Molecular mechanisms underlying Mash1 function in oligodendrogenesis

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Declaration

This project has been completed in the laboratory of François Guillemot, in the Division of Molecular Neurobiology at the National Institute for Medical Research, London. I, Laura Galiñanes-García confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The design of an oligodendrocyte specific array to identify Mash1 bound segments in section 3.4.2 of the results, was work conducted as part of a collaboration with Diogo Castro at the National Institute for Medical Research, London. The gene expression data set from *Mash1* null embryos and the oligodendroglial specific gene list that were cross referenced with segments bound by Mash1 from ChIP-on-chip in this study, in section 3.5.2 of the results, were generated as part of a collaboration with Carlos Parras at CRICM, Paris. I was supported by Boehringer Ingelheim Fonds and the Medical Research Council.

Abstract

Members of the basic helix-loop-helix (bHLH) proneural family of proteins, including Mash1, are crucial transcription factors (TFs) in neurogenesis. More recently, a role for Mash1 in the specification of oligodendrocyte precursor cells (OPCs) has been demonstrated. Here we investigate the role of Mash1 in lineage commitment of neural progenitors and more specifically the mechanisms underlying Mash1 activity in oligodendroglial cell fate specification.

We use an *in vitro* cell culture system to perform Mash1 locational analysis. Mouse OPCs were cultured as oligospheres that expressed Mash1, a proportion of which also coexpressed the early OPC marker platelet-derived growth factor receptor α (PDGFRα) and oligodendrocyte promoting TFs including the bHLH TF Olig2 and the high mobility group (HMG) TF Sox9. We use a chromatin immunoprecipitation (ChIP)-on-chip strategy and found that Mash1 protein binds to proximal genomic regions of early OPC genes such as *Olig1* and *Sox8*, late oligodendrocyte genes including *myelin oligodendrocyte glycoprotein* (*Mog*) and *oligodendrocyte myelin glycoprotein* (*Omg*), and other genes of interest including *Brevican* (*Bcan*), *Notch1* and *Sulfatase1* (*Sulf1*). Mash1 also bound distal genomic regions of *Olig2* and *Sox9* in oligosphere cultures. To formulate a TF combinatorial code for the activation of these putative enhancers, TF synergy were analysed with luciferase reporter assays. Furthermore, to isolate genomic regions with activity in the oligodendroglial lineage *in vivo* we used mouse transient transgenics. We hypothesise that Mash1

interacts with either neuronal- or oligodendroglial-specific cofactors, and that these interactions modulate Mash1 activity. To address this question we performed Sox9 and Olig2 ChIP and found that some Mash1 bound elements were also occupied by these TFs in oligosphere cultures.

In conclusion, using an *in vitro* cellular system and ChIP-on-chip technology to interrogate proximal promoter regions bound by Mash1, we can begin to elucidate the molecular mechanisms of Mash1 function in oligodendroglial cell fate specification.

Acknowledgements

It is fair to say that this work would not have been completed without the support, advice and enthusiasm of a huge number of people. I would like to thank my supervisor François Guillemot for giving me the chance to undertake this project and for his support and guidance throughout the period of this work. I would also like to say a big thank you to present and past lab colleagues. Diogo Castro the ‘ChIP guru’ for all his expert advice, indeed the ChIP experiments would not have been possible without his input. Carlos Parras for his discussions and comments of the thesis and for sharing unpublished data. Hendrik Wildner for always managing to make me laugh. A particular massive thank you to Céline Zimmer whose constant support, discussions and most of all cheeky Friday cider kept me motivated throughout the course of this work.

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Abbreviations

7-AAD	7-Aminoactinomycin D
AEP	Anterior entopeduncular area
ash1	*Achaete-scute* homologue 1
Bcan	Brevican
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CAG	CMV immediate enhancer/β-actin
CAM	Cell adhesion molecule
CGE	Caudal ganglionic eminence
ChIA-PET	Chromatin Interaction Analysis using Paired End Tag
ChIP	Chromatin immunoprecipitation
CM	Conditioned media
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycan
DAPI	4’,6-Diamidino-2-phenylindole
Dll1	Deltalike-1
DMEM	Dulbecco’s Modified Eagle’s Medium
DV	Dorsoventral
E	Embryonic day
EGF	Epidermal growth factor
EM	Electron microscopy
ENCODE	ENCyclopedia Of DNA Elements

ES	Embryonic stem
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
β-gal	β–galactosidase
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCRMA	GeneChip Robust Multiarray Averaging
GFP	Green fluorescent protein
Gna12	Guanine nucleotide binding protein (G protein) alpha 12
GSA	Gene set analysis
HBSS	Hank's buffered salt solution
HDAC1	Histone deacetylase1
Hes	Hairy and enhancer of split
HMG	High mobility group
hnRNA	Heterogeneous nuclear RNA
hnRNP	Heterogeneous nuclear ribonucleoprotein
Hnrpdl1	Heterogeneous nuclear ribonucleoprotein D-like
HSPGs	Heparan sulfate proteoglycan
Id	Inhibitor of differentiation
Insm1	Insulinoma-associated 1, IA-1
IP	Immunoprecipitation
LB	Luria-Bertani
Lfng	Lunatic fringe
LGE	Lateral ganglionic eminence

MBP	Myelin basic protein
MGE	Medial ganglionic eminence
Mog	Myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
Nfasc	Neurofascin
Ngn	Neurogenin
NICD	Notch intracellular domain
NPM	Neurosphere proliferation medium
NSC	Neural stem cell
O-2A	Oligodendrocyte-type-2 astrocyte
OCT	Optimal cutting temperature
Omg	Oligodendrocyte myelin glycoprotein
OPC	Oligodendrocyte precursor cell
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFRα	Platelet-derived growth factor receptor alpha
PFA	Paraformaldehyde
PI	Protease Inhibitor
PLP	Proteolipid protein
pMN	Motorneuron progenitor domain
PNS	Peripheral nervous system
PSANCAM	Polysialylated neural cell adhesion molecule
Ptc1	Patched
QC	Quality control

RLU	Relative luciferase units
RT	Reverse transcriptase
SAM	Significance Analysis of Microarray
SDS	Sodium dodecyl sulfate
Shh	Sonic hedgehog
Smo	Smoothened
Sox	SRY related HMG box
Sulf1	Sulfatase 1
SVZ	Subventricular zone
T3	Thyroid hormone
TAE	Tris-acetate- ethylenediaminetetraacetic acid EDTA
TAT	Transcriptional activator protein
TF	Transcription Factor
TFBS	Transcription Factor Binding Site
TSS	Transcriptional start site
VZ	Ventricular zone
WCE	Whole cell extract
WGA	Whole Genome Amplification
YFP	Yellow fluorescent protein

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Chapter 1

Introduction

1.1 A brief historical and evolutionary perspective

At the beginning of the 20th century in 1921, Pio del Rio Hortega provided the first detailed histological description of oligodendrocytes. Rio Hortega created the word 'oligodendrocyte', from the Greek -oligo- for few, -dendro- for tree, and -cyte- for cell, specifically to describe a cell with less processes compared to other cells of the central nervous system (CNS). A classification system for oligodendrocytes based on their location was devised and consisted of three main subgroups, interfascicular (along axonal tracts), perineural satellite (around neuronal cell bodies), and perivascular (in close proximity to capillaries). Rio Hortega hypothesised that oligodendrocytes played a major role in the generation and maintenance of the myelin sheath. Indeed, electron microscopy (EM) and immunocytochemistry studies have since provided evidence in support of this theory (Bunge et al., 1962; Hirano, 1968; Mori and Leblond, 1970; Ling et al, 1973; Sternberger et al., 1978). Oligodendrocytes mainly function to generate rapid conduction of action potentials in a non-linear manner between interruptions in the insulating myelin membrane that ensheaths the axons of neurons. Prior to the description of oligodendrocytes in 1858 Rudolf Virchow coined the word myelin derived from the Greek myelos, which means marrow. Louis-Antoine Ranvier later described the breaks between sections of myelin as "nodes of Ranvier", in 1878.

The acquisition of the myelin sheath and thus the myelin-synthesising cells of the peripheral nervous system (PNS) and CNS, Schwann cells and oligodendrocytes respectively, have undoubtedly played a crucial role in

vertebrate evolution (reviewed Zalc, 2006; Zalc et al., 2008). Notably, despite being ensheathed by glial cells invertebrate axons are not insulated with compact myelin, and subsequently are only capable of generating action potential that propagate at a mere 1m/s. However, this speed is ample for the survival of small animals in the size range of 0.1-30cm. Invertebrates with a larger body size, such as squids, cuttlefishes and octopods that belong to the Cephalopod class, have adapted by increasing their axonal diameter to 1 mm, and consequently increased the speed at which their action potentials are propagated in order to survive. In contrast, vertebrates have responded to this challenge in the form of the myelin sheath. Notably, this structure facilitates the propagation of action potentials to reach an incredible velocity of 50-100m/s, without increasing the diameter of their axons. However, the ancestral vertebrate axons of lancelets, hagfishes and lampreys that belong to the Agnatha (in Greek, no jaw) class are not myelinated (Bullock et al., 1984). The most ancient living myelinated vertebrate species are the Chondrichthyes or cartilaginous-jawed fish, which include sharks and rays (Kitagawa et al., 1993); it is therefore considered that the acquisition of myelin and a hinged-jaw occurred simultaneously in evolution (Zalc & Coleman, 2000).

The history behind the discovery of oligodendrocytes in the CNS and the great evolutionary feat of myelin acquisition in vertebrates are fascinating. However, our current understandings of the molecular mechanisms that control the generation of oligodendrocytes from neural progenitors in the neuroepithelium of the vertebrate CNS are still in its infancy.

1.2 Oligodendrocytes in disease: Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS that was first described in 1868 by Jean-Martin Charcot. MS has three major classifications: 1) relapsing and remitting, which is exemplified by episodes of neurological dysfunction combined with phases of stability; 2) primary-progressive, in which progressive neurological disability occurs from the beginning; 3) and secondary-progressive, whereby progressive neurological disability arises later in the course of the disease. Axonal loss, a prominent feature of MS not only results from inflammation caused during periods of this disease (Kutzelnigg et al., 2005), but potentially also from neurodegeneration due to a lack of trophic support (Bjartmar et al., 2003). Notably, the progressive loss of axons in MS patients, are a major contributor to the accumulation of irreversible neurological defects associated with this disorder (Trapp and Nave, 2008). The prevalence of MS varies with racial background and geographical location. Indeed, with a prevalence of 1/800, MS is the most common cause of neurological disability in young white adult populations in Europe and North America. Although there are no cures presently for this demyelinating disease, numerous treatments are available to help relieve the symptoms and relapses, as well as slow down the progression of MS (reviewed Nicholas and Chataway, 2007).

Following the pathological loss of myelin in diseases like MS, new myelin sheaths are generated around demyelinated axons of the adult nervous system, a phenomenon that is otherwise referred to as remyelination (reviewed

Franklin and Ffrench-Constant, 2008). The process of remyelination can be partitioned into two core phases, firstly the colonisation of the lesion by OPCs, and secondly the differentiation and maturation of these cells to form functional myelinating oligodendrocytes. Mature oligodendrocytes contact proximal demyelinated axons and generate myelin sheaths, to reinstate rapid propagation of action potential by saltatory conduction (Smith et al., 1979) and rectify neurological deficits (Jeffery and Blakemore, 1997; Liebetanz and Merkler, 2006). Although the vast majority of MS lesions experience remyelination, in more cases than not this process does not restore the myelin sheath completely (Blakemore, 1974), and ultimately results in failure (Ludwin and Maitland, 1984). Failure in remyelination may arise from three principal causes: 1) a deficit in OPC numbers; 2) a defect in the recruitment of OPCs; 3) a defect in the differentiation and maturation of OPCs into myelinating oligodendrocytes (Franklin, 2002).

Presently, there are no corrective therapies in the clinic to actively promote remyelination in the adult nervous system of MS afflicted patients. Therapeutic strategies under investigation in animal models of demyelination include exogenous cell replacement therapies using transplantation, and promotion of endogenous repair with autologous stem and OPC populations. Pioneering experimental studies instigated almost 30 years ago, clearly highlighted the ability of glial cells to myelinate following transplantation into the CNS of demyelination disease rodent models (Duncan et al., 1981; Lachapelle et al., 1983; Blakemore and Crang, 1985). Since then, numerous studies have exploited these methods to introduce a wide range of cell types to

enhance remyelination, these include, primary OPCs (Groves et al., 1993; Zhang et al., 1999; Windrem et al., 2004), Schwann cells (Blakemore and Crang, 1985; Honmou et al., 1996; Bachelin et al., 2005), olfactory ensheathing cells (Imaizumi et al., 1998; Barnett et al., 2000), neural stem cell (NSC) lines (Hammang et al., 1997) and embryonic stem (ES) cell derived glial precursors (Brustle et al., 1999). Nevertheless, there are crucial issues, including the source of cells and the mode of delivery that need to be addressed if these approaches are to be translated into the clinic. Briefly, ES cells offer a number advantages, not only can they provide a limitless supply of OPCs, but individual patient tailored treatment can be delivered. Nonetheless, the *in vitro* differentiation of human ES cells into oligodendrocytes is arduous, requiring an extended period of time and define culture conditions (Nistor et al., 2005).

1.3 Regionalisation and patterning of the ventral telencephalon

The cerebrum is a complex region of the vertebrate CNS, and is derived from the embryonic structure, the telencephalon. The telencephalon commences as a relatively simple neuroepithelium at the most anterior part of the neural plate. In brief, the neural plate forms in the early embryo following neural induction, and ultimately gives rise to the nervous system. Indeed, following these early patterning events, the embryonic telencephalon can be grossly partitioned into the ventral subpallium, which gives rise to the mammalian basal ganglia, and the dorsal pallium, which gives rise to the mammalian cerebral cortex (Figure 1).

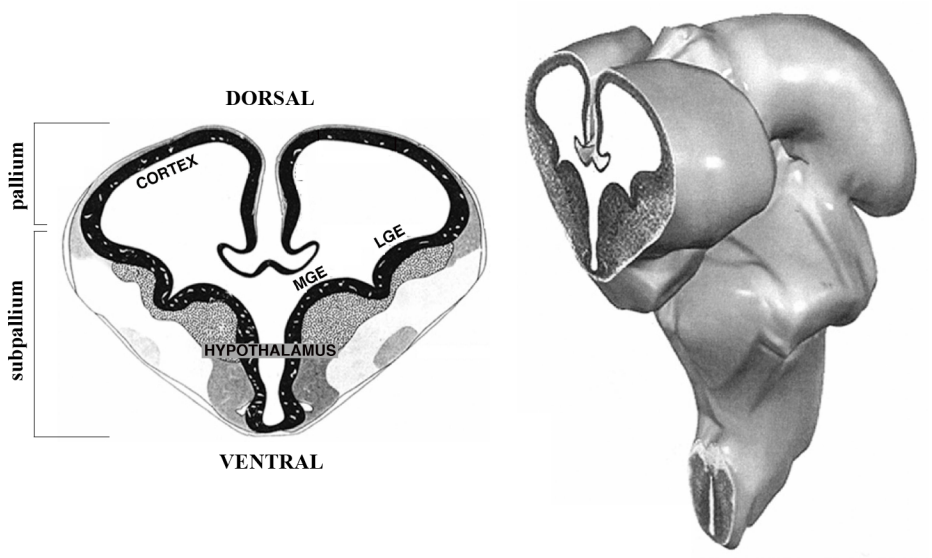


Figure 1. The developing rodent telencephalon. Coronal section through the rodent telencephalon at E12.5. The pallium or dorsal telencephalon develops into the cortex, whilst the subpallium or ventral telencephalon consisting of the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE), generates the striatum and pallidum, respectively. (Altman and Bayer, 1995).

The ventral telencephalon comprises a pair of discrete progenitor domains, the lateral (LGE) and medial (MGE) ganglionic eminences, which form the striatum and pallidum, respectively (Puelles et al., 2000). On the other hand the neocortex the principal element of the dorsal telencephalon, mainly consists of excitatory glutamatergic neurons (which originate from the cortical ventricular zone (VZ), an area bordering the ventricles of the developing cortex) and GABA (γ-aminobutyric acid)-ergic inhibitory interneurons (generated in the VZ of the ganglionic eminences of the ventral telencephalon and subsequently migrating dorsally into the cortex).

In the developing telencephalon, a dorsal source of Wnt and bone morphogenetic protein (BMP), and a ventral supply of Sonic hedgehog (Shh) morphogenetic signals specify positional identity along the dorsoventral (DV) axis, analogous to the scenario in the embryonic spinal cord (Figure 2) (Ulloa and Briscoe, 2007; reviewed Hoch et al., 2009; Lupo et al., 2006; Ciani and Salinas, 2005; Liu and Niswander, 2005; Fuccillo et al., 2006). BMP and Wnt molecules are released from the dorsal midline and paramedial neuroectoderm, whilst ventral regions are responsible for the generation of Shh signal, from the anterior mesendoderm, the ventral hypothalamus and from the rostroventral telencephalon (preoptic area and MGE). Notably, positional identity along the DV axis of the forebrain is highly intricate and is dependent on the interactions and crossregulation between other rostral patterning signals including fibroblast growth factors (Fgfs) (Mason, 2007).

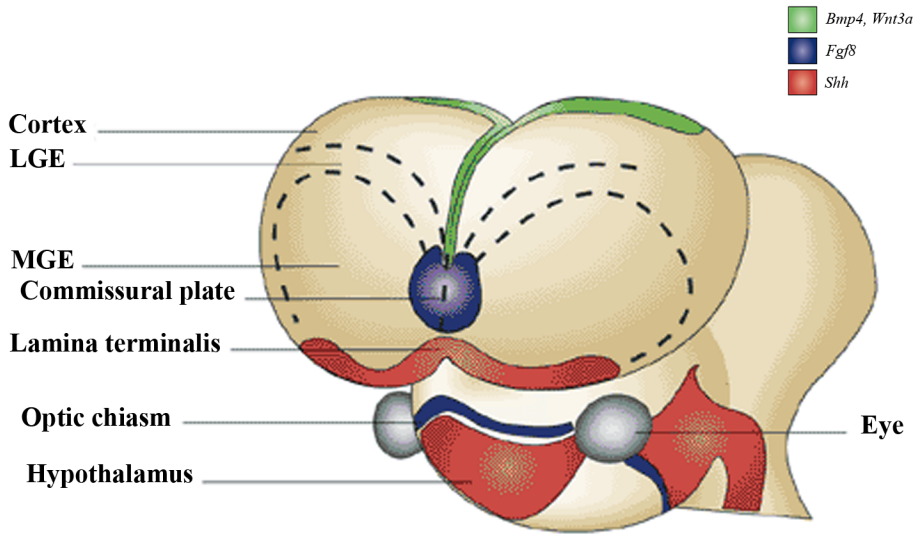


Figure 2. Signalling centres of the deve loping mouse telencephalon. Schematic representation of the mouse brain at mid-gestation with the signalling centres of the telencephalon. Patterning molecules that regulate the regionalisation of the telencephalon during early development, include bone morphogenetic proteins (Bmp4; green) and members of the wingless-type MMTV integration site family (Wnt3a; green), fibroblast growth factors (Fgf8; blue) and sonic hedgehog, (Shh; red) (Marin and Rubenstein, 2001).

Expression of the homeobox TFs Pax6 and Gsh2 in the telencephalon are spatially restricted (reviewed in Hebert and Fishell, 2008; Schuurmans and Guillemot, 2002). Indeed, their domains of expression border the pallial-subpallial boundary in the lateral pallium and dorsal LGE, respectively, and their mutual antagonism is required for its positioning (Yun et al., 2001). These TFs are responsible for creating regional identities of pallial and subpallial domains. Notably, *Pax6* null mice demonstrate a dorsal expansion of gene expression normally associated with the ventral territories of the telencephalon, while *Gsh2* null mice exhibit a ventral expansion of dorsal lateral gene expression (Corbin et al., 2000; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). It is evident that the fully established telencephalon, characteristically subdivided into different regions, with distinct morphologies, connectivities and neurochemical profiles, as well as patterns of gene expression initiated by morphogenetic signals, portrays the primary acquisition of regional identity by progenitor populations (Figure 3) (reviewed in Schuurmans and Guillemot, 2002).

The mammalian telencephalon has been the theme of numerous studies. Notably outstanding breakthroughs have been achieved using the telencephalon as a model system, these include the discovery of neural stem cells and their multipotential properties, as well as the identification of discrete populations of neural progenitors and their adopted modes of division (Davis and Temple, 1994; Doetsch et al., 1999; Morshead et al., 1994; Noctor et al., 2004; Reynolds and Weiss, 1992). Moreover, progenitor cultures are easily established from the telencephalon, and thus this system lends significant advantages for *in vitro* manipulation and experimentation (Conti et al., 2005; Davis and Temple, 1994; Gage et al., 1995; Johe et al., 1996; Reynolds and Weiss, 1992).

