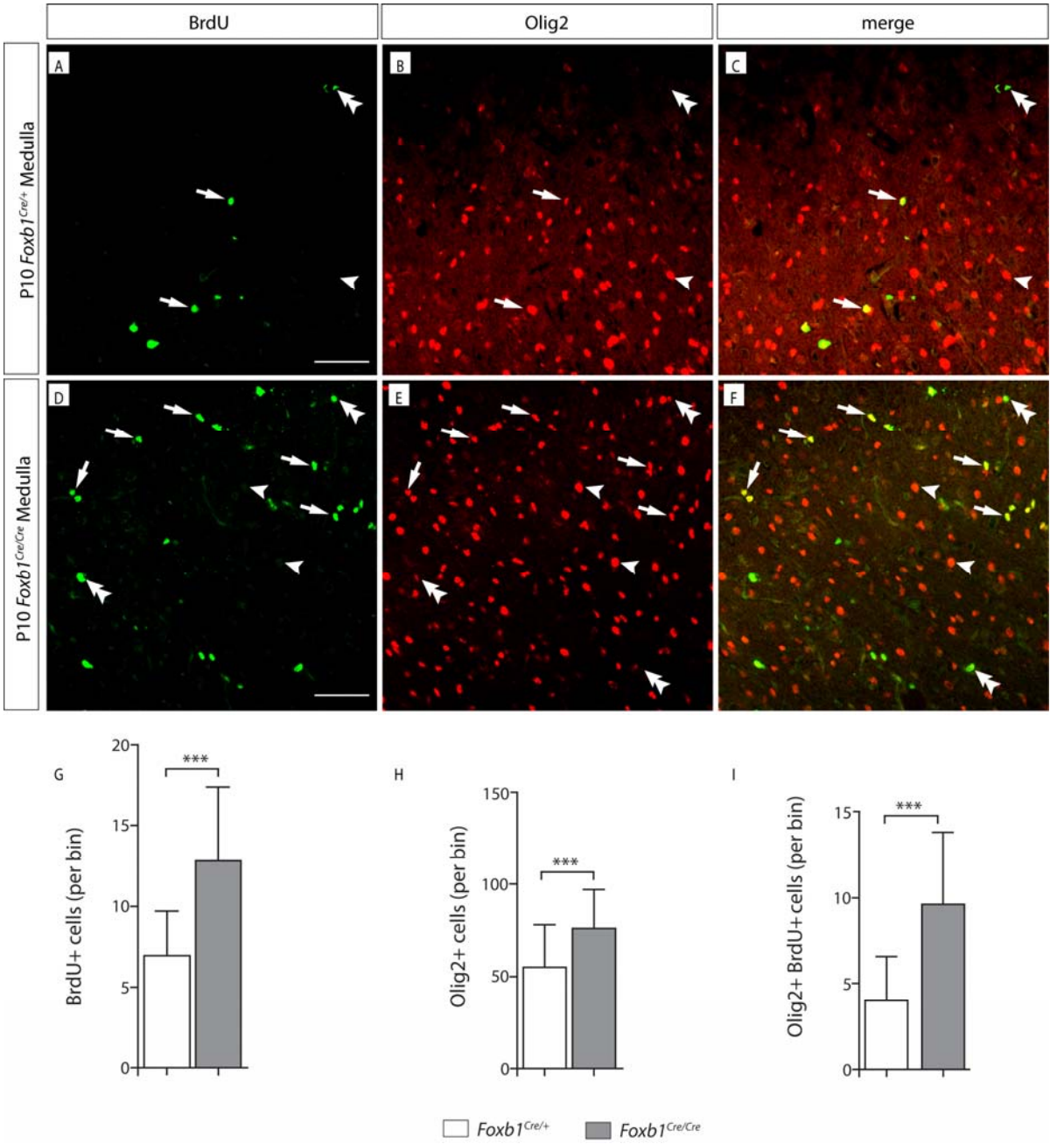


Figure 14. *Foxb1* inactivation increases the proliferating OPC populations.
(A-F) Proliferation of oligodendrocyte lineage in P10 medulla oblongata. BrdU was injected intraperitoneally to label the proliferating cells (green, A and D). The oligodendrocyte lineage are labeled with Olig2 by immunohistochemistry method (red, B and E). The heterozygous results are shown in (A, B and C), and the homozygous proliferation of OPC are shown in (D, E and F). Arrows indicate Olig2+BrdU+ proliferating OPC (yellow, C and F). Both BrdU+ proliferating cells (G) and Olig2+ oligodendrocyte lineage cells (H) are more in homozygous than in heterozygous hindbrain.
There are significantly more proliferating OPC in *Foxb1* homozygotes than in heterozygotes. The coexpressed Olig2+BrdU+ cells are also increased in homozygotes (I).
(For all quantifications, 10 sections per animal and 3 mice each group were counted. All sections were cut sagitally and serially. Mean ± SD; *p<0.05, **p<0.01, ***p<0.005; Nonparametric Mann-Whitney test).

3.7 Myelination in the Hindbrain of Null Mutants Appears Normal

Next, I would like to know if the oligodendrocytes produced in these mice could fulfil normally their main function, i.e. myelination. The G-ratio is the ratio between axon diameter and axon plus myelin sheath diameter, and it is considered a valuable parameter to determine normal myelination function (Chomiak and Hu, 2009; Hildebrand and Hahn, 1978; Little and Heath, 1994; Rushton, 1951; Stidworthy et al., 2003). The normal G-ratio is between 60 and 75% (Chomiak and Hu, 2009; Goldman and Albus, 1968; Jin et al., 2015; Rushton, 1951). Accordingly, I performed electron microscopy analysis on ultrathin sections of the hindbrain of adult (P60) *Foxb1*$^{Cre/Cre}$ mutant mice and found that the mean G-ratio is 69.99 ± 12.07, which is well inside the normal parameters (Fig. 15). This indicates that there is no major loss of function in the oligodendrocytes of the *Foxb1*$^{Cre/Cre}$ mutant mice.

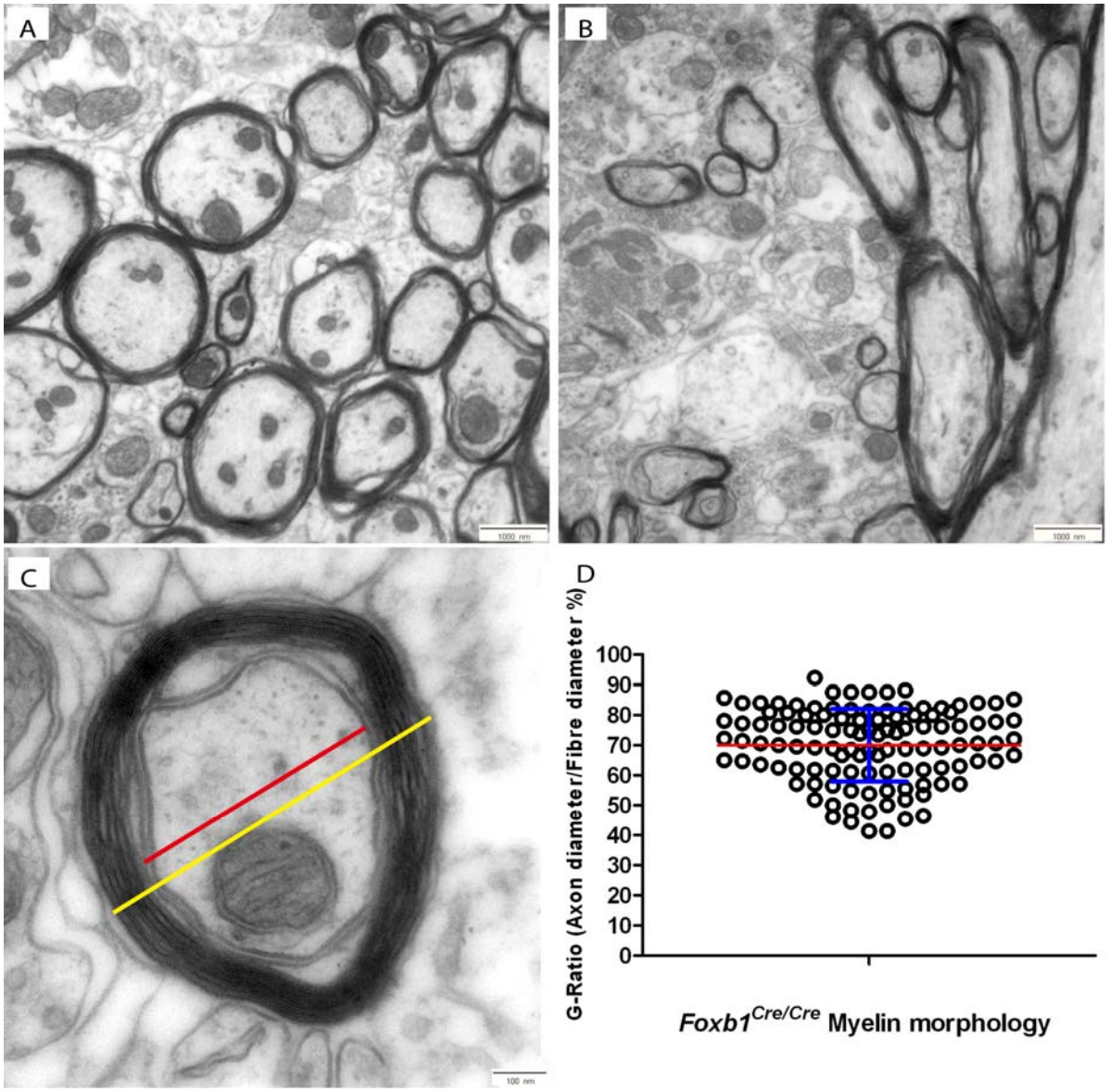


Figure 15. *Foxb1* deficiency doesn't alter the myelination potential of oligodendrocyets.
The morpholgy of *Foxb1*$^{Cre/Cre}$ x Z/AP medulla are presented in details by electro microscope. The myelination are observed in transverse (A) and longitudinal (B) orientation. To evaluate the myelin thickness, G-ratio (axonal diameter divide whole diameter of axon plus myelination) is calculated. The mean G-ration is 69.99% and standard deviation is 12.07%. The value is in the normal range of G-ratio (60%-75%) in wild type mouse.

CHAPTER 4: DISCUSSION

Pathological conditions affecting the maturation of the brain white matter in premature infants are on the rise, and their most common result is mental retardation. The mechanisms regulating the proliferation and differentiation of oligodendrocytes, the cells responsible for the formation of the white matter, are currently under intense scrutiny. Here I approach this “hot area” of investigation through the analysis of a mouse genetic mutation affecting the maturation and proliferation of oligodendrocytes. My research uncovers the transcription factor *Foxb1* involved in oligodendrocyte development and shows that this regulator inhibits the production of oligodendrocyte progenitor cells (OPC) and biases neuroepithelial stem cells in the direction of producing more neurons in detriment of the oligodendrocyte lineage.

Some of my conclusions are relevant to our understanding of how genes and in particular Forkhead transcription factors regulate the generation, maturation and function of specific cell classes in the brain. Some other conclusions are relevant to the OL and as a consequence to therapeutic possibilities for patients with white matter degeneration (see Introduction for details).

Therefore, in what follows I will discuss these points:

- the known functions of *Foxb1* and the novel function uncovered here
- Fox genes as regulators of oligodendrocyte development
- Fox genes as regulators of cell proliferation
- *Foxb1* in the context of the transcriptional regulation of oligodendrocyte proliferation and differentiation
- *Foxb1* in the context of the upstream regulators of OL proliferation and differentiation
- the *Foxb1* lineage in the context of the heterogeneity of OL in the CNS

4.1 Known Functions of *Foxb1*

First of all, I will try to place the regulation of the expansion of the OPC compartment into the framework of the known functions of *Foxb1*. *Foxb1* is essential for the perinatal survival of the cells of one hypothalamic nucleus, the mammillary body (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000a; Alvarez-Bolado et al., 2000b; Zhao et al., 2008; Zhao et al., 2007); previous to their death by apoptosis, the axons of mammillary neurons are unable to specifically branch into the mammillothalamic bundle (Alvarez-Bolado et al., 2000b). Lack of a mammillary body, in turn, results in working memory alterations (Radyushkin et al., 2005). *Foxb1* is also essential for the survival of a subset of spinal cord motoneurons necessary for the appropriate function of the hind legs (Dou et al., 1997). Finally, *Foxb1* is required for the development of the milk ejection reflex (Kloetzli et al., 2001; Labosky et al., 1997). In conclusion, none of the previously published functions of *Foxb1* has a direct relation to oligodendrocytes or to the control of cell proliferation. Therefore, in the present work I uncover a novel function for this gene.

4.2 Forkhead Genes as Regulators of Oligodendrocyte Development

The development and maturation of OL is tightly regulated. It responds to a battery of humoral factors as well as a series of transcription factors. All of these regulators act in a specific temporal sequence taking the OPC and their derivatives through a known series of morphological and biochemical changes ending in the mature OL able to myelinating axons. Although up to now forkhead genes have not been revealed to have important functions in OL development, at least two of them have to my knowledge some involvement, be it indirect or marginal, in this process:

- *Foxa2* is responsible for differentiation of the floor plate and maintenance of *Shh* expression by the floorplate; as a consequence, its deficiency in zebrafish results in failure in the induction and/or differentiation of oligodendrocytes (as well as other cell types) in the ventral zebrafish CNS (Norton et al., 2005).
- *Foxg1* stimulates the expansion of the neural stem cell compartment as well as inhibiting the glial fate choice (including oligodendroglia) (Brancaccio et al., 2010).

However, *Foxb1* is the first forkhead gene specifically expressed in a subpopulation of OL and with specific functions in their development (this report).

4.3 Regulation of Cell Proliferation by Fox Genes

Additionally, my work here uncovers a novel function of *Foxb1* in restricting the expansion of the OPC compartment. Control of cell proliferation, be it positive or negative, is not a novel function for Fox genes. This superfamily of transcription factor genes have several different roles in the developing embryo as well as in the maintenance of the internal milieu of the adult (Lam et al., 2013). Paradoxally, FOX proteins, which show high sequence conservation in the core forkhead motifs, often control divergent processes. Sometimes it is even the case that different FOX proteins control opposing cell fate decisions. They do this by regulating genetic cascades upstream processes like proliferation, indeed, and differentiation, metabolism, aging, survival and apoptosis. Additionally, FOX proteins can act as so-called „pioneer factors“, i.e. proteins able to decondense chromatin facilitating in this way the action of other specific transcription factors. I will briefly discuss here two cases in point: the *Foxo* subfamily and *Foxm1*.

The case of the *Foxo* genes. The *Foxo* subfamily of forkhead genes consists of key negative regulators of cell proliferation and survival. They not only repress the activity of genes crucial for cell cycle progression (like *cyclin D1* and *cyclin D2*), but they also induce the expression of genes with specific cell cycle-inhibition functions like p21, p27, p15 and p19. Additionally, *Foxo* genes regulate G2/M checkpoint genes like 45A, cyclin G2 and PLK1 (Dong et al., 2006; Lam et al., 2013; McGovern et al., 2009; Myatt and Lam, 2007; Paik et al., 2007). Additionally, it is known that *Foxo1* regulates the proliferation and differentiation of neural stem cells through its interaction with the Notch pathway (Hoeck et al., 2010; Kitamura et al., 2007).
The case of *Foxm1*. The main function of *Foxm1* is the regulation of the cell cycle. Additionally, *Foxm1* interacts with β-catenin enabling it to translocate to the nucleus, in this way regulating stem cell maintenance: the expression of downstream Wnt target genes that are key for the survival of stem cells (Francis et al., 2009; Karadedou et al., 2012; Zhang et al., 2011).

In conclusion, the control of OPC proliferation by *Foxb1* is in line with previously known important functions of the Fox superfamily of transcription factors. The fact that

this control is exerted on such a major cellular component of the CNS as the oligodendrocytes is however novel.

4.4 *Foxb1* in the Transcriptional Regulation of OPC Proliferation

OPC proliferation during development and in the adult is tightly regulated; the length of the OPC cell cycle shows differences related to developmental age and region of origin. Proliferation of OPC becomes slower as the CNS develops, from a few hours at early embryonic stages to 1 day per cell cycle perinatally (Calver et al., 1998) and even 1 to 5 weeks per mitosis at P60 (Young et al., 2013) and several months per mitosis in the cortex of very old mice (Psachoulia et al., 2009). Differences in proliferation rate are also pronounced between regions. For instance, OPC proliferation is faster in the corpus callosum than in the cortex, and there are marked differences between CNS axonal bundles. For instance, the OL in the white matter of the spinal cord proliferate faster than those of the optic nerve (Young et al., 2013).

My results here show that *Foxb1* deficiency results in an increase of the proliferation of OPC; this indicates that *Foxb1* represses proliferation. If this is by a direct mechanism, i.e. by repressing genes required for replication (forkhead factors act transcriptional repressors in some models) or indirectly is not yet known.
Another known major repressor of OPC expansion is *Sirt1*. Inactivation of *Sirt1* leads to a similar expansion of the OPC compartment in mice (Rafalski et al., 2013). Interestingly, *Sirt1* acts in other systems through activation of Fox genes (through deacetylation), so that its inactivation would amount to an inactivation of Fox genes downstream (see below).

4.5 Upstream of Fox Genes in the OPC Lineage

This brings us to the next question and opens up a future avenue for research: does Sirt 1 act by deacetylating (activating) *Foxb1* in OPC? Interaction between sirtuins and Forkhead genes (although not with *Foxb1* or other members of the *Foxb* subfamily) has been described. The deacetylation of FOXO transcription factors by SIRT1 is a specifically required step for the transcription of certain genes regulating stress resistance, such as genes that control the repair of damaged DNA (GADD45) and a

regulation of *Foxo* genes by Sirt proteins has been well documented in different model systems ever since it was first found (Araki et al., 2004; Brunet et al., 2004; Calnan and Brunet, 2008; Cohen et al., 2004; Daitoku et al., 2004). This means that a decrease in Sirt1 results in a decrease in *Foxo* activity. Another case of Sirt1 involved in Fox gene regulation results in the inactivation of *Foxp3* through degradation in the proteasome (van Loosdregt et al., 2011; van Loosdregt and Coffer, 2014).

To summarize, although at this point I do not know which upstream regulators control the activity of *Foxb1* in OPC, the known functions of Sirt1 make it a key candidate for this function.