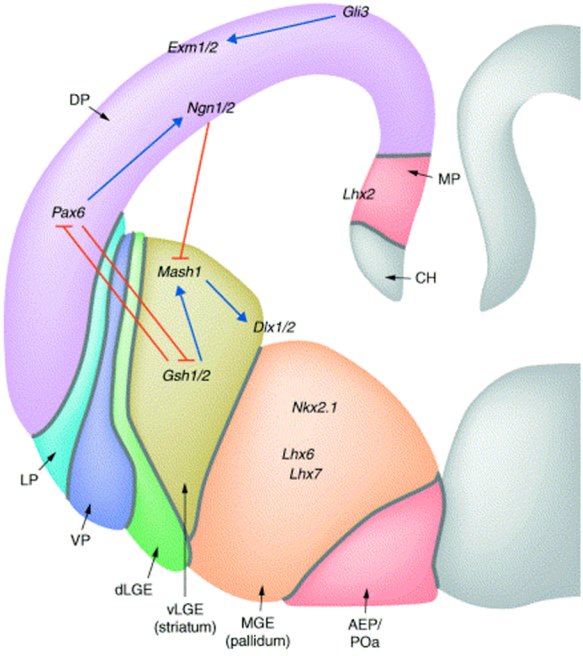


Figure 3. A genetic hierarchy to specify progenitor cell identity in the developing telencephalon. Schematic representation of a coronal section through the telencephalon at E12.5. Progenitors residing in dorsal domains of the telencephalon express the bHLH TFs *Ngn1* and *Ngn2*, and the homeodomain proteins *Emx1*, *Emx2*, *Lhx2* and *Pax6*, whereas progenitors in more ventral regions express the bHLH protein *Mash1* and the homeodomain transcription factors *Gsh1*, *Gsh2*, *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*. Telencephalic progenitor identity is maintained by crucial cross-regulatory interactions between Ngn1/2 and Mash1 and Pax6 and Gsh2. In the scheme arrows and T-bars define positive and inhibitory interactions, respectively. AEP, anterior entopeduncular; dLGE, dorsal LGE; DP, dorsal pallium; LGE, lateral ganglionic eminence; LP, lateral pallium; MGE, medial ganglionic eminence; MP, medial pallium; vLGE, ventral LGE; VP, ventral pallium (Schuurmans and Guillemot, 2002).

1.4 Oligodendrogenesis in the developing telencephalon and spinal cord

1.4.1 The origins of oligodendrocytes

Oligodendrocyte progenitors were first identified in rat optic nerve cell cultures by immunolabeling with antibodies against A2B5 ganglioside, and referred to as oligodendrocyte-type-2 astrocyte progenitor cells (O-2A progenitors; Raff et al., 1983), to indicate their bipotential to differentiate into either oligodendrocytes or astrocytes (type-2 subtype), according to culture medium conditions. In light of the fact that an antigenic phenotype of type-2 astrocytes has not been identified *in vivo*, including in transplantation studies with purified O-2A progenitor cells (Espinosa de los Monteros et al., 1993; Groves et al., 1993), raised the possibility that O-2A progenitors are likely a culture artefact and thus the precursor cells are now more commonly referred to as OPCs. Lineage studies have shown that spinal motorneurons and oligodendrocytes are generated sequentially from a common pool of progenitors in the motorneuron progenitor domain (pMN) in the developing spinal cord (reviewed Richardson et al., 2000 and Rowitch et al., 2002). Moreover, in the brain a precursor for both oligodendrocytes and GABAergic neurons has been proposed (He et al., 2001). It is evident from these data that oligodendrocytes share progenitors with neurons rather than astrocytes in the developing CNS.

The sequential stages of OPC maturation and differentiation are well characterised, and are easily identified by transformations in morphology and gene expression (Figure 4).

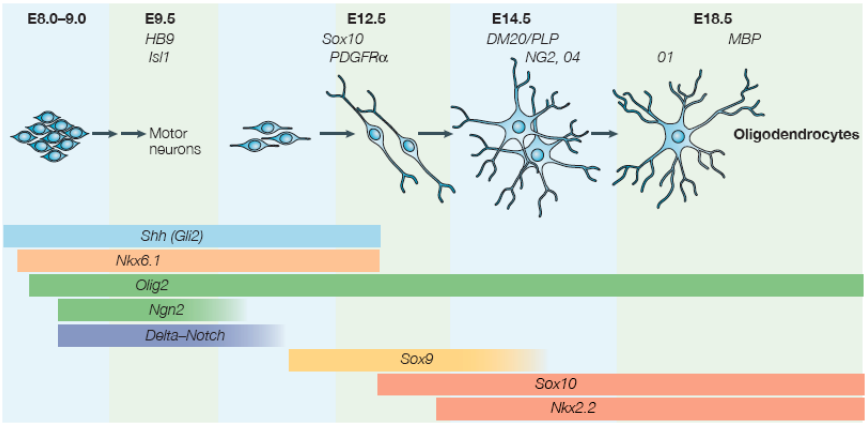


Figure 4. The oligodendroglial lineage: morphology and gene expression. Schematic summary of the oligodendroglial lineage progression in the pMN domain in the developing mouse spinal cord (Rowitch, 2004).

During mouse development, OPCs are first identified in the VZ, of the ventral spinal cord and ventral forebrain at approximately 12.5 days of embryonic development (embryonic day 12.5, E12.5), by their characteristic bipolar morphology and expression of specific early markers, including PDGFRα, a transmembrane protein tyrosine kinase, and the bHLH and Sox (SRY related HMG box) TFs, Olig1 and Sox10, respectively. At this stage, OPCs exhibit a strong migratory behaviour and infiltrate the surrounding parenchyma whilst they continue to proliferate. By E14.5, the expression of the single membrane-spanning chondroitin sulphate proteoglycan, NG2, is induced and these cells develop a multipolar morphology, at which point late precursor markers such as the cell surface O4 marker are upregulated. Mature OPCs eventually exit the cell cycle, they downregulate PDGFRα and NG2 expression and begin to express mature oligodendrocyte markers such as myelin basic protein (MBP), a component of the myelin membrane. Finally, mature oligodendrocytes associate with neighbouring axons and form the myelin sheath.

Oligodendrocytes are generated from a define subset of neural progenitors in multiple locations in the CNS (Figure 5) (reviewed Richardson et al., 2006). In the spinal cord, OPCs are predominantly specified in the VZ of the ventral pMN progenitor domain at E12.5 (Warf et al. 1991; Noll and Miller 1993; Pringle and Richardson 1993; Yu et al. 1994; Timsit et al. 1995). Interestingly, earlier in development this same region is responsible for the formation of motoneurons (Richardson et al. 1997; Sun et al. 1998; Lu et al. 2002; Takebayashi et al. 2002; Zhou and Anderson 2002). A secondary wave of OPCs are generated at more dorsal regions (dI3-5) of the spinal cord at E14.5,

which accounts for about 20% of OPCs (Cai et al. 2005, Vallstedt et al. 2005, Fogarty et al. 2005). In the forebrain, like in the spinal cord, OPCs are mainly produced from a ventral territory extending from the MGE to the anterior entopeduncular area (AEP) at E12.5, these colonise the forebrain by mechanisms of migration and proliferation (Spassky et al. 1998, 2001; Olivier et al. 2001; Tekki-Kessaris et al. 2001). A later source of embryonic OPCs is generated in the LGE and/or caudal ganglionic eminence (CGE) (Kessaris et al. 2006), and this is followed by a final wave of oligodendrogenesis at postnatal stages in the most dorsal domain of the telencephalon, the cortex (Kessaris et al. 2006). Indeed, the progression of OPC specification from ventral to dorsal regions during development is a recurring theme along the length of the vertebrate neuraxis.

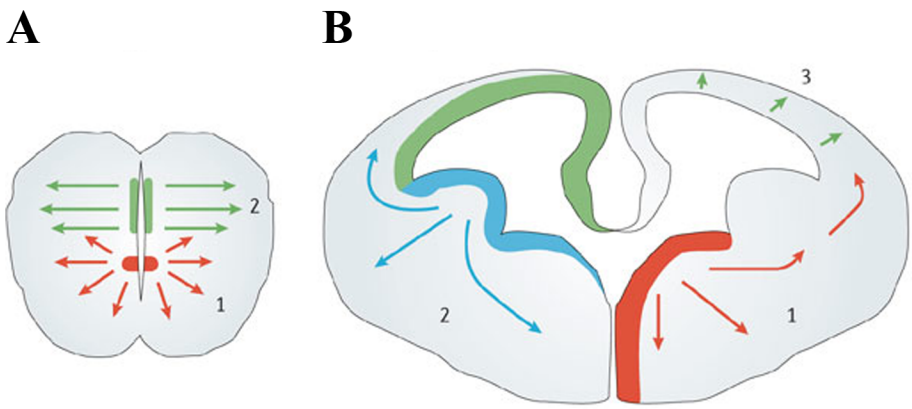


Figure 5. OPC origins in the developing spinal cord and telencephalon. ***A***, In the developing mouse spinal cord, the vast majority of OPCs are generated from pMN in the ventral ventricular zones (1), and commences at E12.5. Later in development, a second wave of OPCs is specified from more dorsal domains (2). ***B***, In the telencephalon, OPCs are specified from the most ventral precursors in the MGE at E12.5 (1), this is followed by the generation of OPCs from the LGE (2), finally OPCs are specified from dorsal progenitors in the cortex after birth (3) (Richardson et al., 2006).

Although region specific OPC populations have been described in the developing telencephalon, on the basis of regionally restricted Nkx2.1, Gsh2, and Emx1 TF expression, it is plausible that the generation of OPCs is in fact not itself regionally restricted but proceeds in a 'Mexican wave' from ventral to dorsal domains (Kessaris et al. 2006). Interestingly, the first OPCs to be specified in the ventral telencephalon are almost completely absent in postnatal animals, instead they are replaced by OPC populations generated later in development and include cells derived from the LGE/CGE throughout the second wave of oligodendrogenesis in the telencephalon (Kessaris et al. 2006). Note that the significance of this cell replacement phenomenon remains unresolved, as does the question of functional heterogeneity of OPC populations specified from molecular distinct regions of the telencephalon. Numerous hypothesis have been proposed in an attempt to explain the substitution of early born OPCs population and include the possibility that they are competitively eliminated by later born OPCs, or simply that an incessant turnover of oligodendrocytes during adulthood may result in the progressive loss of early born OPCs and replacement of cells from stem cells residing in the adult subventricular zone (SVZ). The SVZ is predominately derived from the embryonic LGE/CGE and cortex (Young et al., 2007), and is one source of new oligodendrocytes in the adult (Levison and Goldman 1993, 1997; Luskin and McDermott 1994), the vast majority of which are generated form pre-existing PDGFRA+/NG2 glial cells (Rivers et al., 2008).

1.4.2 Intrinsic and extrinsic regulators of oligodendrocyte cell fate specification

A fundamental question in vertebrate developmental neurobiology is to understand how multipotent neural progenitors, particularly abundant at early stages of neural development, and characterised by their capacity to proliferate, self renew and to generate cells in several neural lineages, are able to generate neurons, oligodendrocytes and astrocytes in a temporal sequence and at specific locations. It is believed that initially NSCs divide symmetrically to enlarge the progenitor pool size. A switch in the mode of division to asymmetric delineates the initiation of neurogenesis, to generate two unequal cell types, a stem cell identical to the parent cell and a committed neuronal progenitor cell. On the contrary the onset of gliogenesis is demarcated by restoration in the mode of division to symmetric. The mechanisms underlying these remarkable changes in progenitor behaviour and fate during CNS development are not fully understood, but are thought to involve a combination and interplay between intrinsic attributes of neural progenitors (including TFs and epigenetic alterations), as well as modifications of their extrinsic signalling environment (such as extracellular factors and their corresponding downstream intracellular signalling pathways) (Temple, 2001).

1.4.2.1 Extrinsic regulators of oligodendrocyte cell fate specification

In the developing telencephalon progenitors must decide whether to adopt a neuronal or glial cell fate, a selection that is influenced by numerous signalling

pathways. Notably, the complexity in the relay of extrinsic signals to a given progenitor are considerable, not only are there extensive interactions between pathways where levels of activity within each pathway are significant, but the same signal may promote different cell fates depending on intrinsic cellular properties. Furthermore, the regulation of gene expression and activity of TFs by extrinsic signals, substantially contribute to the ultimate selection between neuronal or glial cell fates (reviewed in Kessaris et al., 2008; Guillemot, 2007).

The molecular mechanism of the hedgehog signalling pathways have been well characterised in vertebrate and invertebrate species. Briefly, during vertebrate neural development canonical hedgehog signalling is initiated by Shh, one of three homologues of the *Drosophila* hedgehog protein (Echelard et al., 1993; Chiang et al., 1996; Wijgerde et al., 2002). Shh binds to the twelve-pass membrane receptor patched (Ptc1) (Stone et al., 1996; Goodrich et al., 1997; Marigo et al., 1996) to relieve the constitutive repression of the seven-pass G-protein-coupled receptor smoothened (Smo) (Ingham and McMahon, 2006; Chen and Struhl, 1996). Ultimately, Shh signal transduction results in the formation of either repressor or activator types of zinc finger transcription factors that belong to the Gli family (Gli1, Gli2 and Gli3) (Bai et al., 2004; Aza-Blanc et al., 1997).

In the developing telencephalon, neuroepithelial cells proximal to the ventral midline secrete the classical morphogen, Shh, which diffuses to create a concentration gradient. In this system, Shh is responsible for the specification of ventrally derived OPCs (Alberta et al., 2001; Nery et al., 2001; Tekki-Kessaris et al., 2001). The induction of oligodendrogenesis by Shh is mediated through the oligodendrocyte promoting bHLH TFs, Olig1 and Olig2 (Lu et al., 2000; Yung et

al., 2002). Moreover, Shh has been shown to be both necessary and sufficient for the expression of *Olig1* and *Olig2* (Lu et al., 2000; Nery et al., 2001; Zhou et al., 2000).

FGF signalling is transduced through a family of four transmembrane receptor tyrosine kinases (FGFR1–4) in all vertebrates (reviewed Mason, 2007). During telencephalic development the basic FGF2 functions to promote the expansion of neural progenitors in the VZ (Ghosh and Greenberg, 1995; Raballo et al., 2000). However, high level of FGF2 signalling activity support oligodendrogenesis in progenitor cultures, that is independent of Shh function. Indeed, cultures derived from the dorsal telencephalon of *Shh* mutant mice, or cultures subject to inhibition of Shh activity by cyclopamine (11-deoxojervine), retain the capacity to generate OPCs (Chandran et al., 2003; Kessaris et al., 2004; Nery et al., 2001). Moreover, in the embryonic dorsal spinal cord a small population of OPCs are specified in response to FGF signalling, independently of Shh signalling (Cai et al., 2005; Fogarty et al., 2005). More recently a role of FGF receptor signalling in the generation oligodendrocyte progenitors in the zebrafish hindbrain was established (Esain et al., 2010). FGF-receptor signalling in zebrafish ventral hindbrain progenitors not only controls *olig2* expression, in cooperation with Shh, but also promotes the expression of both *sox9a* and *sox9b* during the late gliogenesis phase, and thus promotes oligodendrogenesis in the Olig2 positive domain.

PDGF signalling is required for the *in vitro* differentiation of embryonic multipotent forebrain NSCs into an oligodendroglial lineage that is mediated

through an Erk1/2-dependent signalling pathway and subsequent Olig2 activation (Hu et al., 2008). Interestingly a subset of stem cells in the SVZ of adult rodents express PDGFR, and PDGF signalling in this region has been shown to promote the generation of oligodendrocytes (Jackson et al., 2006). Further functions of PDGF signalling activity in OPC proliferation, survival and migration in animal models and cell culture systems have been described (Barres and Raff, 1994; Fruttiger et al., 1999; Finzsch et al., 2008; Noble et al., 1988; Richardson et al., 1988; Barres et al., 1992; Armstrong et al., 1990; Klinghoffer et al., 2002; Calver et al., 1998).

The core Notch signalling pathway is evolutionarily conserved, and is activated following extracellular interactions between the ligand Delta or Serrate (Jagged) on one cell, with the Notch receptor (a single-pass, transmembrane, heterodimeric protein) on the adjacent cell. Ligand binding activates a succession of proteolytic events, which involve a presenilin–γ-secretase complex (Selkoe and Kopan, 2003), and culminate in the cleavage and release of the Notch intracellular domain (NICD) into the cytoplasm. The NICD translocates into the nucleus (Struhl and Adachi, 1998; Schroeter et al., 1998) aided by nuclear localization signals (Stifani et al., 1992), where it functions to activate and recruit elements of a complex containing the Notch signalling effectors suppressor of hairless (CBF1/RBPjκ) (Fortini and Artavanis-Tsakonas, 1994) and mastermind (Smoller et al., 1990), which subsequently direct the assembly of transcriptional complexes to drive target gene expression. The major effector of the pathway downstream of the Notch receptor is the DNA-binding protein suppressor of hairless.

Indeed, activation of the Notch receptor through its ligands Delta and Serrate is crucial for the control of cell fate choice during CNS development. The generation of neuronal cells precedes that of glial cells and active Notch signalling is vital for the maintenance of proliferative non-committed neural progenitor populations into the gliogenic phase. Evidently, a deficiency in Notch signalling in zebrafish embryos results in a surplus of neurons in the spinal cord at the cost of oligodendroglial cells (Appel et al. 2001; Park and Appel, 2003). On the contrary, an excess in oligodendroglial cells at the expense of motor neurons arises following the expression of a constitutively active form of the Notch receptor (Park and Appel, 2003). In combination these data led to the proposal that Notch signalling is essential for early specification events in the oligodendroglial lineage. However, Notch signalling has also been demonstrated to play an active role during oligodendroglial differentiation. Notably, *in vitro* studies in the developing rat optic nerve showed that oligodendrocyte maturation is inhibited as a result of constitutive activation of Notch signalling (Wang et al. 1998). Furthermore, *in vivo* studies have since shown that a loss of Notch signalling results in the premature differentiation of OPCs into oligodendrocytes (Genoud et al., 2002; Givorgi et al., 2002), while expression of an active form of the Notch receptor in transgenic zebrafish embryos impeded OPC differentiation (Park and Appel, 2003). Collectively, these data indicate that Notch signalling might regulate both the specification of OPCs and their subsequent developmental maturation to oligodendrocytes.

1.4.2.2 Intrinsic regulators of oligodendrocyte cell fate specification

To date numerous key molecular determinants that function to promote oligodendrogenesis have been identified. Although the molecular transcriptional mechanisms controlling oligodendrogenesis have been mostly studied in the context of the developing spinal cord, it is hypothesised that these TFs also regulate oligodendrogenesis in the telencephalon.

1.4.2.2.1 *Olig* genes

The *ato*-related genes characterized by the presence of family-specific residues in their bHLH domain in vertebrates include the *Olig* gene family (Lee, 1997) and contains two major determinants of the oligodendroglial cell fate, namely *Olig1* and *Olig2* genes. In the developing spinal cord these TFs strongly support neuronal and oligodendroglial cell fate specification, whilst actively inhibiting the generation of astrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Olig2 is expressed in the ventral pMN domain of the spinal cord, where it is required for the generation of oligodendrocytes and motorneurons (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002). Note that Olig2 has a number of distinct functions within this progenitor domain. At first Olig2 functions to promote the identity of the pMN domain by actively repressing alternative fates (Mizuguchi et al. 2001; Novitch et al. 2001). Olig2 represses Irx3 a TF involved in the acquisition of V2 interneuron identity in the developing ventral spinal cord. Notably in *Olig2* null mice the p2 domain and thus the V2 interneuron population are subject to a ventral expansion. In addition, Olig2 also

functions to maintain the precursor pool in the pMN domain by repressing the expression of MN specific differentiation factors in the neuroepithelium. Not surprisingly, Olig2 expression in motorneuron progenitors is transient and is subject to rapid down regulation prior to neuronal differentiation. Interestingly, whilst the vast majority of neural bHLH TFs function as activators, Olig2 is known to perform as a transcriptional repressor (Cabrera and Alonso, 1991; Johnson et al., 1992; Mizuguchi e al., 2001; Novitch et al., 2001). Indeed Olig2 strongly inhibits neuronal differentiation in this ventral domain through active competition with Ngn2, firstly for dimerisation with E-proteins and secondly for binding to degenerate E-box elements in the promoter of the post-mitotic motorneuron *Hb9* gene (Lee et al., 2005).

Although the function of these genes in more rostral domains of the neuraxis, such as the telencephalon, are less well characterised it is proposed that its function are analagous. Olig1 expression is specifically restricted to OPCs as soon as they arise in the embryonic ventral telencephalon. In contrast Olig2 expression is significantly broader and is present in ventral neuroepithelial progenitors of the VZ prior to OPC specification that generate both oligodendrocytes and neurons (Furusho et al., 2006; He et al., 2001; Takebayashi et al., 2000; Tekki-Kessaris et al., 2001; Yung et al., 2002). Olig1 and Olig2 expression are maintained throughout oligodendrocyte lineage progression up until the point of terminal differentiation (Lu et al., 2000; Zhou et al., 2000).

Olig1 gain of function data in cortical derived progenitors either from the developing embryo or in culture, results in the generation of ectopic oligodendrocytes, as does Olig2 overexpression in cultured embryonic cortical

progenitors and in the adult SVZ (Lu et al., 2000, 2001; Marshall et al., 2005; Balasubramaniyan et al., 2004; Copray et al., 2006). *Olig2* null embryos present a complete loss of oligodendrocytes in the spinal cord, however a few OPC remnants are evident in specific regions of the developing brain, which are lost following elimination of Olig1 function (Lu et al., 2002; Zhou and Anderson, 2002). Despite the fact that both GABAergic and cholinergic interneuronal subtypes are generated from the ventral telencephalon, *Olig2* null embryos only display a fractional loss of cholinergic neurons (Furusho et al., 2006). In combination, these data suggest that perhaps Olig2 plays a less significant role in neurogenesis in more rostral regions of the developing nervous system, as compared to its function in the specification of oligodendroglial cell fates. In contrast, Olig1 function is required for oligodendrocyte maturation and is essential for physiological myelination (Xin et al., 2005) and remyelination activities in demyelinating lesions (Arnett et al., 2004; Lu et al., 2002).

In the embryonic brain as in the spinal cord Olig2 is involved in the specification of both oligodendroglial and neuronal lineages, suggesting that other factors act in combination with Olig2 to select between the two lineages. Indeed Mash1 and Olig2 interact at a genetic level and act through a common pathway to specify early-born OPCs in the ventral telencephalon during development (Parras et al., 2007). A functional synergy between these TFs has also been identified in neurosphere cultures form rat spinal cord using retroviral overexpression of Mash1 and Olig2 (Sugimori et al., 2007).

1.4.2.2.2 *Sox* genes

Members of the Sox group of TFs are characterised by a conserved HMG DNA-binding domain, approximately 80 amino acids in length that was initially discovered in the mammalian Sry protein (Bowles et al., 2000; Wegner, 1999; Schepers et al., 2002). Based on the phylogenetic analysis of the HMG domains, *Sox* genes can be divided into distinct subgroups, termed SoxA to SoxH, which include the Sox group E comprising *Sox8, Sox9* and *Sox10* genes. Notably, Sox proteins within the same group usually demonstrate an amino acid identity of at least 70%, whilst those from different groups share minimal sequence identity outside their HMG domain (Wegner, 1999). Sox proteins directly bind DNA sequences (A/T)(A/T)CAA(A/T)G and are dependent on cofactors for target gene specificity (reviewed Kamachi et al., 2000; Wilson and Koopman, 2002). Notably Sox10, a key regulator in cell fate specification, lineage progression and terminal differentiation of neural crest derived Schwann cells (Britsch et al., 2001; Schreiner et al., 2007), is dependent on co-regulator interactions for its function during Schwann cell development. Specifically, Sox10 interacts with the class III POU protein Oct6 prior to myelination to induce TF *Krox20* expression (Ghislain and Charnay, 2006). Subsequently, Krox20 functionally synergises with Sox10 to activate expression of specific myelin genes, including *connexin-32* and *myelin protein zero*, during the final phase of terminal differentiation and myelin formation (Bondurand et al., 2001; LeBlanc et al., 2007; Peirano et al., 2000a). Moreover Sox10 and Olig1 functionally synergise to activate *mbp* gene expression in zebrafish (Li et al., 2007). Sox proteins bind the minor groove of DNA, causing significant modifications in its conformation.

It has been suggested that these specific changes may bring proteins on distal gene promoters and enhancers in closer proximity to facilitate interactions.

Members of the same Sox group tend to share expression and function (reviewed Guth and Wegner, 2008; Kiefer, 2007; Wegner and Stolt, 2005). Notably these TFs modulate a range of processes during development, including sex determination, chondrogenesis, neural crest formation, and participate in multiple aspects of CNS development, including gliogenesis. Indeed oligodendrocyte specification and differentiation are dependent on the function of *SoxE* group genes, particularly *Sox9* and *Sox10*. During oligodendrocyte development *SoxE* gene expression is partially overlapping. Sox9 is expressed in a uniform manner along the entire length of the VZ of the embryonic spinal cord, both dorsally and ventrally. Moreover its expression is maintained in OPCs as they emerge from the VZ and migrate into the surrounding parenchyma, and in oligodendrocytes as they differentiate and myelinate, after which its expression is down regulated (Stolt et al., 2003). Sox8 is expressed in progenitors restricted to the ventral domain of the VZ in an oligodendrocyte competent region. In contrast Sox10 expression is exclusively limited to specified OPCs (Stolt et al., 2005). Note Sox8 and Sox10 expression are maintained in mature oligodendrocytes, even after Sox9 expression is down regulated. In the telencephalon, Sox9 is expressed in the ventricular zones in dorsal and ventral territories and its expression is also maintained in OPCs as they invade the surrounding parenchyma following their generation (this study, Figure 11). The role(s) of Sox9 in oligodendrogenesis in these rostral domains are yet to be characterised.

Conditional null mutants of *Sox9*, in which Sox9 function is specifically ablated in neural progenitors, results in a dramatic reduction in the generation oligodendrocytes and astrocytes, with a concomitant increase in the production of motor neurons and to a lesser extent V2 interneurons (Stolt et al., 2003). These data clearly display Sox9 as a major player in the neuron-glia switch (Stolt et al., 2003). Expression of Sox9 in a neuroblastoma cell line, leads to the activation of specific astrocyte and oligodendrocyte markers, at the expense of neuron markers whose expression are repressed (Stolt et al., 2003). In contrast, whilst OPCs are specified as normal in *Sox10* null embryos, the process of differentiation is perturbed, resulting in a significant decrease of mature oligodendrocytes (Britsch 2001; Stolt 2002). Although Sox8 is expressed in both progenitors and oligodendrocytes, *Sox8* mutant mice do not exhibit any apparent defects in glial generation (Stolt et al 2004). Partial functional redundancies between *SoxE* group genes are evident from studies in compound mutants, which demonstrate an increase in the severity of defects associated with the single mutants (Stolt et al., 2003; Stolt et al., 2004; Stolt et al., 2005).

The *SoxD* genes *Sox5* and *Sox6* are expressed just prior to the onset of oligodendroglial cell fate specification and are maintained in OPCs, and down-regulated in terminally differentiating cells (Stolt et al., 2006). *SoxD* genes negatively regulate *SoxE* group genes. Indeed *Sox5* or *Sox6* null mice demonstrate precocious specification of VZ cells to OPCs, a phenotype that is more pronounced in the compound double mutants. Moreover, *Sox5* and *Sox6* double mutant mice exhibit precocious terminal differentiation of oligodendrocyte progenitors. Clearly, SoxD proteins function in the regulation of oligodendrocyte progression during development by inhibiting SoxE function.

1.4.2.2.3 *Nkx2* genes

In the embryonic spinal cord oligodendrogenesis occurs in neighbouring domains of the VZ, which express *Olig2* and the homeobox *Nkx2.2* genes. Although OPC populations from these domains are originally distinct, either expressing Olig2 or Nkx2.2, ultimately simultaneous expression of these genes in the same cell makes it unfeasible to distinguish between these two principal populations (Fu et al., 2002). Notably gain of function studies with Olig2 and Nkx2.2 in the chick spinal cord, demonstrated the capacity of these factors to synergise at a functional level to generate ectopic oligodendrocyte differentiation. The generation of OPCs in *Nkx2.2* null mice appears to be normal, however these mutants display defects in the maturation of OPCs (Qi et al., 2001), to suggest that the functional interaction between Olig2 and Nkx2.2 are likely to play a prominent role in OPC differentiation, rather than in oligodendrocyte specification.

Nkx2.1, a close relative of the *Nkx2.2* gene, is expressed in proliferative progenitors in the developing ventral telencephalon. Although OPCs are absent in the telencephalon of *Nkx2.1* null mice (Nery et al., 2001), this phenotype most likely arises as an indirect consequence of the regulation of Shh expression by Nkx2.1. Certainly, the competency of the *Nkx2.1* mutant telencephalon to produce oligodendrocytes is maintained in progenitor cultures following overexpression of Shh, thus excluding a major role of Nkx2.1 in oligodendrogliogenesis (Nery et al., 2001).

1.4.2.2.4 *Proneural bHLH* genes

Proneural genes were first discovered in *Drosophila* on the basis of their involvement in early phases of neural development (Ghysen and Dambly-Chaudiere, 1988; Garcia-Bellido, 1979). The *achaete-scute* gene complex and *atonal* genes were initially identified by the extent of their sequence similarity (Figure 6A) (Gonzalez et al., 1989; Villares and Cabrera, 1987; Jarman et al., 1993; Goulding et al., 2000a, 2000b; Huang et al., 2000). Further sequence resemblance with other genes, including the oncogene *myc*, the sex-determination gene *daughterless*, and the muscle-determination gene *MyoD* (Villares and Cabrera, 1987; Murre et al., 1989a) eventually resulted in the identification of the bHLH domain, a structural motif that confers specific DNA-binding and dimerisation properties (Figure 6B) (Murre et al., 1989a).

A **B**

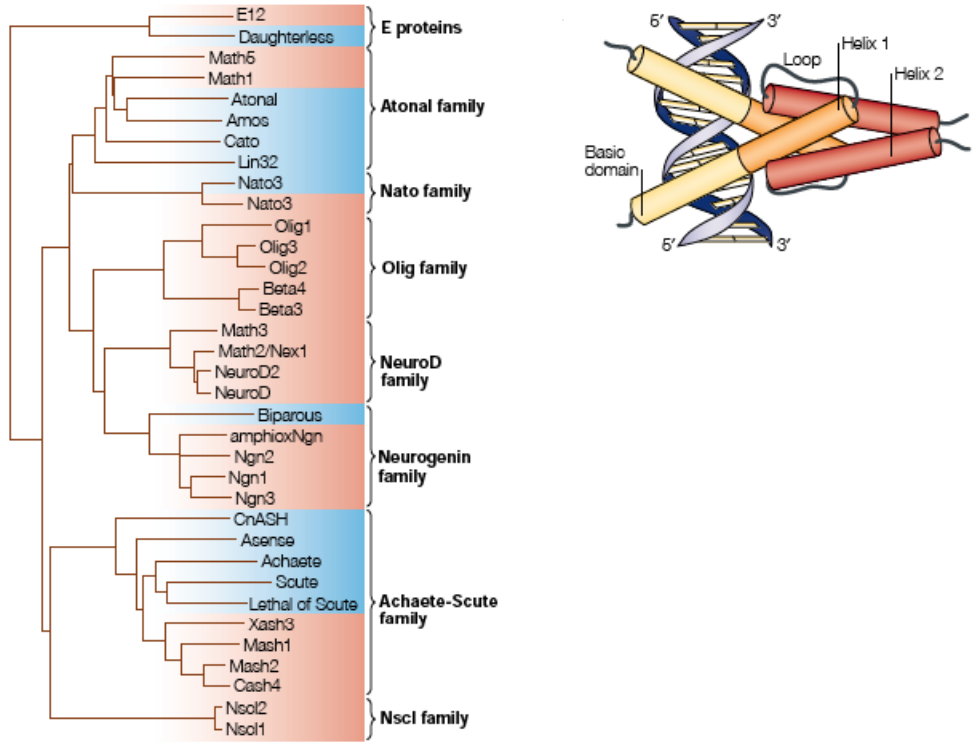


Figure 6. Structure and properties of neural bHLH proteins. ***A***, A dendrogram of the sequence of the bHLH domain of invertebrate (blue) and vertebrate (red) neural bHLH proteins. Note that proteins are categorised into discrete families on the basis of sequence similarities within the bHLH domain. ***B***, Schematic representation of a bHLH dimer complexed to DNA (Bertrand et al., 2002).

Classification of *achaete-scute* and *atonal* genes as proneural in function, was founded on the fact that they were expressed in the ectoderm by groups of cells referred to as 'proneural clusters', as well as their ability to generate neural progenitors (Campuzano and Modolell, 1992; Jan and Jan, 1994; Jimenez and Modolell, 1993). A combination of loss and gain of function analyses have isolated numerous bHLH genes with distinct proneural activity in *Drosophila*. *Achaete-scute* and *atonal* have been well-characterised and together account for the origin of most of the Drosophila PNS. These studies revealed that *achaete-scute* genes function in the development of the fly external sense organs (such as the mechanosensory and chemosensory organs), whilst *atonal* genes were demonstrated to function in the development of internal chordotonal organs (Jarman et al., 1993).

Proneural bHLH proteins function as transcriptional activators (Cabrera and Alonso, 1991; Johnson et al., 1992), and bind to degenerate DNA sequences, known as E-boxes (CANNTG) (Figure 7). Formation of heterodimeric complexes are critical for DNA binding, and this is achieved with the alternative splice variants of the E2A gene, namely E12 and E47 E-proteins, (Murre et al 1989b). Molecules that inhibit proneural gene activity include the vertebrate HLH Id (inhibitor of differentiation) genes, which lack a basic motif for DNA binding and act as repressors of bHLH activity by inhibiting their dimerisation (Massari and Murre, 2000; Campuzano, 2001; Yokota, 2001). Briefly, Ids actively compete for E-proteins, forming heterodimers that are blocked in their ability to bind DNA. Other inhibitors of proneural gene activity include the vertebrate Hes/Her/Esr proteins (the hairy and enhancer of split factors in

Drosophila) (Davis and Turner, 2001; Kageyama and Nakanishi, 1997). These proteins not only function as typical DNA-binding repressors of proneural gene transcription (Chen et al., 1997; Ohsako et al., 1994; Van Doren et al., 1994), but are also thought to inhibit the activity of proneural proteins by interfering with proneural–E-protein complex formation (Davis and Turner, 2001; Kageyama and Nakanishi, 1997). Note that the HLH proteins (ID2 and ID4), as well as negative regulatory bHLH Hes genes (Hes1 and Hes5), inhibit the formation of oligodendrocytes *in vitro* (Kondo and Raff, 2000a, 2000b; Wang et al., 2001). Moreover, in addition to binding to the ubiquitously expressed E2A proteins, ID2 and ID4 also directly interact with OLIG1 and OLIG2 *in vitro* to inhibit oligodendroglial lineage commitment (Samanta and Kessler, 2004).

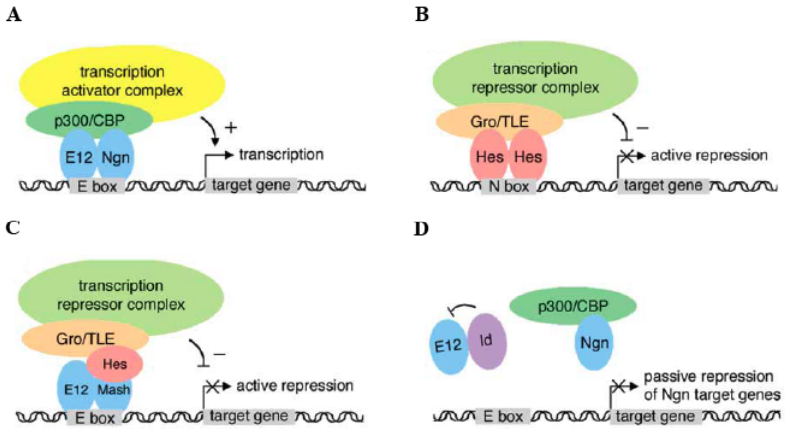


Figure 7. Mechanisms of bHLH TF activity. ***A***, bHLH TFs form heterodimers with E-proteins and bind E-box sequences (CANNTG) to activate the transcription of target genes. ***B***, Hes proteins directly repress the transcription of proneural genes by binding to N-box sequences (CACNAG) in proneural gene promoters. ***C***, Hes proteins repress the activity of proneural proteins through binding to proneural heterodimers and recruiting repressor complexes to target gene promoters. ***D***, Id proteins passively repress proneural protein activity by binding to and sequestering E-proteins, thus inhibiting the formation of proneural heterodimers (Ross et al., 2003).

Critical to the function of proneural genes is the activation of the Notch signalling pathway (Artavanis-Tsakonas, 1999), by a process commonly referred to as lateral inhibition (Figure 8). In this model proneural genes are initially expressed in groups of equivalent neurectodermal cells (Jarman et al., 1993; Campuzano and Modolell, 1992; Blader et al., 1997; Ma et al., 1996; Henrique et al., 1997). Stochastic upregulation of a proneural gene in a target cell directly induces an increase in the levels of the Delta ligand (Figure 8A & B). Consequently, the Notch signalling cascade is activated in adjacent cells, and results in the expression of repressor molecules that belong to the bHLH Enhancer of Split group of genes, which in turn down regulate proneural gene expression in that cell. Notably, through initial establishment of lateral inhibition and subsequent activation of an auto-regulatory loop, proneural gene expression are restricted to single cells which are thus destined for neural differentiation (Artavanis-Tsakonas, 1999; Chitnis and Kintner, 1996; Lewis, 1998).

More recently real-time imaging analysis demonstrated that Notch effectors, proneural genes and Notch ligands are expressed in an oscillatory manner by neural progenitors in the developing mouse brain (Shimojo et al., 2008), raising the question of whether a subtle stochastic difference is gradually amplified and fixed as defined in the classic view of lateral inhibition (review in Kageyama et al., 2008) (Figure 8C). This type of oscillatory expression, that is unsynchronised between neighbouring cells, poses a number of distinct advantages including the maintenance of a group of cells in an undifferentiated state by mutual activation of Notch signalling, in addition to the generation of neural progenitor diversity.

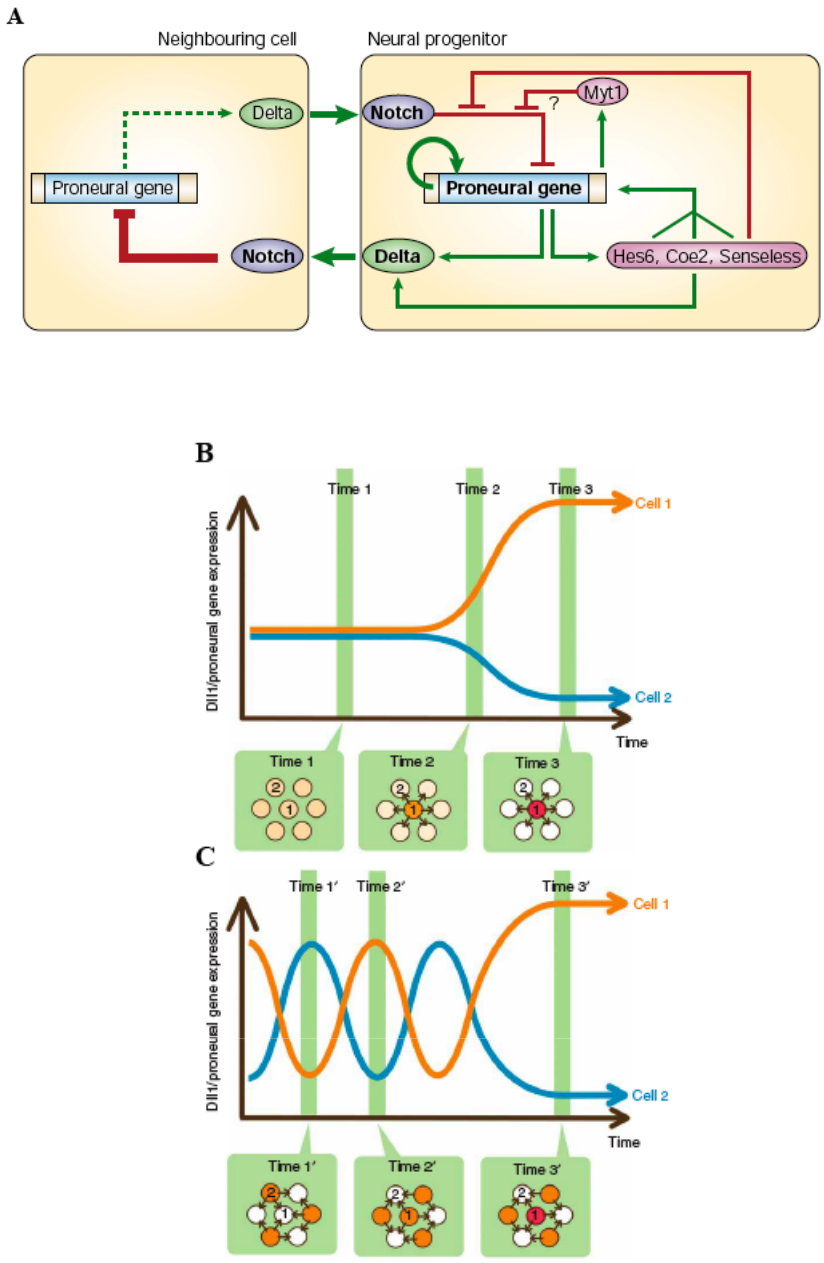

A
Neighbouring cell
Neural progenitor
Delta
Notch
Myt1
?
Proneural gene
Proneural gene
Notch
Delta
Hes6, Coe2, Senseless
B
Time 1
Time 2
Time 3
Cell 1
Cell 2
Dll1/proneural gene expression
Time
C
Time 1′
Time 2′
Time 3′
Cell 1
Cell 2
Dll1/proneural gene expression
Time

Figure 8. Two models for lateral inhibition in vertebrates. ***A & B***, Classic view of lateral inhibition. Initially all neural progenitors are equivalent and express proneural and Notch ligand genes, such as *Dll1*, at similar levels (time 1). A subtle stochastic difference between cells in proneural and Dll1 expression (time 2) is amplified by lateral inhibition, causing subsets of cells to express proneural genes and Dll1 at high levels and to differentiate into postmitotic neurons (cell 1, time 3). These selected cells activate Notch signalling in neighbouring cells, which subsequently become negative for proneural and Dll1 expression and are maintained as neural progenitors (cell 2) (Bertrand et al., 2002; Kageyama et al., 2008). ***C***, Revised view of lateral inhibition. Proneural and Dll1 expression oscillates, as a result of Hes1 oscillation. Note that this pattern of oscillation is dynamic, and therefore it does not facilitate cell fate prediction (Kageyama et al., 2008).