Proteins	Biological process	Key targets‡	Regulation
FOXO1	Angiogenesis	*ANG2*	Down
		VEGFA	Up
	Apoptosis	*BAD* and *TRADD*	Up
	Cell metabolism	*AGRP*, *GPR17*, *MAFA*, *NEUROD*, *NPY*, *PEPCK* and *PGC1*	Up
		PDX1, *PPARG* and *POMC*	Down
	Cell migration and invasion	*MMP1*	Up
		RUNX2	Down
	Differentiation	*MSTN*	Up
	Immune regulation	*CEBPB*, *EOMES*, *IL1B*, *IL7R*, *RAG1* and *RAG2*	Up
	Oxidative stress	*CP* and *SEP*	Up
	Stem cell renewal and differentiation	*OCT4* and *SOX2*	Up
FOXO3A	Angiogenesis	*VEGFA*	Down
	Apoptosis	*BCL10*, *BIM*, *PINK1*, *PI3KIP1* and *PUMA*	Up
	Cell cycle and proliferation	*CCNA1*	Up
	Cell migration and invasion	*CXCR4*	Down
		MMP13	Up
	Oxidative stress	*CAT*, *PRDX3* and *SOD2*	Up
	Signalling	*ESR1* (which encodes ERα)	Up
	Tumour suppression	*MXI1*	Up
FOXO4	Cell migration and invasion	*MMP9*	Up
FOXO1 and FOXO3A	Angiogenesis	*NOS3*	Down
	Apoptosis	*TRAIL* and *FASL*	Up
	Cell cycle and proliferation	*CDKN2B* (which encodes p15), *CDKN2D* (which encodes p19) and *PLK1*	Up
	Cell metabolism	*G6PC* and *IGFBP1*	Up
	Drug resistance	*ABCB1*	Up
	Signalling	*DGKA*, *FOXO1*, *FOXO3A* and *FOXP3*	Up
FOXO1, FOXO3A and FOXO4	Angiogenesis	*SPRY2* and *CITED2*	Up
	Cell cycle and proliferation	*CDKN1A* (which encodes p21), *CDKN1B* (which encodes p27), *CCNG2* and *RBL2* (also known as RB2)	Up
		CCND1 and *CCND2*	Down
	DNA repair	*GADD45A*	Up
	Signalling	*CAV1* and *PDGFRA*	Up
	Tumour suppression	*LKB1*	Up

Figure1. The *Foxo* family participates in cell proliferation and cancer progression. (Adapted from Lam et al., 2013)

4.6 *Foxb1* in the Transcriptional Regulation of OPC Differentiation

The development of the ventral spinal cord and brainstem (and of the corresponding OL) is orchestrated first of all by diffusible signals and among them very particularly by the morphogenetic protein Sonic hedgehog (Shh), secreted by the notochord and floor plate at the ventral midline. Shh acts through the Gli family of transcription factors to stimulate expression of different sets of lineage-determining transcription factor genes. In the pMN neuroepithelial domain, Shh induces the basic helix – loop – helix (bHLH) transcription factor Olig2, required for the sequential production of MNs and OPC. OPC are also generated in the ventrally adjacent p3 neuroepithelial domain, characterized by Nkx2.2 expression. (Agius et al., 2004; Briscoe and Ericson, 2001; Lu et al., 2002; Mekki-Dauriac et al., 2002; Miller et al., 2004; Orentas and Miller, 1996a, b; Poncet et al., 1996; Pringle et al., 1996; Takebayashi et al., 2002; Zhou and Anderson, 2002)

(Although Shh is the model of all diffusible factors, there are many others that have been shown to influence aspects of OL development, especially PDGF and several Fgf family members. The review of these is out of the scope of this work and has been abundantly reviewed elsewhere.)

After being specified to the OL lineage, OPC specifically express a number of transcription factors as they migrate to colonize the CNS and produce OL. Essentially, we can say that there are three phases in the development of OL (OPC, premyelinating OL and myelinating OL) and that each of these phases is associated with intense expression of specific sets of transcription factors. In OPC, transcription factors known to inhibit differentiation and myelination are expressed (*i.e. Hes5, Id2, Id4, Sox5, and Sox6).* These are strongly downregulated as the cells become (or generate) premyelinating OL. However, some level of expression of Olig1 and 2 remain in this second phase, as do Sox10, Nkx2-2, Sox6. Genes newly expressed by premyelinating OL are *Tcf7l2*, *Zfp488* and *Myrf* (Cahoy et al., 2008). Myrf remains in the final, myelinating OL phase, and Zfhx1b, Smad7, and Nkx6-2 appear first or are strongly upregulated in this phase.

After detailed review of current knowledge of transcriptional regulation of OL productoin, I can say that, previous to the present work, no forkhead transcription factor has ever been included in these regulatory cascades.

4.7 Heterogeneity in Oligodendrocytes

The question of the possible heterogeneity of OPC and, therefore, OL is very current because of its relevance for replacement therapy (are there different kinds of OL born from different places in the neuroepithelium? are they interchangeable?). My work here uncovers a specific subpopulation of OPC and OL originated in the ventral brainstem and characterized by belonging to the *Foxb1* lineage and whose proliferation is specifically controlled by *Foxb1*. Nevertheless, my data indicate they not all OL in the brainstem belong to the *Foxb1* lineage.

(According to data from our group not shown in the present work, some forebrain oligodendrocytes, restricted to the dorsal thalamus and the mammillary body of the hypothalamus, belong to the *Foxb1* lineage also).

Already at the end of the XXth century several different morphological classes of OL had been described; these myelinate preferentially either several axons of small caliber or only one or two axons of large calibre (Anderson et al., 1999; Bjartmar et al., 1994; Butt et al., 1998a). Certain molecular differences have also been described between OL subpopulations (Butt et al., 1998b; Kleopa et al., 2004). Nevertheless, it is not clear that those differences arise as a consequence of differential site of origin, i.e., we still do not know if differential markers and eventually functional properties respond to differential place of origin or are shared „transversally“ by OL originated in various points of the ventricular zone.

In the rodent CNS, OPC originate in discrete loci in the ventral neuroepithelium during mid-gestation. In the spinal cord, they emerge between E12.5 and E14.5 from the pMN and the p3 domains. A few days later, OPC are generated from the dorsal neuroepithelium of the brainstem (Huang et al., 2013; Rowitch, 2004; Woodruff et al., 2001). That is, in spinal cord and braistem we have dorsal vs ventral domains. In the developing forebrain, OPC originate sequentially in different domains as well. First they appear in the neuroepithelium of the medial and lateral ganglionic eminences and then, perinatally, in the dorsal neuroepithelium (Kessaris et al., 2006; Rowitch and Kriegstein, 2010).

Are the heterogeneous populations of OL functionally interchangeable? Experiments in which OL from a certain neuroepithelial origin (in the forebrain) have been genetically eliminated show that OL from other regions invade the OL-less areas and repopulate them in days or weeks without apparent problem (no abnormal phenotype develops) (Kessaris et al., 2006). Although spinal cord dorsal and ventral OL seem to be somewhat more specialized (Tripathi et al., 2011; Zhu et al., 2011), these results argue in principle against strong differences between OL lineages, or at least they indicate that the OL have enough plasticity to become functionally identical with those of a different lineage in response to new environments. Moreover, dorsally and ventrally derived OL seem undistinguishable electrophysiologically and antigenically (Clarke et al., 2012; Tripathi et al., 2011).

No functional differences have been found postnatally among OPC cells and OL originated in different neuroepithelial domains. OPC and OL derived from ventral and dorsal germinal zones exhibit similar proliferation rates, cell cycle times, and passive membrane properties, and there are no differences in their response to neurotransmitters GABA and glutamate (Psachoulia et al., 2009; Tripathi et al., 2011).

Future research will tell if OPC or OL of the *Foxb1* lineage present any particular characteristics that can be distinguished from those of other lineages.

4.8 *Foxb1* in the Differentiation of OL (myelination)

The main function of OL is myelination. Since deficiency in *Foxb1* has uncovered a role of this transcription factor in OPC proliferation, a myelination phenotype could in principle be expected. We did not find, however, an alteration of the G-ratio in the brainstem. The *Foxb1* null mutant phenotype has been studied before (Alvarez-Bolado et al., 1999; Alvarez-Bolado et al., 2000a; Alvarez-Bolado et al., 2000b; Dou et al., 1997; Kloetzli et al., 2001; Labosky et al., 1997; Radyushkin et al., 2005; Wehr et al., 1997; Zhao et al., 2008; Zhao et al., 2007) and nothing seems to point towards myelination alterations. Neurological signs to be expected after demyelination (like ataxia, unsteady gait, ocular paralysis, weakness and loss of sensation) have not been observed by us or other authors in these mutants. My conclusion here is that *Foxb1*-deficient OPC produce functionally normal OL.