Indeed, members of the proneural bHLH family of proteins are crucial TFs that play pivotal evolutionary conserved roles in neurogenesis both in vertebrates and invertebrates (reviewed Bertrand et al., 2002; Ross et al., 2003). Vertebrate genes related to *achaete-scute* and *atonal* on the basis of the sequence similarity in the bHLH domain have been identified. *Achaete-scute* homologue 1 (ash1) has been isolated in a range of species, including the mouse (Mash1), chick (Cash1), zebrafish (Zash1), Xenopus (Xash1) and human (hASH1), as have the *atonal*-related genes Neurogenins (Ngns). Proneural TFs are expressed in progenitors of the mammalian telencephalon and include Mash1 in the basal ganglia, and Ngn1/Ngn2 as well as Mash1 (albeit at reduced levels compared to Ngns) in the cortex (Britz et al., 2006). In the developing mammalian CNS, proneural factors Mash1 and Ngn2, regulate the transformation of NSCs into mature neurons, including the acquisition of generic and subtype-specific properties of neurons, Notch signalling activation, cell cycle exit and neuronal migration (Bertrand et al., 2002; Hand et al., 2005; Helms and Johnson, 2003; Schuurmans et al., 2004; Heng et al., 2008), in addition to regulating the commitment of multipotent progenitors to a neuronal or an astroglial fate (Nieto et al., 2001; Sun et al., 2001; Tomita et al., 2000). Involvement in these distinct processes, suggest that proneural proteins have the capacity to activate a large number of target genes in a context dependent manner and in a precisely orchestrated temporal sequence.

Gain of function analysis has demonstrated the potential of proneural genes to induce neural progenitor differentiation to form functional mature neurons (Farah et al., 2000; Mizuguchi et al., 2001; Nakada et al., 2004). These data clearly support the notion that these proneural genes are sufficient to initiate

a complete neuronal program of differentiation. On the other hand, loss of function analysis with *Mash1* null mutant mice (Guillemot et al., 1993), present a severe disruption in the process of neurogenesis. As expected, these defects are most prominent in regions where Mash1 is normally expressed, namely the ventral telencephalon and the olfactory sensory epithelium (Casarosa et al., 1999; Cau et al., 2002; Guillemot et al., 1993; Horton et al., 1999). Undeniably, these defects are inherently linked to a loss of progenitor populations within these domains, in addition to a failure to express the Notch ligands Delta and Serrate/Jagged, and to thus activate Notch signalling. Moreover, loss of proneural bHLH gene activity, leads to the premature emergence of restricted astrocyte precursors and subsequently premature astroglial differentiation (Nieto et al., 2001; Tomita et al., 2000). Notably, *in vitro* clonal analysis clearly suggest that this phenomenon reflects a dual role of proneural proteins in the commitment of multipotent progenitors to the neuronal lineage and the specific inhibition of alternate astroglial cell fates (Nieto et al., 2001; Parras et al., 2004). Interestingly, Mash1 is the only proneural gene to be expressed in the ventral telencephalon, and although there is a loss of SVZ progenitors and of postmitotic neurons in this region (particularly in the globus pallidus), a large fraction of neurons are normally produced particularly in the striatum, to suggest that other genes with specific proneural activity are yet to be discovered in this domain. (Casarosa et al., 1999; Horton et al., 1999). Specification of the striatum depends on the function of the *Gsh1* and *Gsh2* homeobox genes, which are expressed in the VZ of the LGE (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003). Moreover the fact that Gsh2 specifies striatal projection neuron and olfactory bulb interneuron identity at distinct time

points during telencephalic neurogenesis (Waclaw et al., 2009), suggest that Gsh2 may compensate for the loss of Mash1 function in this region.

Although Mash1 is more commonly associated with its function in neurogenesis, this typical proneural bHLH factor also plays an active role in the regulation of oligodendrogenesis. Indeed, Mash1 is widely co-expressed with Olig2, a major determinant of oligodendroglial fate, throughout embryogenesis in the ventral telencephalon where OPCs are specified, as well as in the white matter of the postnatal brain, and in OPC cultures (Gokhan et al., 2005; Kondo and Raff, 2000a; Parras et al., 2004 Parras et al., 2007; Wang et al., 2001). *Mash1* null mutant mice present a reduction in the numbers of OPCs in the olfactory bulb (Parras et al., 2004). In addition clonal analysis of *Mash1* null progenitors in culture demonstrated that mutant progenitors that would otherwise generate neurons and oligodendrocytes instead form astrocytes, while glial progenitors that generate astrocytes and oligodendrocytes were not affected (Parras et al., 2004). Indeed, these data clearly elucidate a role of Mash1 in the specification of a subpopulation of oligodendrocytes. Note however, whether different subsets of OPCs with distinct lineal origins also differ in their requirement for Mash1 *in vivo* remains to be addressed.

In the embryonic ventral telencephalon, a sub population of OPCs expresses Mash1 protein as soon as they emerge in the VZ of the ventral forebrain at E12.5 and E14.5 (Parras et al., 2007). Moreover, a significant proportion of OPCs at E12.5 originate from Mash1 positive progenitors in this domain, as defined by the loss of PDGFRα positive cells in the AEP of *Mash1* null mutant mice (Parras et al., 2007). These data highlight a critical role for

Mash1 function in the generation of the first wave of OPCs in the ventral forebrain between E11.5 and E13.5, whilst its function is dispensable for the production of subsequent waves of OPCs in the embryonic forebrain. Mash1 gain of function in the embryonic dorsal telencephalon results in the induction of *PDGFRα* gene expression (Parras et al., 2007). However other OPC markers fail to be expressed in these ectopically induced cells. These data suggest that Mash1 functions in combination with other TFs in the specification of OPCs. Indeed data from *Mash1* and *Olig2* double mutant mice demonstrated that these TFs genetically synergises in the specification of early born OPCs in the embryonic ventral telencephalon (Parras et al., 2007).

In vivo and *in vitro* gain and loss of function studies in more caudal domains of the developing CNS support the idea that Mash1 acts as an instructive factor for the induction of oligodendroglial cell fate specification (Sugimori et al., 2007). OPCs are severely reduced in number, but not completely absent, in the embryonic spinal cord of *Mash1* null mice. Similar to the situation in more rostral regions, OPC numbers are gradually recovered later in development, to suggest that other factors are likely implicated in the generation of OPCs. More recently a role for Mash1 in the differentiation of OPCs into myelin-expressing oligodendrocytes at late embryonic stages in the spinal cord was demonstrated (Sugimori et al., 2008). Interestingly, retrovirus-mediated overexpression of Mash1, redirected the fate of proliferating adult hippocampal stem/progenitor progeny in their *in vivo* niche, from a neuronal to an exclusive oligodendroglial fate (Jessberger et al., 2008). Altogether, these data suggest that Mash1 activity is modulated at the cellular level in a regional and temporal manner.

Although there is not much information on the molecular mechanisms by which proneural proteins regulate transcription or the co-factors that are involved in the activation of target genes, it is evident that these interactions modulate their transcriptional activity (reviewed Powell and Jarman, 2008). The myogenic bHLH protein, MyoD interacts with a plethora of molecules including DNA binding TFs, Mef2 and Pbx, and chromatin remodelling cofactors such as the histone acetylases CBP/P300 and PCAF and some components of the SWI/SNF complex (Tapscott, 2005). The proneural bHLH Ngn proteins, recruit CBP/P300 to activate target promoters (Ge et al., 2006; Koyano-Nakagawa et al., 1999; Sun et al., 2001). More recently Mash1 and the POU proteins Brn1 and Brn2 were shown to interact on the promoter of the Notch ligand *Delta1* gene and synergistically activate its transcription, a key step in neurogenesis (Castro et al., 2006).

1.5 A model for the transcriptional control of neural cell fates in the telencephalon

In combination, these data provide support for the construction of a simple conceptual model for the transcriptional control of cell fate specification in the developing telencephalon. Similar to its expression in the spinal cord, Sox9 is expressed in the VZ of the telencephalon, and thus may be implicated in the specification of oligodendrocyte cell fates within this domain, as has been demonstrated in more caudal regions of the CNS. Furthermore, a role for Mash1 and Olig2 bHLH transcription factors in the generation of oligodendrocytes in the ventral telencephalon are well established, thus it is plausible to propose that

Sox9 may also contribute to this function. Essentially, this model proposes that modifications in the expression levels of three core TFs, namely Olig2, Sox9 and Mash1 can provide a rationale for the commitment of neural progenitors in the ventral telencephalon to form neurons, oligodendrocytes and subsequently astrocytes in that temporal sequence. Note that an assumption of this model is that ventral neural progenitor cells of the telencephalon generate neurons followed by oligodendrocytes, which has been demonstrated (He et al., 2001). Briefly, the model proposes that Sox9, a repressor of the neuronal fate in the ventral spinal cord, is originally expressed at low levels in telencephalic progenitor cells of the VZ coexpressing Olig2 and Mash1, and thus lead to neuronal fate selection. The mechanisms underlying the initial reduced level of expression of the gliogenic gene *Sox9* are not understood, however Mash1 may repress this gene in neuronal precursors, comparable to the mode of repression of the *Sox10* gene by Mash1 in neural crest-derived neuronal precursors (Kim et al., 2003). Subsequently a progressive increase in the levels of Sox9 expression would trigger a change in the cell fate selection of progenitor cells to generate oligodendrocytes. Down regulation of repressors of the astroglial cell fate, Olig2 and Mash1, in Sox9 expressing progenitors would then mark the end of the oligodendroglial phase and the selection of the astroglial fate. Note that whilst this model details a TF combinatorial code for the generation of Mash1-dependent OPCs, it is clearly evident that Mash1-independent OPC population generated *in vivo* must incorporate other factors for oligodendroglial cell fate specification.

1.6 Promoter occupancy using ChIP-on-chip genomic technology

A key question in trying to understand the molecular mechanisms underlying the activity of a particular TF, is how genomic information is translated into gene regulation. Indeed, over the past decades this issue has been fervently pursued and has led to a conventional opinion of transcriptional regulation whereby *cis*-regulatory elements, including promoters and enhancers, modulate the levels of gene transcription (Lee and Young, 2000; Sandelin et al., 2007). Transcriptional activity is regulated by DNA-binding factors through proximal promoters and distal enhancers. Notably, these site-specific protein-DNA interactions not only help to recruit new factors that are essential for transcriptional activity but moreover function to stabilise pre-existing conformations at the core promoter.

Recent progress in chromatin immunoprecipitation experiments followed by microarray (ChIP-on-chip) or by sequencing (ChIP-seq) techniques (Figure 9), have enabled the creation of specific protein–DNA interaction maps at a genomic scale from a given cell type (Wederell et al., 2008; Xu et al., 2007; Robertson et al., 2007). Briefly, ChIP involves the treatment of cells with formaldehyde to form crosslinks between DNA-binding proteins and DNA, followed by chromatin fragmentation using sonication or enzymatic digestion methods. Immunoprecipitation of crosslinked chromatin with a specific antibody that recognises the factor of interest, enriches for chromatin bound regions and thus the discovery of all the binding sites in the genome for the factor of interest. Precipitated fragments are purified, and using polymerase chain reaction (PCR) techniques, particular genes of interest can be analysed. However, the coverage

of analysis can be extended to a genome-wide scale by ChIP-on-chip or ChIP-seq. Briefly, for ChIP-on-chip the immunoprecipitated sample and control input DNA, are labelled with different fluorescent dyes and subsequently hybridized to microarrays. In this approach, binding sites are identified by the actual intensity of the signal of the immunoprecipitated sample relative to the signal of the input DNA sample for every probe tiled on the microarray, using ChIP-on-chip peak-calling programs (Johnson et al., 2008; Bieda et al., 2006). In ChIP-seq, the immunoprecipitated sample is analysed using high-throughput next-generation sequencers, and binding sites are identified using ChIP-seq peak-calling programs (Robertson et al., 2007; Zhang et al., 2008; Fejes et al., 2008; Jothi et al., 2008; Hoffman et al., 2009). Notably, genome wide profiling of site-specific TFs is an extremely powerful technique, and has made significant contributions to the understanding of the patterns and specificity of TF binding, and stability of interactions between TFs and the chromatin landscape.

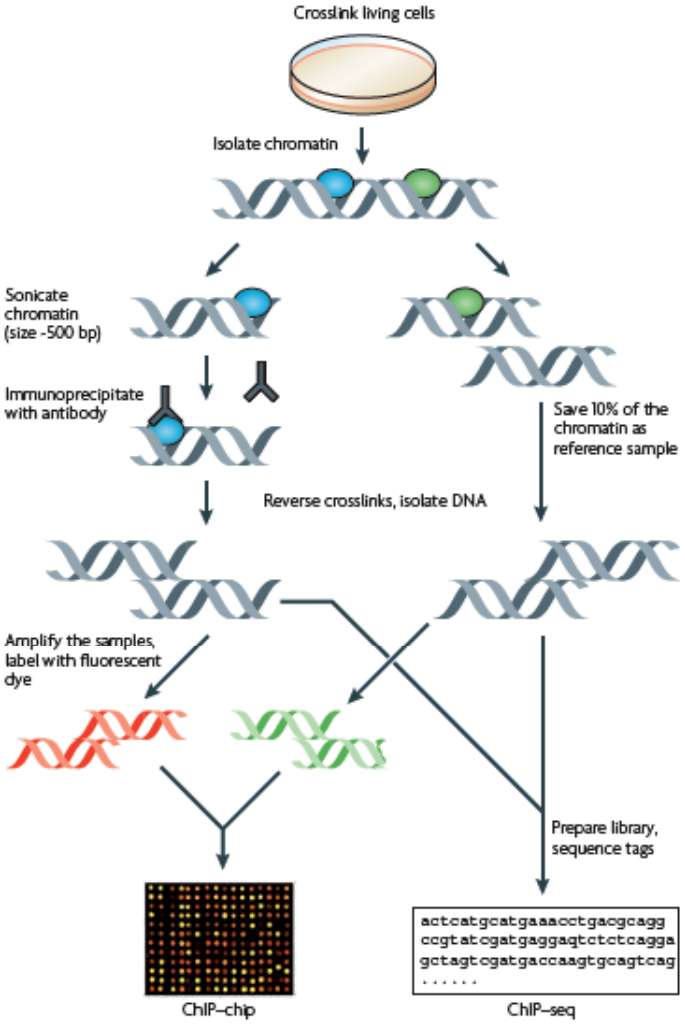


Figure 9. Chromatin immunoprecipitation (ChIP) methods: ChIP-on-chip and ChIP-seq. (Farnham, 2009)

1.7 Neurospheres as a model for studying cell fate specification

Stem cells are defined as undifferentiated cells, which maintain their capacity for self-renewal and multipotentiality. Note that because of the lack of definitive markers stem cells are commonly defined by these functional characteristics. Indeed using a serum-free culture system Reynolds and Weiss demonstrated that a single cell from the adult CNS had the capacity to proliferate and form a non-adherent cluster of undifferentiated cells, otherwise termed as a neurosphere (Reynolds and Weiss., 1992) that was able to generate secondary spheres after dissociation and could differentiate into neurons, astrocytes and oligodendrocytes. Indeed, they demonstrated that the cell they had isolated exhibited critical stem cell attributes, namely extensive self-renewal, the capacity to give rise to a large number of progeny and multipotency (Potten and Loeffler, 1990; Hall and Watt, 1989). These findings lay the foundations for further studies in this field, which have since established a culture system that uses epidermal growth factor (EGF) and FGF2 as mitogens that enable the reliable production of undifferentiated CNS precursors that can be expanded as neurospheres or differentiated into mature cells of the CNS (Reynolds and Weiss., 1992, 1996; Gritti et al., 1995, 1999, 1996; Weiss et al., 1996a, 1996b).

The neurosphere culture system has been used for numerous applications, including in assays to define the presence of stem cells particularly in the CNS (Reynolds and Weiss, 1992; Gritti et al., 1999; Weiss et al., 1996a; Hitoshi et al., 2002; Lu and Wong 2005; Yang and Levison, 2006; Morshead et al., 1994; Maslov et al., 2004; Marshall et al., 2005). Notably, NS cells have been isolated

from various areas of the embryonic brain as well as from proliferative regions in the adult nervous system (Weiss et al., 1996a; Gritti et al., 2002). Furthermore, this culture system has been exploited to study factors and events that regulate stem cell maintenance or differentiation to try to identify a specific molecular signature of 'stem cell identity' (Ivanova et al., 2002; Ramalho-Santos et al., 2002) and to investigate neural development, in particular neurogenesis and gliogenesis (Marshall et al., 2005; Enwere et al., 2004; Kohyama et al., 2005; Pitman et al., 2004; Deleyrolle et al., 2006).

Whilst some studies propose that neurosphere cultures maintain regional identities and intrinsic differences that correspond to the CNS regions they were originally isolated from (Hitoshi et al., 2002; Parmar et al., 2002; Ostenfeld et al., 2002), other studies suggest that the differentiation potential and spatial identity of these cultures are deregulated (Santa-Olalla et al., 2003; Hack et al., 2004; Gabay et al., 2003; Machon et al., 2005). It is therefore not surprising that an ongoing debate ensues regarding the physiological relevance of neurosphere cultures for studies in stem and progenitor cell diversity, phenotype and fate. Moreover neurospheres are not only formed from stem cells but also from progenitor cells with a limited capacity for self-renewal (Reynolds and Rietze, 2005). It is calculated that, less than 10% of neurospheres are derived from *bona fide* NSCs. While the serum-free neurosphere culture system provides an invaluable tool for assaying progenitor cell populations under defined conditions, it is important to note that these cultures are heterogeneous and variable. Nevertheless, this culture system is relevant to model neural development to study oligodendrogenesis from NSC-derived progeny and to identify specific factors involved in these processes.

1.8 Aims of the present work

This project has aimed towards understanding the gene regulatory networks downstream of Mash1 in lineage commitment of neural progenitors and specifically the mechanisms underlying Mash1 activity in oligodendroglial commitment, for which nothing is known. We hypothesise that Mash1 interacts with either neuronal- or oligodendroglial-specific cofactors and that these interactions are responsible for modulating Mash1 activity and subsequent regulation of target promoters in different cells, resulting in the specification of neuronal or oligodendroglial commitment, respectively.

There have been three main components to this work:

1) An *in vitro* cellular system to investigate the molecular mechanisms of Mash1 in oligodendroglial cell fate specification was used to identify genomic regions bound by Mash1 with ChIP-on-chip technology.

2) Mouse transient transgenics were used to interrogate the *in vivo* activity of genomic segments bound by Mash1 in order to isolate regions with activity in the oligodendroglial lineage.

3) ChIP analysis of other oligodendroglial promoting TFs, namely Olig2 and Sox9, were performed to identify TF co-occupancy in genomic regions bound by Mash1.

Chapter 2

Material and Methods

2.1 Animals

Mice were housed, bred and treated according to the guidelines approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986. Transgenic mouse lines, *Rosa26YFP* (Srinivas et al., 2001), *Sox10Cre* (Matsuoka et al., 2005) and *Mash1Δ* (Guillemot et al., 1993), were genotyped as described below. Wild-type Parkes mice were used. Timed-mated mice were set up and midday of the day of vaginal plug discovery was considered as embryonic day E0.5. Embryos were harvested at E12.5-E14.5.

2.1.1 Genotyping

All mice were genotyped by PCR using 1μl of non-quantified extracted DNA. Genomic DNA was extracted from tail or ear biopsies by overnight incubation in 100mM Tris HCl, pH8.5, 200mM NaCl, 5mM EDTA, 0.2% SDS, 100mg/ml proteinase K at 55°C. Samples were centrifuged at 10,000rpm for 10 minutes, the supernatant was precipitated using 1 volume of isopropanol, centrifuged and washed with 70% ethanol. Genomic DNA was resuspended in 50μl of water. PCR were performed using LIM buffer (67mM Tris HCL, pH8.8, 6.7mM $MgCl_2$, 170mg/ml BSA, 16.6mM $(NHS_4)_2SO_4$), 10% DMSO, 1.5mM dNTPs, 22.5ng of each primer reverse and forward, and 1.25 units of Taq DNA polymerase (AB gene).

Rosa26YFP mice were genotyped using forward primer 5'-gctctgagttgttatcagtaagg-3', reverse wild-type locus 5'-gcgaagagtttgtcctcaacc-3', reverse transgenic locus 5'-ggagcgggagaaatggatagt-3'. The PCR program used was 95°C, 2.5 minutes, 35 cycles of 94°C, 30 seconds, 55°C, 30 seconds, 72°C, 45 seconds and a final extension at 72°C, 10 minutes.

Sox10Cre mice were genotyped using forward primer 5'- atccgaaaagaaaacgttga-3' and reverse primer 5'-atccaggttacggatatagt-3'. The PCR program used was 94°C, 3 minutes, 35 cycles of 94°C, 1 minute, 53°C, 1 minute 10 seconds, 72°C, 1 minute and a final extension at 72°C, 5 minutes.

Mash1Δ mice were genotyped using forward primer 5'-ccaggactcaatacgcaggg-3' and reverse primer 5'- gcagcgcatcgccttctatc-3' for the *Mash1Δ* allele, and forward primer 5'-ccaggactcaatacgcaggg-3' and reverse primer 5'-ctccgggagcatgtccccaa-3' for the *Mash1* wild-type allele. The PCR program used was 94°C, 5 minutes, 35 cycles of 94°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute and a final extension at 72°C, 10 minutes.

Transient Transgenic mice were genotyped for the presence of the *lacZ* gene using forward primer 5'-gcacatccccctttcgccagctggcgtaat-3', and reverse primer 5'-cgcgtctggccttcctgtagccagctttca-3'. The PCR program used was 95°C, 5 minutes followed by 33 cycles of 94°C, 1 minute, 58°C, 1 minute, 72°C, 1 minute. Product is approximately 400bp.

2.2 Molecular Biology: Cloning

All plasmids used for cloning were analysed and prepared using standard Qiagen maxiprep and miniprep kits according to manufacturers instructions. Restriction digests were carried out according to Current protocols in Molecular Biology using buffers and restriction enzymes from Roche or New England Biolabs. Restriction digests were purified using a Qiagen gel extraction kit according to the manufacturers instructions or by phenol/chloroform extraction. Plasmids were grown in competent DH5a *Escherichia Coli* bacteria in Luria-Bertani (LB) medium containing 100mg/ml of the antibiotic ampicillin (Sigma). All LB/agar plates used for the isolation of single colonies contained 100mg/ml of ampicillin.

2.2.1 Isolation of RNA and cDNA synthesis

To generate an Engrailed repressor of Mash1, and expression constructs for Sox9 and Olig2 the open reading frame of mouse Mash1 bHLH, Sox9 and Olig2 respectively, were isolated by reverse transcriptase-PCR (RT-PCR). Total RNA was extracted from the heads of two E13 embryos dissected in phosphate buffered saline (PBS). Tissue was transferred to 1ml of TRIzol (Invitrogen) and homogenised on ice. The suspension was incubated at room temperature for 5 minutes before adding 300µl of chloroform and inverting the mixture several times, followed by incubated at room temperature for 3 minutes. Phases were then separated by centrifugation at 4°C, 12,000g for 15 minutes. The aqueous upper phase was removed and precipitated using 500µl isopropanol for 10

minutes at room temperature. The sample was then centrifuged at 4°C, 12,000g for 10 minutes and the pellet washed in 1ml of 75% ethanol and subsequently centrifuging at 4°C, 12,000g for 5 minutes. The pellet was then air-dried and dissolved in 100µl RNase free water. All solutions used throughout this protocol were made using RNase free water. The concentration of the RNA was checked with a spectrophotometer and the integrity of the sample was confirmed by running 3µg of sample in a 1% Tris-acetate- ethylenediaminetetraacetic acid (TA-EDTA or TAE) gel. Single stranded cDNA was synthesised using the High Capacity RNA-to-cDNA Kit according to the manufacturers instructions (Applied Biosystems). Briefly, 2µg of total RNA were used per 20µl reaction, which was incubated at 37°C for 60 minutes and stopped by heating to 95°C for 5 minutes.

2.2.2 Cloning full-length mouse Mash1 bHLH domain, Sox9 and Olig2 expression constructs

Mash1 bHLH open reading frame was amplified with high fidelity PCR using gene specific primers (forward primer 5'-attaccatggtggcgcgccgcaacgagcgc-3' which had an NcoI restriction enzyme site incorporated, and reverse primer 5'-gtatgaattcgtggtgctcgtccagcagctg-3', with an EcoRI site for cloning) and mouse cDNA as template. PCR was performed as follows 95°C, 5 minutes, 30 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°C, 1 minutes and a final extension at 72°C, 10 minutes. The resulting 135bp product was purified by gel extraction and digested using NcoI/EcoRI restriction enzymes. This fragment was then ligated with pSlax EngR vector also digested with NcoI/EcoRI restriction enzymes and

minipreps were sequenced to identify full-length mouse Mash1 bHLH. Subsequently Mash1 bHLH-EngR (the mouse Mash1 bHLH domain in frame with the EngR domain; Smith and Jaynes, 1996) was subcloned upstream of an IRES and an NLS-tagged GFP in the pCAGGS expression vector, otherwise referred to as pCAGGS IRES GFP.

Olig2 and Sox9 expression constructs were cloned from mouse cDNA with high fidelity PCR into the EcoRV/NheI sites of the pCAGGS IRES GFP vector. In brief, the Olig2 open reading frame was amplified using gene specific primers (forward primer 5'-gtacgatatcgccaccatggactcggacgccagcct-3' containing a EcoRV cloning site and a Kozak sequence preceding the start codon, reverse primer 5'-gtccgctagctcacttggcgtcggaggtga-3', containing an NheI restriction site for cloning). PCR was performed as follows 95°C, 5 minutes, 35 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°C, 2 minutes and a final extension at 72°C, 10 minutes. The resulting 972bp product was purified by gel extraction and digested using EcoRV/NheI restriction enzymes. The Sox9 open reading frame was amplified with gene specific primers (forward primer 5'-gtacgatatcgccaccatgaatctcctggacccct-3' containing a EcoRV cloning site and a Kozak sequence preceding the start codon, reverse primer 5'-gtccgctagctcagggtctggtgagctgt-3', containing an NheI restriction site for cloning). PCR was performed as follows 95°C, 5 minutes, 35 cycles of 94°C, 1 minute, 50°C, 1 minute, 72°C, 4 minutes and a final extension at 72°C, 10 minutes. The resulting 2927bp product was purified by gel extraction and digested using EcoRV/NheI restriction enzymes. Olig2 and Sox9 fragments were then ligated with pCAGGS IRES GFP vector also digested with

EcoRV/NheI restriction enzymes and minipreps were sequenced to identify full length mouse Olig2 and Sox9 respectively. Olig2 and Sox9 protein expression was verified by immunofluorescence with antibodies to Olig2 and Sox9, respectively. The mix for all high fidelity PCRs contained LIM buffer, 10% DMSO, 04mM dNTPs, 750ng each primer, 2.5 units of Pfu DNA polymerase and 100ng of cDNA.

2.2.3 Cloning *Sox9, Olig2, Notch1* and *Brevican* putative enhancers for reporter gene assays

For luciferase reporter assays, *Olig2* and *Sox9* putative distal enhancer elements were cloned from mouse genomic DNA with high fidelity PCR into the NheI/SalI sites of the luciferase reporter vector p-βglob-*Luc*. In detail, the *Olig2* genomic element was amplified with gene specific primers (forward primer 5'-gtacgtcgacagacccataaacacatagata-3' containing an SalI cloning site, reverse primer 5'-gtccgctagcagaggtttgcttctggaagct-3' containing an NheI cloning site). PCR was performed as follows 95°C, 5 minutes, 40 cycles of 94°C, 1 minute, 58°C, 1 minute, 72°C, 2 minutes and a final extension at 72°C, 10 minutes. The resulting 848bp product was purified by gel extraction and digested using SalI/NheI restriction enzymes. The *Sox9* genomic element was amplified using gene specific primers (forward primer 5'-gtacgtcgactaaaccaccgggaacattca-3' containing an SalI cloning site, reverse primer 5'-gtccgctagcgcacccctattctgttggg-3' containing an NheI cloning site). PCR was performed as follows 95°C, 5 minutes, 40 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°C, 1 minute and a final extension at 72°C, 10 minutes. The resulting 187bp product was purified by gel

extraction and digested using SalI/NheI restriction enzymes. *Olig2* and *Sox9* fragments were then ligated with p-βglob-*Luc* vector also digested with SalI/NheI restriction enzymes and minipreps were sequenced to identify full length mouse *Olig2* and *Sox9* genomic elements, respectively.

For the generation of transgenic reporter mice, *Olig2*, *Sox9*, *Notch1* and *Brevican* putative enhancer sequences were cloned from mouse genomic DNA with high fidelity PCR into the NotI/SpeI sites as 3' enhancer element into a *lacZ* reporter vector harbouring the basal human β-globin promoter (*BGZA*). In detail, the *Olig2* genomic element was amplified using gene specific primers (forward primer 5'- gtacgcggccgcagacccataaacacatagata-3' containing an NotI cloning site, reverse primer 5'- gtccactagtagaggtttgcttctggaagct-3' containing SpeI cloning site). PCR was performed as follows 95°C, 5 minutes, 40 cycles of 94°C, 1 minute, 58°C, 1 minute, 72°C, 2 minutes and a final extension at 72°C, 10 minutes. The resulting 848bp product was purified by gel extraction and digested using NotI/SpeI restriction enzymes. The *Sox9* genomic element was amplified using gene specific primers (forward primer 5'-gtacgcggccgctaaaccaccgggaacattca-3' containing an NotI cloning site, reverse primer 5'-gtccactagtgcacccctattctgttggg-3' containing SpeI cloning site). PCR was performed as follows 95°C, 5 minutes, 40 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°C, 1 minute and a final extension at 72°C, 10 minutes. The resulting 187bp product was purified by gel extraction and digested using NotI/SpeI restriction enzymes. The *Notch1* genomic element was amplified using gene specific primers (forward primer 5'-gtacgcggccgccaggagtggtggatccctc-3' containing a NotI cloning site, reverse primer 5'-

gtccactagtctaggaaggaaattgacccgtgt-3’ containing a SpeI cloning site). PCR was performed as follows 95°C, 5 minutes, 35 cycles of 94°C, 1 minute, 55°C, 1 minute, 72°C, 2.5 minutes and a final extension at 72°C, 10 minutes. The resulting 1632bp product was purified by gel extraction and digested using NotI/SpeI restriction enzymes. The *Brevican* genomic element was amplified using gene specific primers (forward primer 5’-gtacgcggccgcgacttctcattggttaaaggg-3’ containing a NotI cloning site, reverse primer 5’-gtccactagtcttggtttctttcctagctc-3’ containing a SpeI cloning site). PCR was performed as follows 95°C, 5 minutes, 35 cycles of 94°C, 1 minute, 50°C, 1 minute, 72°C, 2 minutes and a final extension at 72°C, 10 minutes. The resulting 1166bp product was purified by gel extraction and digested using NotI/SpeI restriction enzymes. *Olig2*, *Sox9*, *Notch1* and *Brevican* putative enhancer sequences were then ligated with *BGZA* vector also digested with NotI/SpeI restriction enzymes and minipreps were sequenced to identify corresponding full length mouse genomic elements. The mix for all high fidelity PCRs contained LIM buffer, 10% DMSO, 04mM dNTPs, 750ng each primer, 2.5 units of Pfu DNA polymerase and 100ng of genomic DNA.

2.3 Generation of transgenic mice and LacZ staining

All fragments for pronuclear injection were linearised with NotI restriction enzyme and gel purified on a 1% TAE gel followed by extraction using GFX Gel Band Purification Kit (GE Healthcare) and resuspended in injection buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA). Transgenic mice were generated by standard procedures using fertilized eggs from (CBA x

C57BL/10)F1 embryos and founder animals. Staged transgenic embryos were dissected from the uterus in cold PBS and fixed in 4% paraformaldehyde (PFA) at 4°C for 30 minutes, and washed with PBS. *LacZ* positive embryos, determined by PCR genotype, were cryoprotected in 20% (w/v, in PBS) sucrose overnight at 4°C, frozen in embedding medium, Optimal Cutting Temperature (OCT) (BDH), and stored at -80°C. Sections (10μm) were prepared using a Microm cryostat (Zeiss). Embryo sections were stained at 37°C overnight in X-Gal staining solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 5mM EGTA, 0.01% deoxycolate, 2mM $MgCl_2$, 0.4mg/ml X-gal, 0.02% NP-40 in PBS). The staining reaction was stopped by washing in PBS/0.02% NP-40, sections were post-fixed with 4% PFA for 20 minutes at room temperature, washed with PBS three times for 10 minutes, and mounted with Aquamount (BDH). Images were captured using a ProgRes C14 camera (Jenoptik) linked to an Axioplan II microscope (Zeiss). Images were processed using and Adobe Photoshop CS v8.0 (Adobe Systems) software package.

2.4 Flow cytometric analysis and cell sorting

Sox10Cre/Rosa26YFP embryos (E12.5) were dissected in cold Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) and the ventral telencephalon dissociated using a P200 Gilson to obtain a single cell suspension and passed through a 40μm cell strainer (VWR). Cells were subsequently sorted for GFP expression and 7-Aminoactinomycin D (7-AAD), to exclude dead cells, using a MoFlo cytometer (Dako UK, Ely, United Kingdom). Flow cytometry data were analysed on FlowJo 8.8.6 software package.

2.5 *In vitro* cell culture

2.5.1 NS5 cells

NS5 cells were cultured as described previously (Conti et al., 2005), with the following modification; cells were propagated on laminin-coated flasks (10μg/ml, Sigma) rather than on gelatin.

2.5.2 Neurosphere formation and neurosphere-derived NSCs as monolayer cultures

Parkes embryos (E13.5) were dissected in DMEM:F12 (Gibco) supplemented with 100 IU/ml Penicillin and 100μg/ml Streptomycin (Gibco) on ice. Ventral telencephalic tissues were isolated and transferred into 0.5ml neurosphere proliferation medium (NPM). Briefly, for 100ml of NPM; DMEM-F12, -L-Glutamine (Gibco), 93.5ml; 40% Glucose (Sigma), 1.5ml; final concentration 100U/ml Penicillin, 1ml; final concentration 100μg/ml streptomycin sulphate, 1ml; L- Glutamine (200mM stock), 1ml; N2 supplement 10X (Invitrogen), 1ml; B27 5X (Invitrogen), 2ml; Human recombinant bFGF (R&D Systems) 1μg/μl stock, 2μl; Human recombinant EGF (R&D Systems) 1μg/μl stock, 2μl. The tissues were mechanically dissociated using a P200 Gilson to obtain a single cell suspension and passed through a 40μm cell strainer (VWR). The numbers of cells were counted using a haemocytometer, and cells were then plated at a density of 5 x 10^4 cells/ml in NPM in tissue culture dishes. Cells were cultured at 37°C, 5% CO_2 and 24 hours later cells were again

dissociated and passed through a cell strainer. Seventy-two hours after initial seeding the medium was replaced with fresh NPM. Primary neurospheres formed in this assay were maintained in culture for a total of 10 days, with a medium change every day.

For NSC monolayer cultures primary neurospheres were mechanically dissociated after 10 days of neurosphere formation using a P200 Gilson in the presence of Accutase (Sigma) and passed through a 40μm cell strainer (VWR) to obtain a single cell suspension. Cultures were propagated with NPM as described above on poly-ornithine (10μg/ml, Sigma) and laminin (5μg/ml, Sigma) coated culture flasks. Cultures were thereafter maintained as adherent cultures for a total of 10 passages after which fresh cultures were started.

2.5.2.1 Neurosphere differentiation

Primary neurospheres at day 10 of neurosphere formation were transferred into neurosphere differentiation medium (same as NPM, but without EGF and FGF mitogens) on poly-ornithine (10μg/ml, Sigma) and laminin (5μg/ml,Sigma) coated glass coverslips. Neurospheres were allowed to differentiate for 5 days at 37°C, 5% CO_2, and half of the medium changed every day with fresh neurosphere differentiation medium.

2.5.3 Oligospheres Cultures

Oligospheres were cultured as described previously (Chen et al., 2007).

2.6 Immunohistochemistry and Immunocytochemistry

Mouse embryos were dissected in ice-cold PBS and heads were fixed in 4% PFA for 30 minutes at 4°C, washed with PBS, transferred into 15% sucrose in phosphate buffer (PB) pH 7.2 overnight at 4°C, embedded in 7.5% gelatin, 15% sucrose in PB at 42°C, frozen in –40°C isopentane and stored at –80°C. Sections (10μm) were prepared using a Microm cryostat (Zeiss). For immunohistochemistry, frozen sections were air dried, washed in PBS at 42°C to remove the gelatin and processed for immunofluorescence. At least three embryos were analysed per condition, unless specified otherwise. Sections were treated with a blocking solution (PBS plus 10% normal goat or donkey serum and 0.1% TritonX-100, PBS-T) for 30 minutes at room temperature and incubated with primary antibodies overnight at 4°C. Oligospheres were allowed to pellet by gravity in a microcentrifuge tube, rinsed briefly with PBS, fixed with 4% PFA at 4°C for 10 minutes, washed three times with PBS and further processed as described for embryonic tissue above. At least five oligospheres were analysed per condition, unless specified otherwise. For immunocytochemistry, cells were fixed for 10 minutes with 4% PFA at room temperature, washed with PBS, blocked for 15 minutes at room temperature and incubated with primary antibodies overnight at 4°C.

Primary antibodies; rabbit anti-β-galactosidase (1:20,000; Cappel), rabbit anti-GFAP (1:1,000; DAKO), mouse anti-Tuj1 (1:1,000; Sigma), mouse anti-HuC/D (1:200; Molecular Probes), rabbit anti-GFP (1:1,000; Molecular Probes), sheep anti-GFP (1:1,000; Molecular Probes), rabbit anti-MBP (1:1,000; Abcam),

goat anti-Sox9 (1:500; R&D Systems), rat anti-PDGFRα (1:800; BD Pharmigen), mouse anti-O4 (a kind gift from B. Zalc), mouse anti-Mash1 (1:100; Hybridoma supernatant, generated in-house), rabbit anti-Sox2 (1:800; Chemicon) and rabbit anti-Olig2 (1:1,000; Chemicon). Corresponding conjugated fluorescent secondary antibodies, Alexa 488 (green 1:1,000; Millipore) or Cy3 or Cy5 (red; 1:500; and blue; 1:500 respectively; Jackson ImmunoResearch), were incubated for 2 hours or 1 hour at room temperature for tissue sections or monolayer cell cultures, respectively. Sections were washed in PBS, incubated at room temperature with 4',6-Diamidino-2-phenylindole (0.1μg/ml, DAPI, Sigma) for 15 minutes, and washed again with PBS before mounting with fluorescent mounting medium Aquamount (BDH). Note that some oligosphere sections the nuclei were counterstained with TOTO-3 (1:1,000) for 2 hours at room temperature (simultaneously with the secondary antibodies). Images were acquired with an epifluorescent microscope (Axioplan II, Zeiss) equipped with a CCD (charge-coupled device) digital camera (ProgRes C14, Jenoptik) and Openlab software (Improvision), or a laser scanning confocal microscope (Radiance 2100, BioRad). Cell counts were performed on representative fields. Images were processed using and Adobe Photoshop CS v8.0 (Adobe Systems) software package.

2.7 Cell Transfections

Neurosphere-derived NSCs were transfected using the mouse NSCs Nucleofector™ kit and optimised protocols provided by the manufacturer (Lonza). Briefly, 5×10^6 cells were resuspended in 100μl of the mouse NSC

Nucleofector™ solution (pre-warmed to room temperature). Cells were mixed with 5µg of DNA, transferred into an Amaxa certified cuvette and transfected with the program A-33. Immediately after transfection, 500µl of the 37°C pre-warmed culture medium was added, and cells were plated onto PORN/laminin coated coverslips at a final concentration of 200,000 cells/ml (100,000 cells/well) in differentiation medium. All cDNAs were expressed from pCAGGS IRES GFP. Medium was changed three hours after plating, when the majority of cells had adhered, to remove cellular debris. Data are represented as means of triplicates, and experiments were repeated three times.