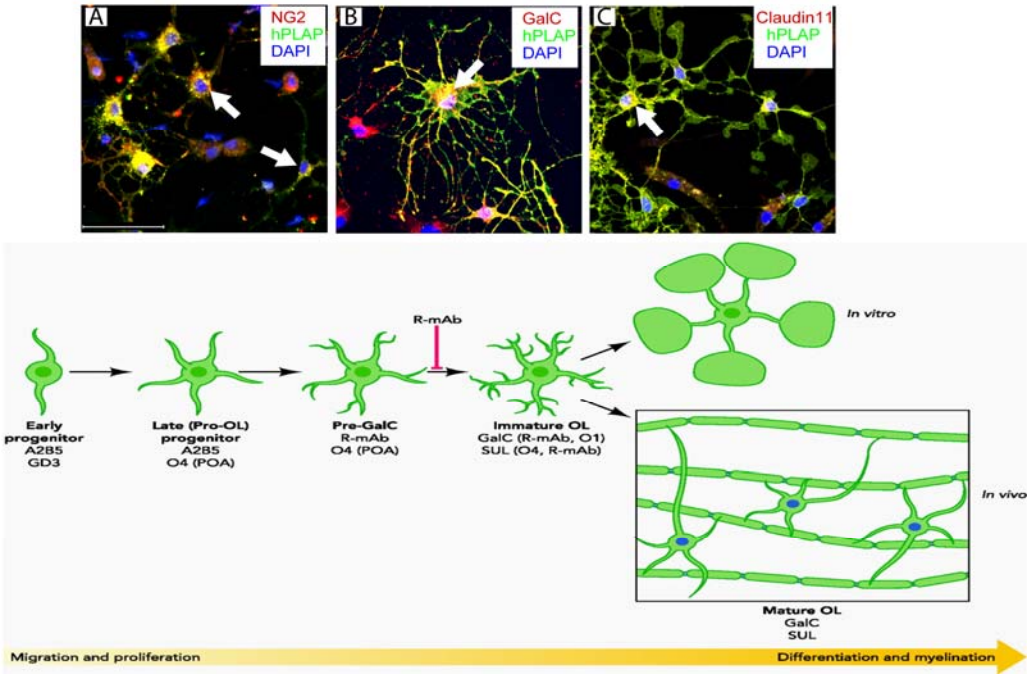


Figure 2. Comparison of *Foxb1*-lineage OL morphology to schematic oligodendrocyte development in vitro.
Foxb1 lineage OL and precursors show similar morphology to that reported in the literature. (Schematic image are adapted from Jackman, Ishii and Bansal, 2009)

Conclusions

- *Foxb1*-expressing neuroepithelial cells contribute abundant OPC and OL to the mouse brain.
- The function of *Foxb1* consists of inhibiting the proliferation of OPC and biasing the production of the *Foxb1*-expressing neuroepithelium towards neurons and astrocytes.
- *Foxb1*-lineage OL are a novel subpopulation of these cells whose specific properties as well as their ability to replace other subpopulations (for instance, telencephalic OL) as part of a therapeutic approach are from this moment on open questions.
- *Foxb1* is a novel and important player in the development of the oligodendrocyte lineage.

REFERENCES

Agius, E., Soukkarieh, C., Danesin, C., Kan, P., Takebayashi, H., Soula, C., and Cochard, P. (2004). Converse control of oligodendrocyte and astrocyte lineage development by Sonic hedgehog in the chick spinal cord. Dev Biol *270*, 308-321.

Aharoni, R. (2014). Immunomodulation neuroprotection and remyelination - the fundamental therapeutic effects of glatiramer acetate: a critical review. J Autoimmun *54*, 81-92.

Alexander, J.E., Hunt, D.F., Lee, M.K., Shabanowitz, J., Michel, H., Berlin, S.C., MacDonald, T.L., Sundberg, R.J., Rebhun, L.I., and Frankfurter, A. (1991). Characterization of posttranslational modifications in neuron-specific class III beta-tubulin by mass spectrometry. Proc Natl Acad Sci U S A *88*, 4685-4689.

Alizadeh, A., Dyck, S.M., and Karimi-Abdolrezaee, S. (2015). Myelin damage and repair in pathologic CNS: challenges and prospects. Front Mol Neurosci *8*, 35.

Alvarez-Bolado, G., Cecconi, F., Wehr, R., and Gruss, P. (1999). The fork head transcription factor Fkh5/Mf3 is a developmental marker gene for superior colliculus layers and derivatives of the hindbrain somatic afferent zone. Brain Res Dev Brain Res *112*, 205-215.

Alvarez-Bolado, G., Zhou, X., Cecconi, F., and Gruss, P. (2000a). Expression of Foxb1 reveals two strategies for the formation of nuclei in the developing ventral diencephalon. Dev Neurosci *22*, 197-206.

Alvarez-Bolado, G., Zhou, X., Voss, A.K., Thomas, T., and Gruss, P. (2000b). Winged helix transcription factor Foxb1 is essential for access of mammillothalamic axons to the thalamus. Development *127*, 1029-1038.

Anderson, E.S., Bjartmar, C., Westermark, G., and Hildebrand, C. (1999). Molecular heterogeneity of oligodendrocytes in chicken white matter. Glia *27*, 15-21.

Ang, S.L., Wierda, A., Wong, D., Stevens, K.A., Cascio, S., Rossant, J., and Zaret, K.S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development *119*, 1301-1315.

-REFERENCES-

Anitei, M., Ifrim, M., Ewart, M.A., Cowan, A.E., Carson, J.H., Bansal, R., and Pfeiffer, S.E. (2006). A role for Sec8 in oligodendrocyte morphological differentiation. J Cell Sci *119*, 807-818.

Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.

Baracskay, K.L., Kidd, G.J., Miller, R.H., and Trapp, B.D. (2007). NG2-positive cells generate A2B5-positive oligodendrocyte precursor cells. Glia *55*, 1001-1010.

Barratt, H.E., Budnick, H.C., Parra, R., Lolley, R.J., Perry, C.N., and Nesic, O. (2016). Tamoxifen promotes differentiation of oligodendrocyte progenitors in vitro. Neuroscience *319*, 146-154.

Barrette, B., Nave, K.A., and Edgar, J.M. (2013). Molecular triggers of neuroinflammation in mouse models of demyelinating diseases. Biol Chem *394*, 1571-1581.

Bilella, A., Alvarez-Bolado, G., and Celio, M.R. (2014). Coaxiality of Foxb1- and parvalbumin-expressing neurons in the lateral hypothalamic PV1-nucleus. Neurosci Lett *566*, 111-114.

Bjartmar, C., Hildebrand, C., and Loinder, K. (1994). Morphological heterogeneity of rat oligodendrocytes: electron microscopic studies on serial sections. Glia *11*, 235-244.

Borit, A., and McIntosh, G.C. (1981). Myelin basic protein and glial fibrillary acidic protein in human fetal brain. Neuropathol Appl Neurobiol *7*, 279-287.

Bosma, M.M. (2010). Timing and mechanism of a window of spontaneous activity in embryonic mouse hindbrain development. Ann N Y Acad Sci *1198*, 182-191.

Boulanger, J.J., and Messier, C. (2014). From precursors to myelinating oligodendrocytes: contribution of intrinsic and extrinsic factors to white matter plasticity in the adult brain. Neuroscience *269*, 343-366.

Brancaccio, M., Pivetta, C., Granzotto, M., Filippis, C., and Mallamaci, A. (2010). Emx2 and Foxg1 inhibit gliogenesis and promote neuronogenesis. Stem Cells *28*, 1206-1218.

-REFERENCES-

Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol *11*, 43-49.

Bronstein, J.M., Chen, K., Tiwari-Woodruff, S., and Kornblum, H.I. (2000). Developmental expression of OSP/claudin-11. J Neurosci Res *60*, 284-290.

Bronstein, J.M., Micevych, P.E., and Chen, K. (1997). Oligodendrocyte-specific protein (OSP) is a major component of CNS myelin. J Neurosci Res *50*, 713-720.

Bronstein, J.M., Popper, P., Micevych, P.E., and Farber, D.B. (1996). Isolation and characterization of a novel oligodendrocyte-specific protein. Neurology *47*, 772-778.

Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y.*, et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Bu, J., Banki, A., Wu, Q., and Nishiyama, A. (2004). Increased NG2(+) glial cell proliferation and oligodendrocyte generation in the hypomyelinating mutant shiverer. Glia *48*, 51-63.

Butler, A.B., and Hodos, W. (2005). Comparative vertebrate neuroanatomy : evolution and adaptation, 2nd edn (Hoboken, N.J., Wiley-Interscience).

Butt, A.M., Ibrahim, M., and Berry, M. (1998a). Axon-myelin sheath relations of oligodendrocyte unit phenotypes in the adult rat anterior medullary velum. J Neurocytol *27*, 259-269.

Butt, A.M., Ibrahim, M., Gregson, N., and Berry, M. (1998b). Differential expression of the L- and S-isoforms of myelin associated glycoprotein (MAG) in oligodendrocyte unit phenotypes in the adult rat anterior medullary velum. J Neurocytol *27*, 271-280.

Caccamo, D., Katsetos, C.D., Herman, M.M., Frankfurter, A., Collins, V.P., and Rubinstein, L.J. (1989). Immunohistochemistry of a spontaneous murine ovarian teratoma with neuroepithelial differentiation. Neuron-associated beta-tubulin as a marker for primitive neuroepithelium. Lab Invest *60*, 390-398.

Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubischer, J.L., Krieg, P.A., Krupenko, S.A.*, et al.* (2008). A transcriptome

database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci *28*, 264-278.

Calnan, D.R., and Brunet, A. (2008). The FoxO code. Oncogene *27*, 2276-2288.

Calver, A.R., Hall, A.C., Yu, W.P., Walsh, F.S., Heath, J.K., Betsholtz, C., and Richardson, W.D. (1998). Oligodendrocyte population dynamics and the role of PDGF in vivo. Neuron *20*, 869-882.

Carlson, N.R. (2013). Physiology of behavior, Eleventh edn (Boston, Pearson).