2.9 Luciferase assays

NS5 cells were seeded 1 day earlier onto 48-well plates (Nunc) at a density of $1.2x10^5$cells/well and maintained under propagation culture conditions (as described in Conti et al., 2005). Transient transfections were performed using lipofectamine transfection reagent according to the manufacturers instructions (Invitrogen). All cDNAs were expressed from pCAGGS IRES GFP (Sox9EngR expression construct, transactivation domain of chick Sox9 containing amino acid 265–495 replaced by Engrailed transcriptional repressor domain; Cheung et al., 2005; and the Mash1 expression construct; Geoffroy et al., 2009). The luciferase reporter constructs used the β-globin minimal promoter. The amount of expression and reporter plasmid used for each transfection was 375ng/well. Cells were harvested 24 hours after transfection and extracts were assayed according to manufacturers instructions using the Promega luciferase system. Values (relative luciferase units, RLU) were corrected for protein expression

through co-transfection with CMV-β-gal plasmid and measurement of β-Galactosidase (250ng plasmid/well). Data is shown as the mean of quadruplicate values obtained from representative experiments. The error bars represent the standard deviation of the mean, which was calculated from quadruplicates.

2.10 Quantitative RT-PCR

For quantitative real-time RT-PCR analysis, RNA was extracted from oligosphere and neurosphere cultures with TRIzol (Invitrogen) as described above, followed by Qiagen RNeasy kit according to the manufacturers instructions. The integrity of the RNA was assessed by spectrophotometry. A total of 25ng of RNA was used for reverse transcription with the High Capacity RNA-to-cDNA Kit (Applied Biosystems) to synthesis cDNA as described above. Final volumes of cDNA were diluted 1:3 and 2μl were used as template for amplification. The following program was used 50°C, 2 minutes, 95°C, 10 minutes, 40 cycles of 95°C, 15 seconds, 60°C, 1 minute, 72°C, 1 minute. To quantitate mRNA levels, cDNA samples were analyzed in triplicate by Real-Time PCR using TaqMan Gene Expression Assays for the appropriate genes, on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Transcript levels of targets were normalized to levels of housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, as an internal control. Assay ID; Sox10, Mm01300162_m1; PDGFRα, Mm01211694_m1; Olig2, Mm01210556_m1; Sox9, Mm00448840_m1; Mash1, Mm03058063_m1; GAPDH, Mm03302249_g1. Data is shown as the mean of triplicate values

obtained from representative experiments. The error bars represent the standard deviation of the mean, which was calculated from triplicates.

2.11 Chromatin Immunoprecipitation Assays

ChIP assays were performed with oligosphere culture material, with a monoclonal mouse anti-Mash1 antibody (Hybridoma supernatant, generated in-house), polyclonal rabbit anti-Sox9 (AB5535, Chemicon), polyclonal rabbit anti-Olig2 (AB9610, Chemicon) or a purified mouse IgG1, κ isotype antibody (BioLegend) as a negative control. Chromatin from oligosphere cultures were prepared at either 5 or 14 days of oligosphere formation. Briefly, oligospheres were washed in Hank's buffered salt solution (HBSS, Gibco), fixed with 1% formaldehyde (Sigma) for 10 minutes at 4°C, quenched with 125mM of glycine (Sigma) for a further 8 minutes at room temperature, and washed with PBS supplemented with a Protease Inhibitor cocktail (PI, Roche). Oligospheres were then lysed with sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8.0) for 30 minutes at 4°C. Chromatin fragmentation was carried out using sonication with a Bioruptor (UCD-200, Diagenode) for 30 minutes, with an interval cycle time of 30 seconds on, 30 seconds off. The concentration of the chromatin was checked by spectrophotometry and the integrity and size of fragments confirmed by running 1-3μg of sample in a 2% TAE gel.

Chromatin immunoprecipitation (IP) assays with anti-Mash1 antibody were performed with 30μg of chromatin per IP. Initially, chromatin was pre-

cleared with 50µl of Dynabeads Sheep anti-Mouse IgG (110-31, Invitrogen) in IP buffer (1X IP buffer [10X IP Buffer, 0.2M HEPES pH8.0, 2M NaCl; 0.02M EDTA]; 0.1% Na-DOC; 1% Triton X-100, 1mg/ml BSA; 25X PI) for 1.5 hours at 4°C. Pre-cleared chromatin was then incubated with 3µl of Mash1 antibody overnight at 4°C rocking (for control IP 3µl of purified mouse IgG1, κ isotype antibody were used). The following day 50µl of pre-blocked beads were added per IP for 2 hours at 4°C. Beads were then captured with a magnet and 50µl of chromatin from the negative control sample set aside (to be used at 5% input chromatin), beads were washed 5 times with RIPA buffer containing 0.5M LiCl, and once with TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA). Note all washes were done for 4 minutes at room temperature in rotation. Beads were then resuspended in elution buffer (10mM Tris pH8.0, 1% SDS) for 10 minutes at 65°C. The supernatant was then incubated with proteinase K for 2 hours at 42°C, and crosslinks were reversed overnight at 65°C. Chromatin was then extracted by phenol/chloroform, and precipitated with NaAc and ispopropanol, and the pellet washed with 75% ethanol, after which it was air dried and re-suspended in 120µl of water.

Chromatin IP assays with anti-Olig2 (AB9610, Chemicon) and anti-Sox9 (AB5809, Chemicon) antibodies were performed essentially as above, with the following modifications: (i) chromatin was diluted in reduced SDS buffer (50mM HEPES-KOH pH7.5, 150mM NaCl, 2mM EDTA, 1% TritonX-100, 0.1% NaDOC and 0.1% SDS) rather than IP buffer; (ii) 5µl of anti-Olig2 and anti-Sox9 antibodies were added per IP, and were incubated overnight with chromatin and 50µl of pre-blocked beads; (iii) after Olig2 or Sox9 IPs, beads were washed three times with reduced SDS buffer, once with reduced SDS

buffer supplemented with 0.35M NaCl, twice with NP40 wash buffer (10mM Tris-HCl pH8.0, 0.25M LiCl, 1mM EDTA, 0.5% NP40 and 0.5% NaDOC) and once with TE buffer; (iv) to retrieve immunoprecipitates, magnetic beads Dynabeads ProteinG (100-04D, Invitrogen) for Olig2 and Sox9 have been used.

Immunoprecipitated DNA sequences were quantified by real-time PCR (primers are listed below; primers were designed with Primer3 Tool [http://frodo.wi.mit.edu/primer3/] and amplicon sequences where checked by BLAST against the mouse genome to ensure specificity) by using the iCycler iQ Real-Time PCR Detection System (BioRad) and a SYBR-Green-based kit for quantitative PCR (iQ Supermix, BioRad). Quantities of immunoprecipitated DNA were calculated by comparison to a standard curve generated by serial dilutions of input DNA. The data were plotted as means of at least two independent ChIP assays and error bars represent standard deviations. IP efficiency was calculated as the ratio of precipitated sequence over total amount of sequence in the input chromatin.

The primers used for amplification: ActinB forward, 5'-gccatgttcaatggggtact-3', reverse, 5'-ggtgctaagaaggctgttcc-3'; DeltaM forward, 5'-gcgtggctgtcattaagg-3', reverse, 5'-ggtgctgtctgcattacc-3'; Delta3 forward, 5'-atttcctgtccgtttgcctctc-3', reverse, 5'-gtaaatgtcgccatctgc-3'; Lfng forward, 5'-ttaaccaggccagctgtatg-3', reverse, 5'-cattgtccgccagctttg-3'; Fbxw7 forward, 5'-cagctatgttcctgctgtgc-3', reverse, 5'-caacttctgcctgcttcctc-3'; Delta1 ORF forward, 5'-gtctcaggaccttcacagtag-3', reverse, 5'-gagcaaccttctccgtagtag-3'; Fbxw7 ORF forward, 5'-ctcgtcacattggagagtgg-3', reverse, 5'-caggagcttggtttcctcag-3'; Sox9 forward, 5'-gagtaaaccaccgggaacat-3', reverse, 5'-aacgctaactgtggaatcgaa-3';

Olig2 forward, 5’-acaatgtcaggccattagtatgtt -3’, reverse, 5’-acagaatggtgctgttcagg-3’; Dll4 forward, 5’-ggccaggatgaggatatg-3’, reverse, 5’-ggtcctcagctgtatggtaatg-3’; Tubb3 forward, 5’-aggggacgaagcaaagagta-3’, reverse, 5’-aggaacctccacccaagag-3’; Map2 forward, 5’-cgaatgactgccttccctat-3’, reverse, 5’-cccctccttacacaccaaac-3’; Brevican forward, 5’-ttcccactttcctcctcttg-3’, reverse, 5’-gtaaggtcacagcccccac-3’; Cspg4 forward, 5’-ggtacacagatggggctcac-3’, reverse, 5’-cagctactcctgcccttcc-3’; Ascl1 forward, 5’-tccctttgtaggggttgaa-3’, reverse, 5’-ctgttcccgttcctgttcc-3’; Olig1 forward, 5’-ccacccggaactctcttct-3’, reverse, 5’-acttcatcagccccttcttg-3’; Mog forward, 5’-aacccttcgcttcaggct-3’, reverse, 5’-tccctttccaggtcttgatg-3’; Sox8 forward, 5’-ctgtccccgtacccatctta-3’, reverse, 5’-ggctcaccattcctagttgg-3’; Sulf1 forward, 5’-gggagcagatggatgtaattc-3’, reverse, 5’-tgcagcgactttaatgatcc-3’; Sulf1 forward, 5’-tctgcaaaccaccattagga-3’, reverse, 5’-gatgaggaagatgtgggctt-3’; Nfasc forward, 5’-gccaagtagcagaagtgacg-3’, reverse 5’-tttcctgccaatgtgtttct-3’; Notch1 forward, 5’-ttaatcgcctcccaacaata-3’, reverse 5’-cttttctccccttgcag-3’; Tyro3 forward, 5’-tgctgtgtggttaaagaggg-3’, reverse 5’-tgggtgtccctgatctcata-3’; Pld1 forward, 5’-tgcagaagttaaggaaataagcc-3’, reverse 5’-ttgttgctatgtctaccccg-3’; Hmgcs2 forward, 5’-cccttcagtctcgtccaagt-3’, reverse 5’-taagtggaaaggaagccctg-3’; Hnrpdl1 forward, 5’-ccacgcgaactcaaaactta-3’, reverse 5’-gggggaatcggtgtaaaag-3’; Gna12 forward, 5’-gttgggaaatagcgatgagg-3’, reverse 5’-tgaaaatgatgacccaccc-3’; Ptpro forward, 5’-ggcctgacttctctctgctt-3’, reverse 5’-actctgccattaccctcca-3’; BB128963 forward, 5’-ggggtttccagtgtttgttc-3’, reverse 5’-catgaaatcgttgcactcaaa-3’; Gab1 forward, 5’-gaccctagattcccgatgag-3’, reverse 5’-caagatgctgattgtgagatga-3’; Nf1 forward, 5’-caggcaggaagacattttca-3’, reverse 5’-atgctaactgacccatgcag-3’; Sox9 forward, 5’-tcaccacaccagcttcgt-3’, reverse 5’-tgcttagaaatggtcttttgga-3’; Cmtm5 forward, 5’-ttgtctttggcatctgtggt-3’, reverse 5’-

aaggggaagggtggatgt-3’; Fstl1 forward, 5’-cacactgggaacatttgagttt-3’, reverse 5’-tgagctgaacactttaacccc-3’; Olig1 forward, 5’-cttccagaaccctcagcc-3’, reverse 5’-gctgccaaaccttcagtcta-3’; Sox8 forward, 5’-ggtttgggtctacatggacag-3’, reverse 5’-aggtctgagtgtgccaagc-3’; Sema6a forward, 5’-tgctgagaacgtggttaaagat-3’, reverse 5’-ccgggttccttttggtat-3’.

For the preparation of chromatin material for hybridisation to the oligodendrocyte specific array, whole genome amplification was performed with GenomePlex complete Whole Genome Amplification (WGA) Kit (Sigma), with modifications previously described (O’Geen et al., 2006). Technical replicates of Mash1 coprecipitated chromatin and control IgG coprecipitated chromatin were fluorescently labelled with cyanine 5-dUTP dye and input chromatin with cyanine 3-dUTP according to Agilent instructions. The oligodendrocyte specific array was designed using eArray Agilent software (Diogo Castro, NIMR), and printed using SurePrint technology (Agilent). Labelled immunoprecipitated chromatin were hybridised onto the custom designed oligodendrocyte specific array, scanned on an Agilent DNA Microarray Scanner and the initial data extraction performed using Agilent Feature Extractor Software, version 9.5.1 (Doug King, Windeyer Institute, UCL). Further binding data analyses were performed using ChIP Analytics software package (Agilent).

2.12 Statistics

All statistical comparisons were carried out using the ‘Student's’ t-Test.

Chapter 3

Results

3.1 Mash1 and OPCs

Mash1 is a bHLH TF essential for neural differentiation and specification in the nervous system. Indeed, genetic fate-mapping studies using inducible Cre-lox technology have shown that Mash1 is present in a range of cell populations throughout different domains of the CNS, which include proliferating progenitor cells that are already committed to differentiate (Battiste et al., 2007; Kim et al., 2008). Consistent with a broad role in neural specification during CNS development, specific progenitors expressing Mash1 initially generate neurons, and then switch to promote an oligodendroglial cell fate.

Previous results have shown that the function of the proneural gene *Mash1* is necessary for the specification of an oligodendroglial fate in the developing brain and spinal cord, as well as in the postnatal brain (Parras et al., 2004, 2007; Sugimori et al., 2007). Briefly, a subpopulation of OPCs generated from localised ventral domains of the embryonic telencephalon requires Mash1 for their specification (Parras et al., 2007). Notably, Mash1 expression is detected in a subset of OPCs as they emerge in the VZ during the first wave of oligodendrogenesis in the telencephalon at E12.5-E14.5, and this particular population is lost in *Mash1* null mutant mice (Parras et al., 2007).

The fact that Mash1 is involved in regulating the acquisition of distinct cell fates, namely neurons and oligodendrocytes in the CNS, suggests that it functions cooperatively with distinct factors to specify neurogenesis and oligodendrogenesis, respectively. Indeed, Olig2 has been shown to synergise

with Mash1 in the generation of OPCs in loss and gain of function analyses, in the developing mouse ventral telencephalon during the first wave of oligodendrogenesis and in rat neurosphere primary cultures, respectively (Parras et al., 2007; Sugimori et al., 2007). Other oligodendrocyte promoting factors in the CNS include Sox9 (Stolt et al., 2003). However, whether Sox9 is capable of functionally synergising with Mash1 to specify an oligodendroglial cell fate, remains to be established. To further investigate the relationship between Mash1 and Sox9 in OPC specification, we first examined their expression during the first wave of oligodendrogenesis using PDGFRα, one of the first known markers to be expressed in OPCs in the CNS (Woodruff et al., 2001). At E12.5 a subset of cells in the VZ of the ventral telencephalon coexpressed Mash1 and Sox9 (Figure 10A and A1, white arrows), and a smaller number also coexpressed PDGFRα (Figure 10A and A1, white arrowhead). Mash1 expression was maintained in cells away from the VZ, although at low levels, and some of these cells coexpressed Sox9 and PDGFRα (Figure 10A and A2, white arrowheads), whilst others coexpressed Sox9 alone (Figure 10A, white arrow). At E13.5 a subset of cells in the VZ and away from the VZ of the ventral telencephalon coexpressed Mash1 and Sox9 (Figure 10B, B1 and B2, white arrows), and some of these also expressed PDGFRα (Figure 10B, B1 and B2, white arrowheads). In conclusion, these preliminary spatial and temporal analysis of Mash1 and Sox9 expression with the early OPC marker PDGFRα, highlight the potential for these TFs to cooperate in the specification of early born OPCs in the developing ventral telencephalon.

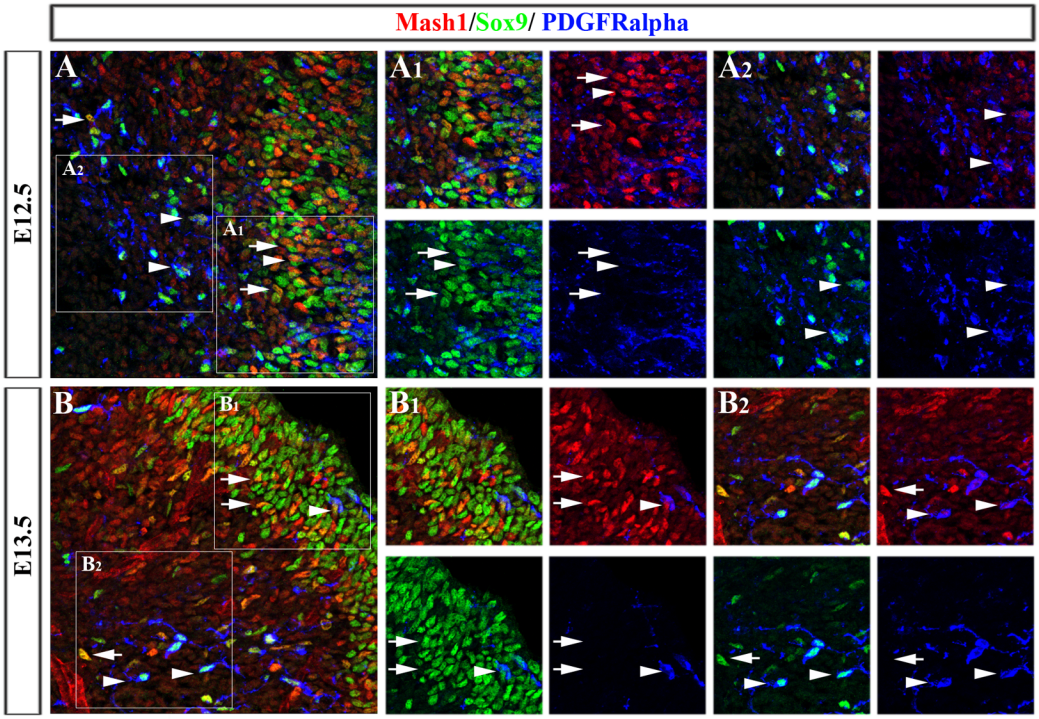


Figure 10. Mash1 and Sox9 are coexpressed in OPCs in the VZ and in OPCs migrating away fr om the V Z in the vent ral telencephalon. ***A***, Wild-type E12.5 mouse coronal section though the ventral telencephalon, Mash1 and Sox9 are coexpressed (white arrows) in the VZ (***A1***) and in OPCs migrating away from the VZ. In addition a subpopulation of these cells also express PDGFRα (white arrowheads) in the VZ (***A1***) and migrating away from the VZ (***A2***). ***B***, At E13.5, in the same region, many Mash1+ cells also express Sox9 (white arrows) in the VZ (***B1***) and migrating away from the VZ (***B2***), a subset of these cells also express PDGFRα in both the VZ (***B1***) and migrating away form the VZ (***B2***) (white arrowheads).

Certainly, we do not understand the precise molecular mechanisms through which Mash1 functions in oligodendroglial cell fate specification. We propose the following, firstly that Mash1 activity is modulated in a regional temporal manner, secondly that Mash1 forms part of a combinatorial network of TFs necessary for OPC specification, and finally that Mash1 behaves in a cell autonomous manner to generate oligodendroglial cells in the CNS.

3.2 *In vivo* cellular system to assay Mash1 function in oligodendrogenesis

To begin to address the mechanisms underlying the role of Mash1 in the specification of oligodendroglial cells in the ventral telencephalon, we first searched for a suitable cellular model system amenable to molecular and genomic analysis using a ChIP-on-chip approach. The Sox10Cre/Rosa26YFP transgenic mouse line irreversibly marks the progeny of Sox10+ cells with the yellow fluorescent protein (YFP), including oligodendrocytes in the developing ventral telencephalon (Figure 11A) (Matsuoka et al., 2005; Srinivas et al., 2001). At E12.5 a sub-population of GFP+ OPCs expressed Mash1 in the VZ and in cells migrating away from the VZ (Figure 11A1, white arrows), some of which also expressed PDGFRα (Figure 11A1, white arrowheads). Fluorescence activated cell-sorting (FACS) analysis of Sox10 Cre/Rosa26YFP ventral telencephalon at E12.5 revealed a very small fraction of GFP+ cells (2000 cells/ ventral telencephalon), amounting to approximately 1.49% of the entire tissue (Figure 11B). Note that these numbers are insufficient for a genomic strategy, such as ChIP-on-chip, where a minimum of 1 million FACS sorted cells per ChIP replica are required (Carlos Parras and Diogo Castro, personal

communication). We concluded that in the developing embryo oligodendrocyte progenitor populations are relatively small, thus their isolation for the purpose of genomic analysis unfeasible. We therefore opted for an *in vitro* cellular approach to interrogate Mash1 function in oligodendrogenesis.