Carlson, N.R., and Birkett, M.A. (2013). Physiology of behavior, 12th edition / edn.

Carlsson, P., and Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. Dev Biol *250*, 1-23.

Chekenya, M., Hjelstuen, M., Enger, P.O., Thorsen, F., Jacob, A.L., Probst, B., Haraldseth, O., Pilkington, G., Butt, A., Levine, J.M*., et al.* (2002). NG2 proteoglycan promotes angiogenesis-dependent tumor growth in CNS by sequestering angiostatin. FASEB J *16*, 586-588.

Chomiak, T., and Hu, B. (2009). What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. PLoS One *4*, e7754.

Clarke, L.E., Young, K.M., Hamilton, N.B., Li, H., Richardson, W.D., and Attwell, D. (2012). Properties and fate of oligodendrocyte progenitor cells in the corpus callosum, motor cortex, and piriform cortex of the mouse. J Neurosci *32*, 8173-8185.

Cobb, J.A., O'Neill, K., Milner, J., Mahajan, G.J., Lawrence, T.J., May, W.L., Miguel-Hidalgo, J., Rajkowska, G., and Stockmeier, C.A. (2016). Density of GFAP-immunoreactive astrocytes is decreased in left hippocampi in major depressive disorder. Neuroscience *316*, 209-220.

Cohen, H.Y., Miller, C., Bitterman, K.J., Wall, N.R., Hekking, B., Kessler, B., Howitz, K.T., Gorospe, M., de Cabo, R., and Sinclair, D.A. (2004). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science *305*, 390-392.

Cohen, J., Woodhams, P.L., and Balazs, R. (1979). Preparation of viable astrocytes from the developing cerebellum. Brain Res *161*, 503-514.

-REFERENCES-

Dai, J., Bercury, K.K., Ahrendsen, J.T., and Macklin, W.B. (2015). Olig1 function is required for oligodendrocyte differentiation in the mouse brain. J Neurosci *35*, 4386-4402.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., Nakajima, T., and Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A *101*, 10042-10047.

Davies, J.E., and Miller, R.H. (2001). Local sonic hedgehog signaling regulates oligodendrocyte precursor appearance in multiple ventricular zone domains in the chick metencephalon. Dev Biol *233*, 513-525.

de Castro, F., Bribian, A., and Ortega, M.C. (2013). Regulation of oligodendrocyte precursor migration during development, in adulthood and in pathology. Cell Mol Life Sci *70*, 4355-4368.

De Groote, G., De Waele, P., Van de Voorde, A., De Broe, M., and Fiers, W. (1983). Use of monoclonal antibodies to detect human placental alkaline phosphatase. Clinical chemistry *29*, 115-119.

Dimou, L., and Gallo, V. (2015). NG2-glia and their functions in the central nervous system. Glia *63*, 1429-1451.

Dimou, L., and Gotz, M. (2014). Glial cells as progenitors and stem cells: new roles in the healthy and diseased brain. Physiol Rev *94*, 709-737.

Dong, X.Y., Chen, C., Sun, X., Guo, P., Vessella, R.L., Wang, R.X., Chung, L.W., Zhou, W., and Dong, J.T. (2006). FOXO1A is a candidate for the 13q14 tumor suppressor gene inhibiting androgen receptor signaling in prostate cancer. Cancer Res *66*, 6998-7006.

Dou, C., Ye, X., Stewart, C., Lai, E., and Li, S.C. (1997). TWH regulates the development of subsets of spinal cord neurons. Neuron *18*, 539-551.

Douvaras, P., and Fossati, V. (2015). Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. Nat Protoc *10*, 1143-1154.

-REFERENCES-

Dromard, C., Bartolami, S., Deleyrolle, L., Takebayashi, H., Ripoll, C., Simonneau, L., Prome, S., Puech, S., Tran, V.B., Duperray, C., *et al.* (2007). NG2 and Olig2 expression provides evidence for phenotypic deregulation of cultured central nervous system and peripheral nervous system neural precursor cells. Stem Cells *25*, 340-353.

Duffy, P.E. (1982). Glial fibrillary acidic protein and induced differentiation of glia in vitro. J Neurol Sci *53*, 443-460.

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. Science *330*, 779-782.

Emery, B., and Lu, Q.R. (2015). Transcriptional and Epigenetic Regulation of Oligodendrocyte Development and Myelination in the Central Nervous System. Cold Spring Harb Perspect Biol *7*, a020461.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., and Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. Nat Med *4*, 1313-1317.

Fancy, S.P., Zhao, C., and Franklin, R.J. (2004). Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. Mol Cell Neurosci *27*, 247-254.

Felts, P.A., Baker, T.A., and Smith, K.J. (1997). Conduction in segmentally demyelinated mammalian central axons. J Neurosci *17*, 7267-7277.

Ferreira, A., and Caceres, A. (1992). Expression of the class III beta-tubulin isotype in developing neurons in culture. J Neurosci Res *32*, 516-529.

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

Fortino, V.R., Chen, R.S., Pelaez, D., and Cheung, H.S. (2014). Neurogenesis of neural crest-derived periodontal ligament stem cells by EGF and bFGF. J Cell Physiol *229*, 479-488.

-REFERENCES-

Francis, R.E., Myatt, S.S., Krol, J., Hartman, J., Peck, B., McGovern, U.B., Wang, J., Guest, S.K., Filipovic, A., Gojis, O.*, et al.* (2009). FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. Int J Oncol *35*, 57-68.

Franklin, R.J., and Ffrench-Constant, C. (2008). Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci *9*, 839-855.

Gierut, J.J., Jacks, T.E., and Haigis, K.M. (2014). Whole-mount X-Gal staining of mouse tissues. In Cold Spring Harbor protocols, pp. 417-419.

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., and Huber, T. (2013). Origin and differentiation of microglia. Front Cell Neurosci *7*, 45.

Goldman, L., and Albus, J.S. (1968). Computation of impulse conduction in myelinated fibers; theoretical basis of the velocity-diameter relation. Biophys J *8*, 596-607.

Grako, K.A., and Stallcup, W.B. (1995). Participation of the NG2 proteoglycan in rat aortic smooth muscle cell responses to platelet-derived growth factor. Exp Cell Res *221*, 231-240.

Guichet, P.O., Guelfi, S., Teigell, M., Hoppe, L., Bakalara, N., Bauchet, L., Duffau, H., Lamszus, K., Rothhut, B., and Hugnot, J.P. (2015). Notch1 stimulation induces a vascularization switch with pericyte-like cell differentiation of glioblastoma stem cells. Stem Cells *33*, 21-34.

Hardy, R.J., and Friedrich, V.L., Jr. (1996). Oligodendrocyte progenitors are generated throughout the embryonic mouse brain, but differentiate in restricted foci. Development *122*, 2059-2069.

Hart, I.K., Richardson, W.D., Heldin, C.H., Westermark, B., and Raff, M.C. (1989). PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. Development *105*, 595-603.

Hartfuss, E., Galli, R., Heins, N., and Gotz, M. (2001). Characterization of CNS precursor subtypes and radial glia. Dev Biol *229*, 15-30.

-REFERENCES-

Hildebrand, C., and Hahn, R. (1978). Relation between myelin sheath thickness and axon size in spinal cord white matter of some vertebrate species. J Neurol Sci *38*, 421-434.

Hill, R.A., and Nishiyama, A. (2014). NG2 cells (polydendrocytes): listeners to the neural network with diverse properties. Glia *62*, 1195-1210.

Hill, R.A., Patel, K.D., Goncalves, C.M., Grutzendler, J., and Nishiyama, A. (2014). Modulation of oligodendrocyte generation during a critical temporal window after NG2 cell division. Nat Neurosci *17*, 1518-1527.

Hoeck, J.D., Jandke, A., Blake, S.M., Nye, E., Spencer-Dene, B., Brandner, S., and Behrens, A. (2010). Fbw7 controls neural stem cell differentiation and progenitor apoptosis via Notch and c-Jun. Nat Neurosci *13*, 1365-1372.

Hsieh, J., Aimone, J.B., Kaspar, B.K., Kuwabara, T., Nakashima, K., and Gage, F.H. (2004). IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J Cell Biol *164*, 111-122.

Huang, H., Zhao, X.F., Zheng, K., and Qiu, M. (2013). Regulation of the timing of oligodendrocyte differentiation: mechanisms and perspectives. Neurosci Bull *29*, 155-164.

Ikeda, Y., and Ikeda, M.A. (2015). Cyclin E marks quiescent neural stem cells and caspase-3-positive newborn cells during adult hippocampal neurogenesis in mice. Neurosci Lett *607*, 90-96.

Ito, D., Tanaka, K., Suzuki, S., Dembo, T., and Fukuuchi, Y. (2001). Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke *32*, 1208-1215.

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., and Zecevic, N. (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. Front Neuroanat *3*, 5.

Jessen, K.R., and Mirsky, R. (1980). Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature *286*, 736-737.

-REFERENCES-

Jin, J., Peng, Q., Hou, Z., Jiang, M., Wang, X., Langseth, A.J., Tao, M., Barker, P.B., Mori, S., Bergles, D.E.*, et al.* (2015). Early white matter abnormalities, progressive brain pathology and motor deficits in a novel knock-in mouse model of Huntington's disease. Hum Mol Genet *24*, 2508-2527.

Jonsson, H., and Peng, S.L. (2005). Forkhead transcription factors in immunology. Cell Mol Life Sci *62*, 397-409.

Kadowaki, T., Nakadate, K., Sakakibara, S., Hirata, K., and Ueda, S. (2007). Expression of Iba1 protein in microglial cells of zitter mutant rat. Neurosci Lett *411*, 26-31.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nat Protoc *1*, 2406-2415.

Kaestner, K.H., Bleckmann, S.C., Monaghan, A.P., Schlondorff, J., Mincheva, A., Lichter, P., and Schutz, G. (1996). Clustered arrangement of winged helix genes fkh-6 and MFH-1: possible implications for mesoderm development. Development *122*, 1751-1758.

Kaestner, K.H., Lee, K.H., Schlondorff, J., Hiemisch, H., Monaghan, A.P., and Schutz, G. (1993). Six members of the mouse forkhead gene family are developmentally regulated. Proc Natl Acad Sci U S A *90*, 7628-7631.

Karadedou, C.T., Gomes, A.R., Chen, J., Petkovic, M., Ho, K.K., Zwolinska, A.K., Feltes, A., Wong, S.Y., Chan, K.Y., Cheung, Y.N.*, et al.* (2012). FOXO3a represses VEGF expression through FOXM1-dependent and -independent mechanisms in breast cancer. Oncogene *31*, 1845-1858.

Kaufmann, E., and Knochel, W. (1996). Five years on the wings of fork head. Mech Dev *57*, 3-20.

Keough, M.B., and Yong, V.W. (2013). Remyelination therapy for multiple sclerosis. Neurotherapeutics *10*, 44-54.

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

Kessaris, N., Pringle, N., and Richardson, W.D. (2008). Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. Philos Trans R Soc Lond B Biol Sci *363*, 71-85.

Kim, H., Shin, J., Kim, S., Poling, J., Park, H.C., and Appel, B. (2008). Notch-regulated oligodendrocyte specification from radial glia in the spinal cord of zebrafish embryos. Dev Dyn *237*, 2081-2089.

Kipp, M. (2016). Remyelination strategies in multiple sclerosis: a critical reflection. Expert Rev Neurother *16*, 1-3.

Kitamura, T., Kitamura, Y.I., Funahashi, Y., Shawber, C.J., Castrillon, D.H., Kollipara, R., DePinho, R.A., Kitajewski, J., and Accili, D. (2007). A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. J Clin Invest *117*, 2477-2485.

Kleopa, K.A., Orthmann, J.L., Enriquez, A., Paul, D.L., and Scherer, S.S. (2004). Unique distributions of the gap junction proteins connexin29, connexin32, and connexin47 in oligodendrocytes. Glia *47*, 346-357.

Kloetzli, J.M., Fontaine-Glover, I.A., Brown, E.R., Kuo, M., and Labosky, P.A. (2001). The winged helix gene, Foxb1, controls development of mammary glands and regions of the CNS that regulate the milk-ejection reflex. Genesis *29*, 60-71.

Kohelet, D., Shochat, R., Lusky, A., and Reichman, B. (2006). Risk factors for seizures in very low birthweight infants with periventricular leukomalacia. J Child Neurol *21*, 965-970.

Konopaske, G.T., Dorph-Petersen, K.A., Sweet, R.A., Pierri, J.N., Zhang, W., Sampson, A.R., and Lewis, D.A. (2008). Effect of chronic antipsychotic exposure on astrocyte and oligodendrocyte numbers in macaque monkeys. Biol Psychiatry *63*, 759-765.

Kucharova, K., and Stallcup, W.B. (2010). The NG2 proteoglycan promotes oligodendrocyte progenitor proliferation and developmental myelination. Neuroscience *166*, 185-194.

-REFERENCES-

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

Kuspert, M., and Wegner, M. (2015). SomethiNG 2 talk about-Transcriptional regulation in embryonic and adult oligodendrocyte precursors. Brain Res.

Kutzelnigg, A., and Lassmann, H. (2014). Pathology of multiple sclerosis and related inflammatory demyelinating diseases. Handb Clin Neurol *122*, 15-58.

Labosky, P.A., Winnier, G.E., Jetton, T.L., Hargett, L., Ryan, A.K., Rosenfeld, M.G., Parlow, A.F., and Hogan, B.L. (1997). The winged helix gene, Mf3, is required for normal development of the diencephalon and midbrain, postnatal growth and the milk-ejection reflex. Development *124*, 1263-1274.

Lam, E.W., Brosens, J.J., Gomes, A.R., and Koo, C.Y. (2013). Forkhead box proteins: tuning forks for transcriptional harmony. Nat Rev Cancer *13*, 482-495.

Lasiene, J., Shupe, L., Perlmutter, S., and Horner, P. (2008). No evidence for chronic demyelination in spared axons after spinal cord injury in a mouse. J Neurosci *28*, 3887-3896.

Lehmann, O.J., Sowden, J.C., Carlsson, P., Jordan, T., and Bhattacharya, S.S. (2003). Fox's in development and disease. Trends Genet *19*, 339-344.

Li, H., and Richardson, W.D. (2015). Evolution of the CNS myelin gene regulatory program. Brain Res.

Ligon, K.L., Fancy, S.P., Franklin, R.J., and Rowitch, D.H. (2006). Olig gene function in CNS development and disease. Glia *54*, 1-10.

Little, G.J., and Heath, J.W. (1994). Morphometric analysis of axons myelinated during adult life in the mouse superior cervical ganglion. J Anat *184 (Pt 2)*, 387-398.

Liu, X.B., and Schumann, C.M. (2014). Optimization of electron microscopy for human brains with long-term fixation and fixed-frozen sections. Acta neuropathologica communications *2*, 42.

-REFERENCES-

Lobe, C.G., Koop, K.E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. Dev Biol *208*, 281-292.

Lolait, S.J., Harmer, J.H., Auteri, G., Pedersen, J.S., and Toh, B.H. (1983). Expression of glial fibrillary acidic protein, actin, fibronectin and factor VIII antigen in human astrocytomas. Pathology *15*, 373-378.

Lourenco, T., Paes de Faria, J., Bippes, C.A., Maia, J., Lopes-da-Silva, J.A., Relvas, J.B., and Graos, M. (2016). Modulation of oligodendrocyte differentiation and maturation by combined biochemical and mechanical cues. Sci Rep *6*, 21563.

Lu, Q.R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C.D., and Rowitch, D.H. (2002). Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell *109*, 75-86.

Ludwin, S.K., and Maitland, M. (1984). Long-term remyelination fails to reconstitute normal thickness of central myelin sheaths. J Neurol Sci *64*, 193-198.

Magavi, S.S., Leavitt, B.R., and Macklis, J.D. (2000). Induction of neurogenesis in the neocortex of adult mice. Nature *405*, 951-955.

Malatesta, P., Hack, M.A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., and Gotz, M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. Neuron *37*, 751-764.

Martenson, R.E., Deibler, G.E., and Kies, M.W. (1969). Extraction of rat myelin basic protein free of other basic proteins of whole central nervous system tissue. An analysis of its electrophoretic heterogeneity. J Biol Chem *244*, 4268-4272.

McGovern, U.B., Francis, R.E., Peck, B., Guest, S.K., Wang, J., Myatt, S.S., Krol, J., Kwok, J.M., Polychronis, A., Coombes, R.C.*, et al.* (2009). Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer. Mol Cancer Ther *8*, 582-591.

McTigue, D.M., and Tripathi, R.B. (2008). The life, death, and replacement of oligodendrocytes in the adult CNS. J Neurochem *107*, 1-19.

Meijer, D.H., Kane, M.F., Mehta, S., Liu, H., Harrington, E., Taylor, C.M., Stiles, C.D., and Rowitch, D.H. (2012). Separated at birth? The functional and molecular divergence of OLIG1 and OLIG2. Nat Rev Neurosci *13*, 819-831.

Mekki-Dauriac, S., Agius, E., Kan, P., and Cochard, P. (2002). Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord. Development *129*, 5117-5130.

Meyer, M., Gonzalez Deniselle, M.C., Garay, L., Sitruk-Ware, R., Guennoun, R., Schumacher, M., and De Nicola, A.F. (2015). The progesterone receptor agonist Nestorone holds back proinflammatory mediators and neuropathology in the wobbler mouse model of motoneuron degeneration. Neuroscience *308*, 51-63.

Miller, R.H. (2002). Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol *67*, 451-467.

Miller, R.H. (2005). Dorsally derived oligodendrocytes come of age. Neuron *45*, 1-3.

Miller, R.H., Dinsio, K., Wang, R., Geertman, R., Maier, C.E., and Hall, A.K. (2004). Patterning of spinal cord oligodendrocyte development by dorsally derived BMP4. J Neurosci Res *76*, 9-19.

Miller, R.H., and Ono, K. (1998). Morphological analysis of the early stages of oligodendrocyte development in the vertebrate central nervous system. Microsc Res Tech *41*, 441-453.

Mitew, S., Hay, C.M., Peckham, H., Xiao, J., Koenning, M., and Emery, B. (2014). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. Neuroscience *276*, 29-47.

Mo, Z., and Zecevic, N. (2009). Human fetal radial glia cells generate oligodendrocytes in vitro. Glia *57*, 490-498.

Morita, K., Sasaki, H., Fujimoto, K., Furuse, M., and Tsukita, S. (1999). Claudin-11/OSP-based tight junctions of myelin sheaths in brain and Sertoli cells in testis. J Cell Biol *145*, 579-588.