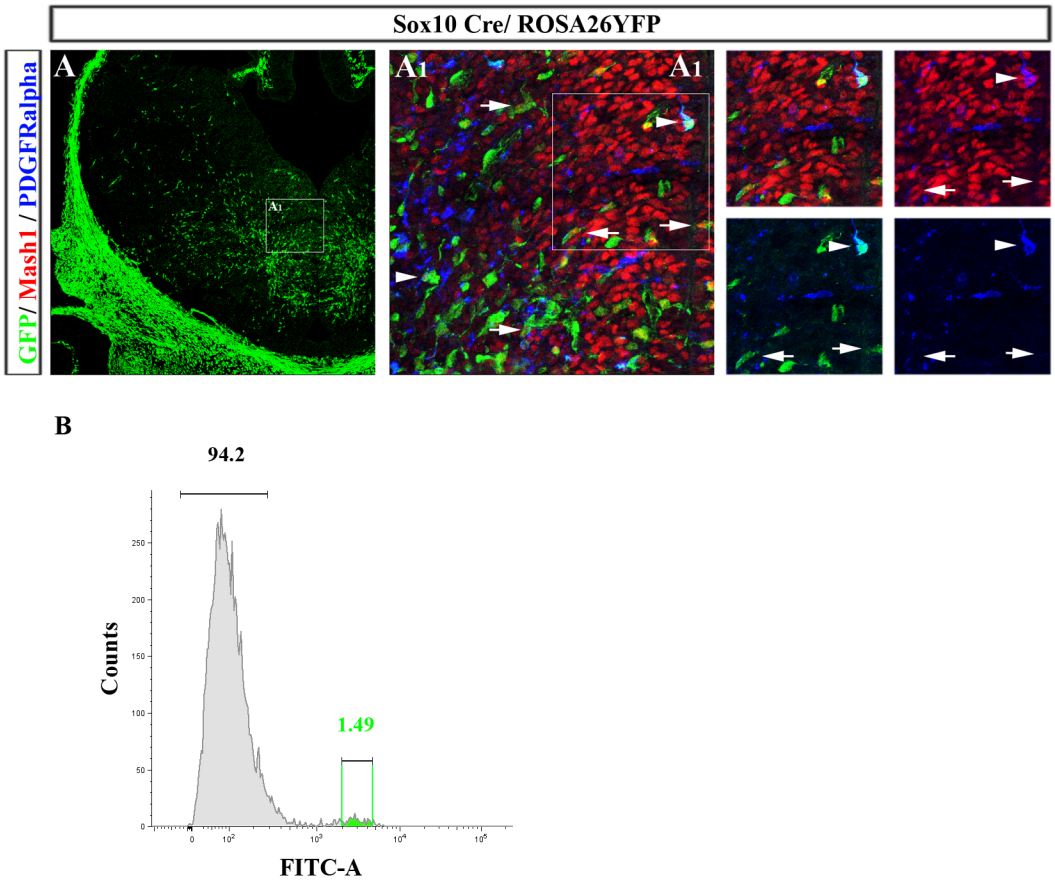


Figure 11. Sox10Cre/ROSA26YFP marks OPCs in the ventral telencephalon at E12.5. ***A***, A coronal section through the telencephalon of a Sox10Cre/Rosa26YFP mouse embryo, immunostained with an anti-GFP antibody. GFP is detected in OPCs in the ventral telencephalon, and is maintained in migrating cells. Inset ***A1*** is an enlargement of the area outlined by the square in ***A***. ***A1***, A small population of Mash1+ cells coexpress GFP in cells of the VZ and migrating away from the VZ (white arrows). A fraction of Mash1+/ GFP+ cells also coexpress PDGFRα in both the VZ and migrating away from the VZ (white arrowheads). ***B***, Histogram representation of E12.5 Sox10Cre/Rosa26YFP ventral telencephalon cell sorting analysis. Only a small fraction (1.49%) of total telencephalic tissue is GFP+.

3.3 *In vitro* cellular system to assay Mash1 function in oligodendrogenesis

3.3.1 CG-4 cell line is a cell fate restricted model

CG-4 cells are a stable cell line derived from rat primary cultures of bipotential O-2A progenitor cells (Louis et al., 1992). They are maintained in a proliferative state in serum-free culture medium supplemented with mitogens FGF-2 and PDGF, and have the capacity to differentiate into either oligodendrocytes or type 2-astrocytes (Louis et al., 1992). Although this cell line has been extensively used to study oligodendrocyte differentiation and maturation, in addition to the process of myelination successfully (Wang et al., 2009; Hoshina et al, 2007; Wei et al., 2007; Zhao et al., 2006; Miskimins et al., 2002; Schnädelbach et al., 2001; Espinosa de los Monteros et al., 1997; Solly et al., 1997; Ranjan and Hudson, 1996; Bhat and Zhang, 1996; Franklin et al., 1995; Tontsch et al., 1994), it represents an already cell fate restricted model that is not appropriate to study the step of specification of multipotent progenitors to the oligodendroglial lineage. Indeed, this cellular model does not recapitulate the *in vivo* differentiation potential of Mash1+ progenitors in the ventral telencephalon, which generate neurons and oligodendrocytes in a sequential manner. We therefore chose not to pursue further with this cell line.

3.3.2 NS5 cell line represents a late stage neural progenitor with a highly restricted differentiation potential

The adherent NS5 cell line, derived from ES cells in the presence of EGF and FGF-2 mitogens, constitutes a pure and homogenous tripotential neural stem cell line that has the capacity to differentiate efficiently into neurons, astrocytes, and oligodendrocytes (Conti et al., 2005; Glaser et al., 2007). Furthermore, these cells can be expanded indefinitely, and thus present a reliable source of neural progenitors for dissecting the mechanisms of neural differentiation. Established protocols for the generation of oligodendrocytes, that consist of an initial phase of proliferation with FGF2, PDGF and forskolin, followed by a differentiation period in the presence of thyroid hormone (T3) and ascorbic acid, have demonstrated that this differentiated cell type can be efficiently generated from NS5 cells (Glaser et al., 2007). Although, approximately 20% of cells in these differentiated cultures contain O4+ oligodendrocytes (Glaser et al., 2007), we have been unable to generate oligodendrocytes from the NS5, and thus have not been capable of replicating these data successfully. In the presence of foetal calf serum (FCS), NS5 cells preferentially differentiated into astrocytes and produce only few neurons (data not shown). We thus propose that these cells represent a later stage neural progenitor, and therefore have a limited neurogenic and oligodendrogenic potential.

To determine whether NS5 cells are nevertheless an appropriate model to study oligodendroglial lineage specification by TFs we used a gain of function strategy and introduced single or combinations of TFs. To date a number of key

molecular determinants of OPC specification have been identified, these include the bHLH transcription factors Olig1 and Olig2 (Lu et al., 2002; Takebayashi et al., 2002; Zhou et al., 2002) and the HMG transcription factor Sox9 (Stolt et al., 2003). Moreover a synergy between Mash1 and Olig2 in the specification of the oligodendroglial cell fate has been well documented both *in vivo* and *in vitro* (Parras et al., 2007; Sugimori et al., 2007). We first analyse the endogenous basal level of expression of these oligodendrocyte promoting TFs in propagating NS5 cells (Figure 12A). Mash1 was expressed in a non-uniform pattern with ranging levels of protein expression in the NS5 cells (Figure 12B and E,) and both Olig2 and Sox9 were expressed in all cells albeit at variable levels (Figure 12C and F, respectively). Note that a large proportion of Olig2 and Sox9 expressing cells also coexpressed Mash1 (white arrowheads in Figure 12D and G, respectively). Endogenous overlapping expression patterns of these TFs in the NS5 cells raised the possibility that this line may retain intrinsic cellular properties important for oligodendroglial cell fate specification. Notably, although the neuronal differentiation of NS5 cells is poor and eventually ends in significant cell death following EGF withdrawal, Mash1 overexpression under conditions of propagation results in the efficient generation of Tuj1+ neurons after 48 hours (Ben Martynoga, personal communication). These data clearly highlight the inherent neuronal differentiation competence of NS5 cells.

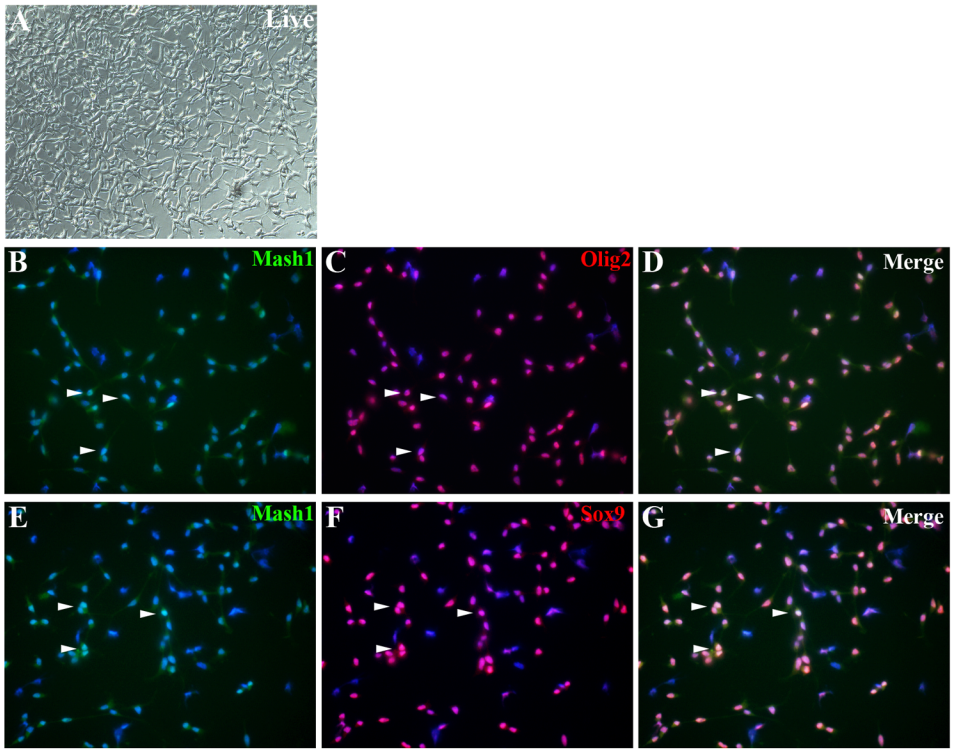


Figure 12. **Mash1, Sox9 and Olig2 are expressed in NS5 cells.** ***A***, NS5 cells propagated in EGF and FGF-2, express Mash1 in a non-uniform manner at variable levels (***B*** **&** ***E***). ***C***, All cells in culture express Olig2, although at different levels. ***D***, A large number of NS5 cells coexpress both Mash1 and Olig2 (white arrowheads). ***F***, All cells express Sox9, although at different levels. ***G***, A large proportion of NS5 cells coexpress both Mash1 and Sox9 (white arrowheads). Note that all nuclei are counterstained with DAPI.

To test the idea that NS5 cells retain the competence to generate oligodendrocytes, Mash1, Olig2 and Sox9 TFs were expressed individually and in combination under propagation conditions. Briefly, we use Nucleofector technology that lends high efficiency and robust transfection with high cell viability in cells that are difficult to transfect, such as the NS5, to transport DNA directly into the nucleus. A bicistronic vector (pCAGGS-IRES-nls-GFP) that encoded either Mash1, Olig2 or Sox9 and nuclear targeted version of the jellyfish green fluorescent protein (GFP, as a marker to identify transfected cells; pCAGGS-Mash1-IRES-nls-GFP; pCAGGS-Olig2-IRES-nls-GFP; and pCAGGS-Sox9-IRES-nls-GFP, respectively), under the control of the CMV immediate enhancer/β-actin (CAG) promoter, was used to attain high transgene levels of expression (Niwa et al., 1991). Seventy-two hours after nucleofection, cultures were analysed by immunocytochemistry to identify differentiated cell types, Tuj1+ neurons, O4+ oligodendroglia and GFAP+ astrocytes, respectively. We found that no single factor or indeed combination of factors were sufficient to drive oligodendroglial cell fate specification in NS5 cells. Moreover whilst overexpression of Mash1 alone generated Tuj1+ neurons (data not shown), neither Olig2 nor Sox9 TFs induced the expression of the differentiated cell markers analysed. In conclusion, these data demonstrate that the exogenous addition of Olig2 and Sox9 TFs alone are insufficient to drive differentiation in NS5 cells.

Notably, these results were unexpected since a specific role for these factors in oligodendroglial cell fate specification in culture have been well-documented. Briefly, ectopic expression of Sox9 in the mouse Neuro2a neuroblastoma cells, leads to the induction of several oligodendrocyte markers,

including Sox10, proteolipid protein (PLP) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (Stolt et al., 2003). A function of Olig2 in oligodendroglial cell fate when overexpressed in both human and rodent progenitors has been well established (Hwang et al., 2009; Maire et al., 2009; Sugimori et al., 2007; Copray et al., 2006; Marshall et al, 2005). Neurosphere cultures established from E13.5 rat spinal cords, infected with a recombinant retrovirus to overexpress Olig2 induce the generation of O4+ oligodendrocytes (Sugimori et al., 2007). In addition, neurosphere-derived NSCs isolated from the embryonic mouse brain (E14), transfected with Olig2 expression vector using the Nucleofector system, induces the development of fully mature oligodendrocytes after 4 days in culture, and functional oligodendrocytes in the demyelinated corpus callosum of cuprizone treated animals (Copray et al., 2006). However, on the contrary exposure of NS5 cells to a recombinant Olig2 protein variant containing the transduction domain from the human immunodeficiency virus-1, transcriptional activator protein (TAT), under oligodendrocyte promoting culture conditions (Glaser et al., 2007) are insufficient to instruct oligodendroglial fate specification (Kristin Stock, personal communication).

Olig2 activity is modulated according to the cellular context. In the developing spinal Olig2 functions sequentially in motorneuron and oligodendrocyte fate specification. This dual action is facilitated by spatio-temporal alterations in the expression domains of other TFs with which Olig2 functionally interacts (Zhou et al 2001). Just prior to OPC formation, the domains of Olig2 and Nkx2.2 expression switch from being mutually exclusive to overlapping, and Neurogenins 1 and 2 are extinguished within this region. Indeed, coexpression of Olig2 and Nkx2.2, promotes ectopic and premature

oligodendrocyte differentiation in the embryonic chick spinal cord (Zhou et al 2001; Sun et a., 2001) and are necessary and sufficient for oligodendrocyte differentiation and myelin gene expression (Fu et al., 2002).

Together, these data raise the possibility that perhaps Olig2 protein levels are already saturating, or that cofactors required for its function are limiting, and thus increasing the levels of Olig2 are simply insufficient to promote oligodendrogenesis in NS5 cells. These hypothetical scenarios could also account for the inability of Sox9 to induce an oligodendroglial cell fate in NS5 cells. In conclusion, we propose that NS5 cells represent a late stage progenitor with a highly restricted differentiation potential, which can only be overruled with an instructive differentiation factor, such as Mash1. Consequently, this *in vitro* cellular system was regarded as unsuitable for investigating the role of Mash1 and potential cofactors in oligodendroglial cell fate specification.

3.3.3 Primary neurosphere-derived NSC cultures

The fact that primary multipotent neural progenitor cells generate oligodendroglial lineage-restricted precursors following overexpression of specific TFs (Sugimori et al., 2007: Copray et al., 2006; Balasubramaniyan et al., 2004; Lu et al., 2000), suggests that primary cultures may have an enhanced intrinsic ability to generate oligodendroglial cells in response to TFs. Primary neurosphere-derived NSC cultures were therefore established from E13.5 ventral telencephalon. In brief, non-adherent spherical clusters of cells otherwise referred to as neurospheres formed after 10 days *in vitro* in the presence of EGF and FGF-2 (Figure 13A). Neurospheres were tripotential, and 5 days after growth factor withdrawal gave rise to neurons, astrocytes and oligodendrocytes (Figure 13B & C). Dissociated neurospheres were pooled and expanded as monolayer cultures (Figure 14A), which expressed the neuroepithelial marker Sox2 (Figure 14B). We first examine basal endogenous levels of Mash1, Olig2 and Sox9 TFs. Primary cultures expressed Mash1 in a non-uniform pattern with ranging levels of protein expression (Figure 14C, F). Olig2 and Sox9 were expressed in all cells albeit at variable levels (Figure 14D and G, respectively), and a large proportion coexpressed Mash1 (Figure 14E and H, respectively, white arrowheads). Notably, neurosphere-derived NSC cultures exhibited some degree of heterogeneity, and contained small numbers of GFAP+ astrocytes (Figure 14I), Tuj1+ neurons (Figure 14J) and O4+ oligodendroglial cells (Figure 14K).

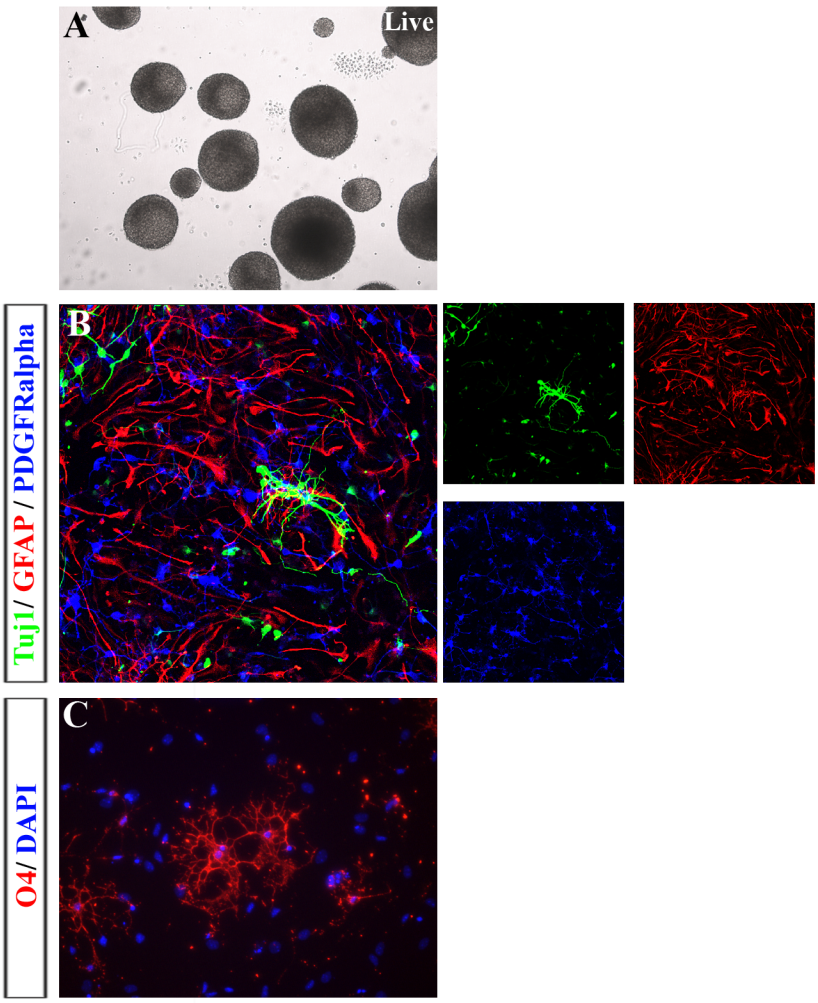


Figure 13. **Neurospheres are tripotential.** ***A***, Dissociated E13.5 ventral telencephalon cultured for 10 days in the presence of EGF and FGF-2, results in the formation of non-adherent neurospheres. ***B***, Neurospheres plated on poly-ornithine/laminin coated surfaces and cultured for 5 days in the absence of growth factors give rise to Tuj1+ neurons, GFAP+ astrocytes and PDGFα+ OPCs. ***C***, O4+ oligodendroglial cells are also generated under these differentiation conditions.