Motomura, K., Natsume, A., Watanabe, R., Ito, I., Kato, Y., Momota, H., Nishikawa, R., Mishima, K., Nakasu, Y., Abe, T.*, et al.* (2012). Immunohistochemical analysis-based

proteomic subclassification of newly diagnosed glioblastomas. Cancer Sci *103*, 1871-1879.

Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development *116*, 201-211.

Murtie, J.C., Zhou, Y.X., Le, T.Q., Vana, A.C., and Armstrong, R.C. (2005). PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. Neurobiol Dis *19*, 171-182.

Myatt, S.S., and Lam, E.W. (2007). The emerging roles of forkhead box (Fox) proteins in cancer. Nat Rev Cancer *7*, 847-859.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A *90*, 8424-8428.

Naruse, M., Nakahira, E., Miyata, T., Hitoshi, S., Ikenaka, K., and Bansal, R. (2006). Induction of oligodendrocyte progenitors in dorsal forebrain by intraventricular microinjection of FGF-2. Dev Biol *297*, 262-273.

Ness, J.K., Valentino, M., McIver, S.R., and Goldberg, M.P. (2005). Identification of oligodendrocytes in experimental disease models. Glia *50*, 321-328.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401-413.

Nishiyama, A. (2007). Polydendrocytes: NG2 cells with many roles in development and repair of the CNS. Neuroscientist *13*, 62-76.

Nishiyama, A., Komitova, M., Suzuki, R., and Zhu, X. (2009). Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat Rev Neurosci *10*, 9-22.

Nishiyama, A., Watanabe, M., Yang, Z., and Bu, J. (2002). Identity, distribution, and development of polydendrocytes: NG2-expressing glial cells. J Neurocytol *31*, 437-455.

-REFERENCES-

Noll, E., and Miller, R.H. (1993). Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord. Development *118*, 563-573.

Norton, W.H., Mangoli, M., Lele, Z., Pogoda, H.M., Diamond, B., Mercurio, S., Russell, C., Teraoka, H., Stickney, H.L., Rauch, G.J.*, et al.* (2005). Monorail/Foxa2 regulates floorplate differentiation and specification of oligodendrocytes, serotonergic raphe neurones and cranial motoneurones. Development *132*, 645-658.

Olivier, C., Cobos, I., Perez Villegas, E.M., Spassky, N., Zalc, B., Martinez, S., and Thomas, J.L. (2001). Monofocal origin of telencephalic oligodendrocytes in the anterior entopeduncular area of the chick embryo. Development *128*, 1757-1769.

Olofsson, I.A., Skov, L., and Miranda, M.J. (2015). [Demyelinating diseases in children with acute neurological symptoms]. Ugeskr Laeger *177*.

Ono, K., Fujisawa, H., Hirano, S., Norita, M., Tsumori, T., and Yasui, Y. (1997). Early development of the oligodendrocyte in the embryonic chick metencephalon. J Neurosci Res *48*, 212-225.

Orentas, D.M., and Miller, R.H. (1996a). A novel form of migration of glial precursors. Glia *16*, 27-39.

Orentas, D.M., and Miller, R.H. (1996b). The origin of spinal cord oligodendrocytes is dependent on local influences from the notochord. Dev Biol *177*, 43-53.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R.*, et al.* (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309-323.

Parras, C.M., Hunt, C., Sugimori, M., Nakafuku, M., Rowitch, D., and Guillemot, F. (2007). The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. J Neurosci *27*, 4233-4242.

Patel, J.R., and Klein, R.S. (2011). Mediators of oligodendrocyte differentiation during remyelination. FEBS Lett *585*, 3730-3737.

Paus, T., and Toro, R. (2009). Could Sex Differences in White Matter be Explained by g ratio? Front Neuroanat *3*, 14.

Perez-Cerda, F., Sanchez-Gomez, M.V., and Matute, C. (2015). Pio del Rio Hortega and the discovery of the oligodendrocytes. Front Neuroanat *9*, 92.

Perez Villegas, E.M., Olivier, C., Spassky, N., Poncet, C., Cochard, P., Zalc, B., Thomas, J.L., and Martinez, S. (1999). Early specification of oligodendrocytes in the chick embryonic brain. Dev Biol *216*, 98-113.

Petryniak, M.A., Potter, G.B., Rowitch, D.H., and Rubenstein, J.L. (2007). Dlx1 and Dlx2 control neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. Neuron *55*, 417-433.

Poduslo, J.F., and Braun, P.E. (1975). Topographical arrangement of membrane proteins in the intact myelin sheath. Lactoperoxidase incorproation of iodine into myelin surface proteins. J Biol Chem *250*, 1099-1105.

Poncet, C., Soula, C., Trousse, F., Kan, P., Hirsinger, E., Pourquie, O., Duprat, A.M., and Cochard, P. (1996). Induction of oligodendrocyte progenitors in the trunk neural tube by ventralizing signals: effects of notochord and floor plate grafts, and of sonic hedgehog. Mech Dev *60*, 13-32.

Pouly, S., Prat, A., Blain, M., Olivier, A., and Antel, J. (2001). NG2 immunoreactivity on human brain endothelial cells. Acta Neuropathol *102*, 313-320.

Pringle, N., Collarini, E.J., Mosley, M.J., Heldin, C.H., Westermark, B., and Richardson, W.D. (1989). PDGF A chain homodimers drive proliferation of bipotential (O-2A) glial progenitor cells in the developing rat optic nerve. EMBO J *8*, 1049-1056.

Pringle, N.P., Mudhar, H.S., Collarini, E.J., and Richardson, W.D. (1992). PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development *115*, 535-551.

Pringle, N.P., Yu, W.P., Guthrie, S., Roelink, H., Lumsden, A., Peterson, A.C., and Richardson, W.D. (1996). Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog. Dev Biol *177*, 30-42.

-REFERENCES-

Psachoulia, K., Jamen, F., Young, K.M., and Richardson, W.D. (2009). Cell cycle dynamics of NG2 cells in the postnatal and ageing brain. Neuron Glia Biol *5*, 57-67.

Radyushkin, K., Anokhin, K., Meyer, B.I., Jiang, Q., Alvarez-Bolado, G., and Gruss, P. (2005). Genetic ablation of the mammillary bodies in the Foxb1 mutant mouse leads to selective deficit of spatial working memory. Eur J Neurosci *21*, 219-229.

Rafalski, V.A., Ho, P.P., Brett, J.O., Ucar, D., Dugas, J.C., Pollina, E.A., Chow, L.M., Ibrahim, A., Baker, S.J., Barres, B.A.*, et al.* (2013). Expansion of oligodendrocyte progenitor cells following SIRT1 inactivation in the adult brain. Nat Cell Biol *15*, 614-624.

Raff, M.C., Mirsky, R., Fields, K.L., Lisak, R.P., Dorfman, S.H., Silberberg, D.H., Gregson, N.A., Leibowitz, S., and Kennedy, M.C. (1978). Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature *274*, 813-816.

Ranscht, B., Clapshaw, P.A., Price, J., Noble, M., and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc Natl Acad Sci U S A *79*, 2709-2713.

Rapport, M.M., Graf, L., Autilio, L.A., and Norton, W.T. (1964). Immunochemical Studies of Organ and Tumour Lipids. Xiv. Galactocerebroside Determinants in the Myelin Sheath of the Central Nervous System. J Neurochem *11*, 855-864.

Redwine, J.M., and Armstrong, R.C. (1998). In vivo proliferation of oligodendrocyte progenitors expressing PDGFalphaR during early remyelination. J Neurobiol *37*, 413-428.

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

Rivers, L.E., Young, K.M., Rizzi, M., Jamen, F., Psachoulia, K., Wade, A., Kessaris, N., and Richardson, W.D. (2008). PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat Neurosci *11*, 1392-1401.

Ross, S.E., Greenberg, M.E., and Stiles, C.D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13-25.

Rovainen, C.M. (1985). Respiratory bursts at the midline of the rostral medulla of the lamprey. J Comp Physiol A *157*, 303-309.

Rowitch, D.H. (2004). Glial specification in the vertebrate neural tube. Nat Rev Neurosci *5*, 409-419.

Rowitch, D.H., and Kriegstein, A.R. (2010). Developmental genetics of vertebrate glial-cell specification. Nature *468*, 214-222.

Rushton, W.A. (1951). A theory of the effects of fibre size in medullated nerve. J Physiol *115*, 101-122.

Sakry, D., and Trotter, J. (2015). The role of the NG2 proteoglycan in OPC and CNS network function. Brain Res.

Sarnat, H.B., Nochlin, D., and Born, D.E. (1998). Neuronal nuclear antigen (NeuN): a marker of neuronal maturation in early human fetal nervous system. Brain Dev *20*, 88-94.

Scheller, N.M., Svanstrom, H., Pasternak, B., Arnheim-Dahlstrom, L., Sundstrom, K., Fink, K., and Hviid, A. (2015). Quadrivalent HPV vaccination and risk of multiple sclerosis and other demyelinating diseases of the central nervous system. JAMA *313*, 54-61.

Schonrock, L.M., Kuhlmann, T., Adler, S., Bitsch, A., and Bruck, W. (1998). Identification of glial cell proliferation in early multiple sclerosis lesions. Neuropathol Appl Neurobiol *24*, 320-330.

Shapiro, L.A., Perez, Z.D., Foresti, M.L., Arisi, G.M., and Ribak, C.E. (2009). Morphological and ultrastructural features of Iba1-immunolabeled microglial cells in the hippocampal dentate gyrus. Brain Res *1266*, 29-36.

Silbereis, J.C., Nobuta, H., Tsai, H.H., Heine, V.M., McKinsey, G.L., Meijer, D.H., Howard, M.A., Petryniak, M.A., Potter, G.B., Alberta, J.A.*, et al.* (2014). Olig1 function is required to repress dlx1/2 and interneuron production in Mammalian brain. Neuron *81*, 574-587.

-REFERENCES-

Sloane, J.A., Batt, C., Ma, Y., Harris, Z.M., Trapp, B., and Vartanian, T. (2010). Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. Proc Natl Acad Sci U S A *107*, 11555-11560.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nature genetics *21*, 70-71.

Steiner, J., Martins-de-Souza, D., Schiltz, K., Sarnyai, Z., Westphal, S., Isermann, B., Dobrowolny, H., Turck, C.W., Bogerts, B., Bernstein, H.G.*, et al.* (2014). Clozapine promotes glycolysis and myelin lipid synthesis in cultured oligodendrocytes. Front Cell Neurosci *8*, 384.

Stidworthy, M.F., Genoud, S., Suter, U., Mantei, N., and Franklin, R.J. (2003). Quantifying the early stages of remyelination following cuprizone-induced demyelination. Brain Pathol *13*, 329-339.

Swaiman, K.F. (2012). Pediatric neurology : principles & practice, 5th edn (Philadelphia, Elsevier/Saunders).

Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O., and Ikenaka, K. (2002). The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr Biol *12*, 1157-1163.

Tassetto, M., and Gao, F.B. (2006). Transcriptional control of dendritic patterning in Drosophila neurons. Genome Biol *7*, 225.

Tekki-Kessaris, N., Woodruff, R., Hall, A.C., Gaffield, W., Kimura, S., Stiles, C.D., Rowitch, D.H., and Richardson, W.D. (2001). Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. Development *128*, 2545-2554.

Tripathi, R.B., Clarke, L.E., Burzomato, V., Kessaris, N., Anderson, P.N., Attwell, D., and Richardson, W.D. (2011). Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts. J Neurosci *31*, 6809-6819.

Ueno, T., Ito, J., Hoshikawa, S., Ohori, Y., Fujiwara, S., Yamamoto, S., Ohtsuka, T., Kageyama, R., Akai, M., Nakamura, K.*, et al.* (2012). The identification of transcriptional targets of Ascl1 in oligodendrocyte development. Glia *60*, 1495-1505.

-REFERENCES-

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

van Loosdregt, J., Brunen, D., Fleskens, V., Pals, C.E., Lam, E.W., and Coffer, P.J. (2011). Rapid temporal control of Foxp3 protein degradation by sirtuin-1. PLoS One *6*, e19047.

van Loosdregt, J., and Coffer, P.J. (2014). Post-translational modification networks regulating FOXP3 function. Trends Immunol *35*, 368-378.

Vana, A.C., Lucchinetti, C.F., Le, T.Q., and Armstrong, R.C. (2007). Myelin transcription factor 1 (Myt1) expression in demyelinated lesions of rodent and human CNS. Glia *55*, 687-697.

Vanella, L., Raciti, G., Barbagallo, I., Bonfanti, R., Abraham, N., and Campisi, A. (2015). Tissue transglutaminase expression during neural differentiation of human mesenchymal stem cells. CNS Neurol Disord Drug Targets *14*, 24-32.

von Budingen, H.C., Mei, F., Greenfield, A., Jahn, S., Shen, Y.A., Reid, H.H., McKemy, D.D., and Chan, J.R. (2015). The myelin oligodendrocyte glycoprotein directly binds nerve growth factor to modulate central axon circuitry. J Cell Biol *210*, 891-898.

Wehr, R., Mansouri, A., de Maeyer, T., and Gruss, P. (1997). Fkh5-deficient mice show dysgenesis in the caudal midbrain and hypothalamic mammillary body. Development *124*, 4447-4456.

Weigel, D., and Jackle, H. (1990). The fork head domain: a novel DNA binding motif of eukaryotic transcription factors? Cell *63*, 455-456.

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E., and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell *57*, 645-658.

Wilson, H.C., Scolding, N.J., and Raine, C.S. (2006). Co-expression of PDGF alpha receptor and NG2 by oligodendrocyte precursors in human CNS and multiple sclerosis lesions. J Neuroimmunol *176*, 162-173.

-REFERENCES-

Wolf, H.K., Buslei, R., Schmidt-Kastner, R., Schmidt-Kastner, P.K., Pietsch, T., Wiestler, O.D., and Blumcke, I. (1996). NeuN: a useful neuronal marker for diagnostic histopathology. J Histochem Cytochem *44*, 1167-1171.

Woodruff, R.H., Tekki-Kessaris, N., Stiles, C.D., Rowitch, D.H., and Richardson, W.D. (2001). Oligodendrocyte development in the spinal cord and telencephalon: common themes and new perspectives. Int J Dev Neurosci *19*, 379-385.

Yeung, M.S., Zdunek, S., Bergmann, O., Bernard, S., Salehpour, M., Alkass, K., Perl, S., Tisdale, J., Possnert, G., Brundin, L.*, et al.* (2014). Dynamics of oligodendrocyte generation and myelination in the human brain. Cell *159*, 766-774.

Young, K.M., Psachoulia, K., Tripathi, R.B., Dunn, S.J., Cossell, L., Attwell, D., Tohyama, K., and Richardson, W.D. (2013). Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. Neuron *77*, 873-885.

Yu, Y., Chen, Y., Kim, B., Wang, H., Zhao, C., He, X., Liu, L., Liu, W., Wu, L.M., Mao, M.*, et al.* (2013). Olig2 targets chromatin remodelers to enhancers to initiate oligodendrocyte differentiation. Cell *152*, 248-261.

Yuelling, L.W., Waggener, C.T., Afshari, F.S., Lister, J.A., and Fuss, B. (2012). Autotaxin/ENPP2 regulates oligodendrocyte differentiation in vivo in the developing zebrafish hindbrain. Glia *60*, 1605-1618.

Zannino, D.A., and Appel, B. (2009). Olig2+ precursors produce abducens motor neurons and oligodendrocytes in the zebrafish hindbrain. J Neurosci *29*, 2322-2333.

Zhang, N., Wei, P., Gong, A., Chiu, W.T., Lee, H.T., Colman, H., Huang, H., Xue, J., Liu, M., Wang, Y.*, et al.* (2011). FoxM1 promotes beta-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer Cell *20*, 427-442.

Zhao, T., Szabo, N., Ma, J., Luo, L., Zhou, X., and Alvarez-Bolado, G. (2008). Genetic mapping of Foxb1-cell lineage shows migration from caudal diencephalon to telencephalon and lateral hypothalamus. Eur J Neurosci *28*, 1941-1955.

-REFERENCES-

Zhao, T., Zhou, X., Szabo, N., Leitges, M., and Alvarez-Bolado, G. (2007). Foxb1-driven Cre expression in somites and the neuroepithelium of diencephalon, brainstem, and spinal cord. Genesis *45*, 781-787.

Zhou, Q., and Anderson, D.J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell *109*, 61-73.

Zhou, Q., Wang, S., and Anderson, D.J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron *25*, 331-343.

Zhou, Y.X., Flint, N.C., Murtie, J.C., Le, T.Q., and Armstrong, R.C. (2006). Retroviral lineage analysis of fibroblast growth factor receptor signaling in FGF2 inhibition of oligodendrocyte progenitor differentiation. Glia *54*, 578-590.

Zhu, Q., Whittemore, S.R., Devries, W.H., Zhao, X., Kuypers, N.J., and Qiu, M. (2011). Dorsally-derived oligodendrocytes in the spinal cord contribute to axonal myelination during development and remyelination following focal demyelination. Glia *59*, 1612-1621.

Zoellner, H.F., and Hunter, N. (1989). Histochemical identification of the vascular endothelial isoenzyme of alkaline phosphatase. J Histochem Cytochem *37*, 1893-1898.

-PUBLICATIONS-

LIST OF PUBLICATIONS BY THE CANDIDATE

1. Haddad-Tóvolli R, Paul FA, **Zhang Yuanfeng**, Zhou X, Theil T, Puelles L, Blaess S and Alvarez-Bolado G, Differential requirements for Gli2 and Gli3 in the regional specification of the mouse hypothalamus. Front. Neuroanat. 2015. 9:34.

2. Michael Heide, **Yuanfeng Zhang**, Xunlei Zhou, Tianyu Zhao, Amaya Miquelajauregui, Alfredo Varela-Echavarria, Gonzalo Alvarez-Bolado, Lhx5 controls mamillary differentiation in the developing hypothalamus of the mouse. Front Neuroanat. 2015 Aug 14;9:113.

3. **Yuanfeng Zhang**, Gonzalo Alvarez-Bolado, Differential developmental strategies by Sonic hedgehog in thalamus and hypothalamus. J Chem Neuroanat. 2015 Dec 12. pii: S0891-0618(15)00098-8.

4. **Yuanfeng Zhang**, Elti Hoxha, Tianyu Zhao, Xunlei Zhou, Gonzalo Alvarez-Bolado, The role of transcription factor *Foxb1* Gene in Oligodendrocyte Development, Glia (2016 manuscript)

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