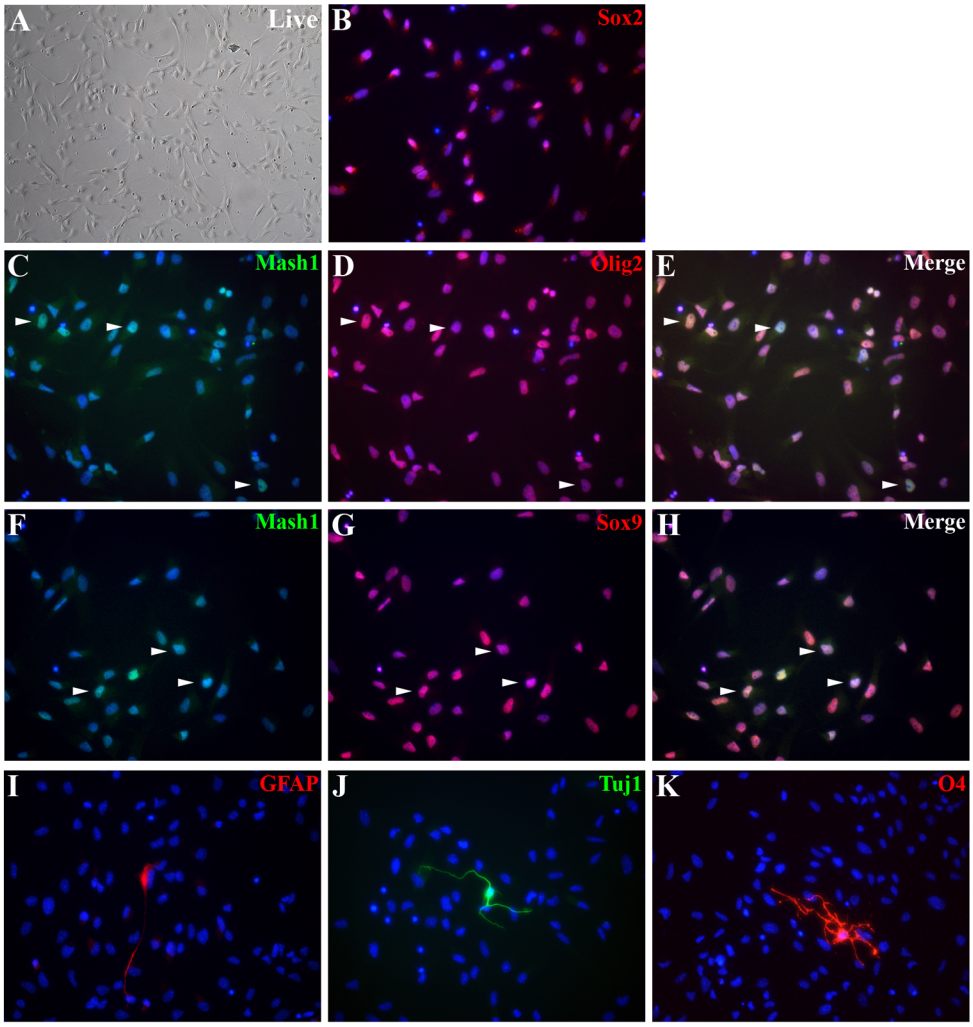


Figure 14. **Monolayer Neurosphere-derived NSCs express Mash1 Olig2 and Sox9, and display some degree of heterogeneity.** ***A***, Dissociated neurospheres plated on poly-ornithine/laminin surfaces form monolayer cultures that can be propagated in the presence of EGF and FGF-2. ***B***, Primary cultures express the neuroepithelial marker, Sox2. ***C & F***, Mash1 is expressed in a non-uniform pattern and at variable levels. ***D***, Olig2 is expressed in all cells although at different levels and there is a significant degree of overlap with Mash1 protein (***E*** white arrowheads). ***G***, Sox9 is expressed in all cells although at different levels and there is a significant degree of overlap with Mash1 protein (***H*** white arrowheads). Neurosphere-derived NSC cultures are heterogeneous and have a small number of contaminants, GFAP+ astrocytes (***I***), Tuj1+ neurons (***J***) and O4+ oligodendrocytes (***O***). Note that all nuclei are counterstained with DAPI.

To promote the generation of oligodendroglial cells we used a gain of function approach, and introduced single or combinations of TFs, Mash1, Olig2, and Sox9, as described previously. We analysed the effect of these TFs 3 days after nucleofection under propagation conditions (in the presence of mitogens, EGF and bFGF-2). Mash1 overexpression resulted in the exclusive generation of Tuj1+ neurons (Figure 15E white arrowheads & Figure 16B, in 43% of GFP+ cells). Sox9 overexpression induced the formation of O4+ oligodendroglial cells (Figure 15I white arrowheads & Figure 16C, in 29% of GFP+ cells) and less efficiently GFAP+ astrocytes (Figure 15G white arrowhead & Figure 16A, in 4% of GFP+ cells), in accordance with its role as a general promoter of gliogenesis (Stolt et al., 2003). Interestingly, Olig2 overexpression was not a strong inducer of oligodendrogenesis (Figure 15L white arrowhead & Figure 16C, in 3% of GFP+ cells). We propose that crucial co-factor(s) and or stimuli required for Olig2 function in OPC specification are absent in these cultures, perhaps similar to the NS5 cell line.

The combined overexpression of Mash1 and Sox9 generated Tuj1+ neurons (Figure 15N white arrowheads & Figure 16B, in 38% of GFP+ cells) and significantly more O4+ oligodendroglial cells than Sox9 alone (Figure 15O white arrowheads & Figure 16C, in 58% of GFP+ cells). These data suggest that Mash1 and Sox9 cooperate to specify O4+ oligodendroglail cells . Interestingly, these O4+ oligodendroglial cells demonstrated a highly branched and elaborate morphology in comparison to those generated by expression of Sox9 alone that may reflect the previously reported role of Mash1 in oligodendrocyte differentiation (Sugimori et al., 2008). The 5% reduction in the production of Tuj1+ neurons resulting from Mash1 and Sox9 coexpression as compared to

expression of Mash1 alone, may result from the potential repressive activities of Sox9 on neuronal specification (Stolt et al., 2003). Nevertheless, coexpression of Mash1 and Sox9 fails to direct uncommitted progenitors towards one specific cell fate choice, thus making the contribution of Mash1 activity in oligodendroglial or neuronal cell fate acquisition non dissociable. Surprisingly, the combined overexpression of Mash1 and Olig2 did not synergise to generate O4+ oligodendroglial cells, as has been previously reported in rat spinal cord primary cultures (Sugimori et al., 2007), and neurons were exclusively generated with this combination of TFs (Figure 15Q white arrowheads & Figure 16B, in 37% of GFP+ cells). Contrary to the Nucleofector specifications, we were only capable of attaining very low transfection efficiencies (1.5-2%), with a concomitant high percentage of cell death (approximately 50%), in adherent primary neurosphere-derived NSCs. All together, we concluded that this *in vitro* system was not amenable for genomic analyses with ChIP-on-chip.

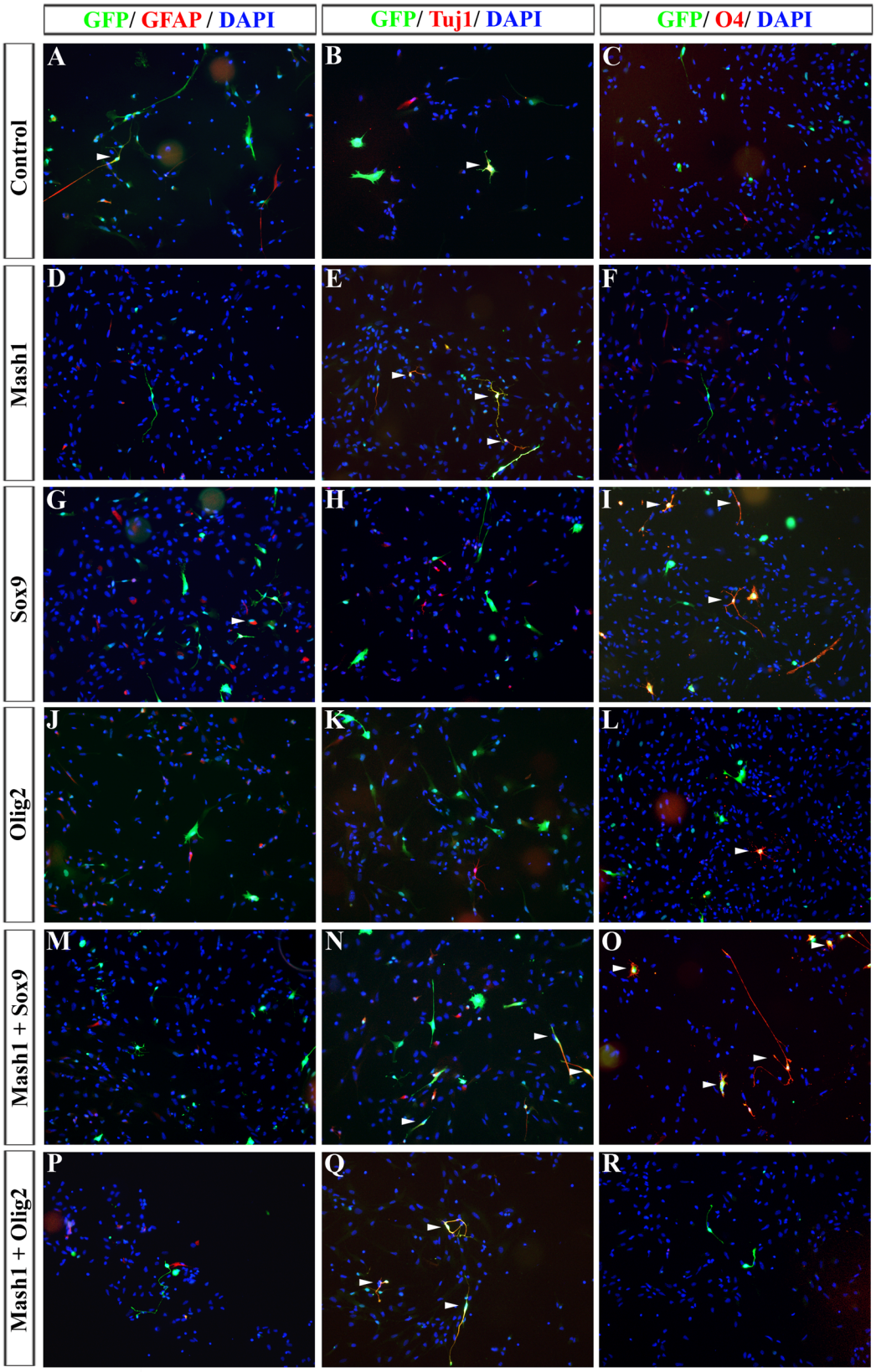

GFP/ GFAP / DAPI
GFP/ Tuj1/ DAPI
GFP/ O4/ DAPI
Control
Mash1
Sox9
Olig2
Mash1 + Sox9
Mash1 + Olig2
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

Figure 15. **Cell-fate specification activities of Mash1 Sox9 and Olig2 in monolayer neurosphere-derived NSCs.** Overexpression of single or combination of transcription factors, analysed 3 days after nucleofection in the presence of growth factors EGF and FGF-2. Immunostained with an anti-GFP antibody, and anti-GFAP, anti-Tuj1 and anti-O4 antibodies, to detect astrocytes, neurons and oligodendroglial cells, respectively. ***A-C***, Control cultures transfected with CAGGS empty vector give rise to small numbers of GFAP+ astrocytes (***A***, white arrowhead) and Tuj1+ neurons (***B***, white arrowhead), but no oligodendroglial cells (***C***). ***D-F***, Mash1 gain of function results in the generation of Tuj1+ neurons (***E***, white arrowheads) but no astrocytes (***D***), or oligodendroglial cells (***F***). ***G-I***, Overexpression of Sox9 gives rise to a small number of GFAP+ astrocytes (***G***, white arrowhead) and a larger number of O4+ oligodendroglial cells (***I***, white arrowheads). ***J-L***, Olig2 gain of function does not have a strong effect on cell fate specification, and generates a small number of O4+ oligodendroglial cells (***L***, white arrowhead). ***M-O***, Combined overexpression of Mash1 and Sox9 results in the generation of Tuj1+ neurons (***N***, white arrowheads) and O4+ oligodendroglial cells (***O***, white arrowheads) but no astrocytes (***M***). ***P-R***, Mash1 and Olig2 gain of function gives rise to Tuj1 + neurons (***Q***, white arrowheads), but no oligodendroglial cells (***R***). Note that nuclei are counterstained with DAPI.

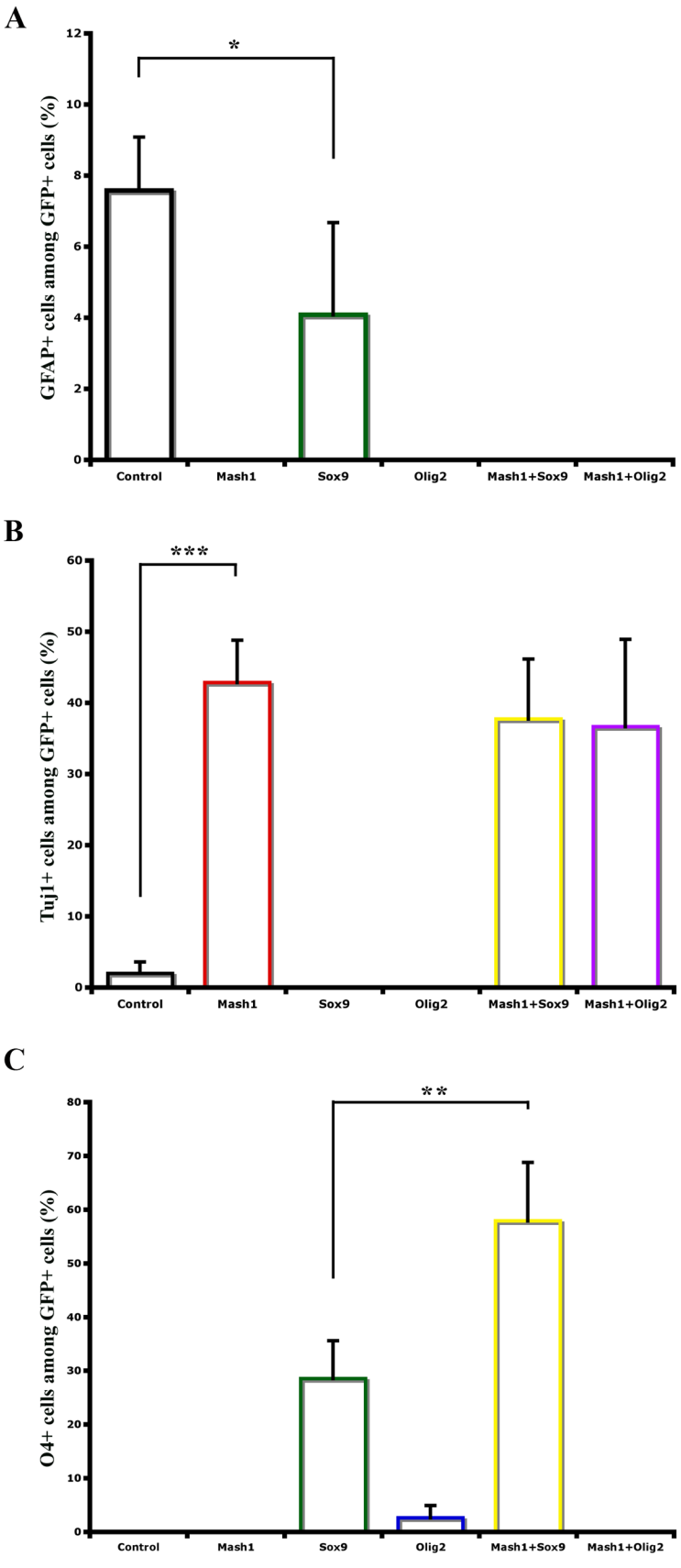

A
GFAP+ cells among GFP+ cells (%)
*
Control
Mash1
Sox9
Olig2
Mash1+Sox9
Mash1+Olig2
B
Tuj1+ cells among GFP+ cells (%)
***
C
O4+ cells among GFP+ cells (%)
**

Figure 16. Quantification of cell-fate specification activities of Mash1 Sox9 and Olig2 in monolayer neurosphere-derived NSCs. ***A-C***, Percentages of GFAP+ cells (***A***), Tuj1+ cells (***B***) and O4+ cells (***C***) among GFP+ cells. Transcription factors used for nucleofection are shown on the x-axis (mean ± standard deviations from three technical replicates). Statistical analysis using Students t-test; * P<0.05, ** P<0.01, *** P<0.001.

3.3.4 Mouse oligosphere cultures coexpress Mash1, oligodendrocyte promoting TFs and an early OPC marker

Evidently the generation of oligodendroglial cells following exogenous additions of a combinatorial set of TFs was problematic. We therefore focused on an alternate *in vitro* cellular system to study the function of Mash1 in oligodendrogenesis. Methods for the isolation and purification of rat OPCs from the CNS have been described, and include immunopanning (Gard et al., 1993, 1995; Barres and Raff, 1993), FACS by exploiting cell surface-specific antigens (Gard et al., 1995; Behar et al., 1988), differential gradient centrifugation (Vitry et al., 2001; Duncan et al., 1992; Goldman et al., 1986) or a shaking method based on differential adherent properties of glia (McCarthy and de Vellis, 1980; Szuchet and Yim, 1984), which permits the separation of rat OPCs from the astroglial cells in the mixed glial culture by shearing forces. In contrast to rat OPCs, mouse OPCs have proven more difficult to isolate. Mouse OPCs do not share all of the cell surface antigens with their rat counterparts such as A2B5 (Fanarraga et al., 1995), impeding approaches such as immunopanning and cell sorting using FACS as described for rat OPC isolation. In addition, mouse OPCs tend to differentiate in mixed glial cultures *in vitro* and are also relatively difficult to separate from astrocytes by shaking methods.

Several studies described methods to generate self-renewing OPCs from neural progenitor/stem cells in different species such as dog and rodents (Avellana-Adalid et al., 1996; Zhang et al., 1998a, 1998b; Vitry et al, 1999). More recently, a simple procedure to prepare a large, highly enriched population

of OPCs from embryonic multipotent cortical progenitor cells of the mouse was described (Chen et al., 2007). These methods allow generation of large numbers of mouse OPCs through formation of ‘‘oligospheres’’ from neurospheres. OPCs isolated by these procedures can be induced to differentiate into mature oligodendrocytes (Chen et al., 2007). Specifically, neurospheres were first generated from dissociated E14.5 cortex for a period of 4 days in the presence of EGF and FGF-2 mitogens (Figure 17A and B), followed by gradual replacement of the media with neuroblastoma B104 conditioned media (CM) to induce oligosphere formation for a further 14 days (Figure 17A and C). Oligospheres analysed at day 14, expressed Mash1 protein in 52% of all the cells in the oligospheres (Fig. 18A and B). Mash1+ cells also coexpressed Olig2 and PDGFRα (Figure 18C and E, in 40% of total Mash1+ cells), as well as Sox9 and PDGFRα (Figure 18D and E, in 34% of total Mash1+ cells). However these cultures are heterogeneous and demonstrate variable proportions of Tuj1+ neurons (Figure 19A) and GFAP+ astrocytes (Figure 19B), in addition to smaller numbers of HuC/D+ immature neurons (Figure 19C) and MBP+ mature oligodendrocytes (Figure 19D).

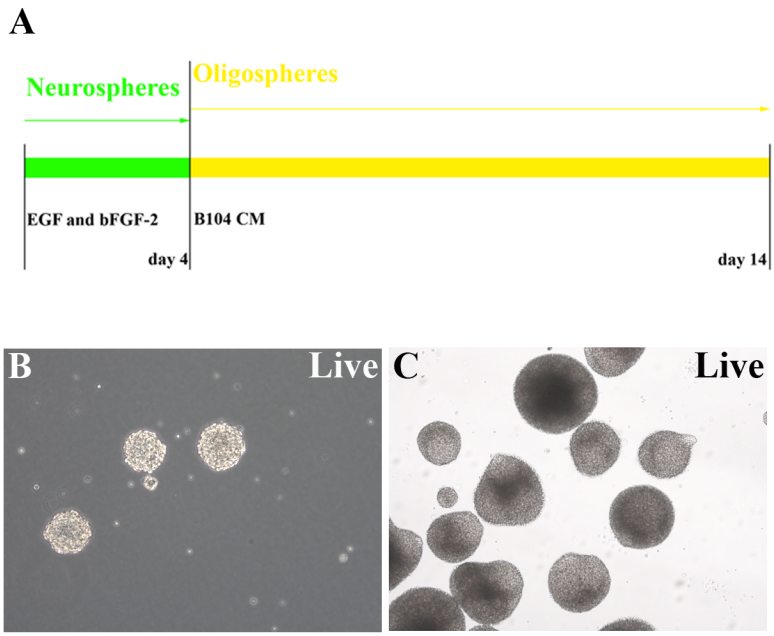


Figure 17. Mouse Oligospheres cultures. ***A***, Schematic of culture conditions and length of time to generate neurospheres and subsequently oligospheres. ***A-C***, Dissociated E14.5 cortex cultured in the presence of EGF and FGF for 4 days generates neurospheres (***B***). Neurospheres cultured for a further 14 days, gradually replacing the media with B104 CM, results in the formation of oligospheres (***C***).

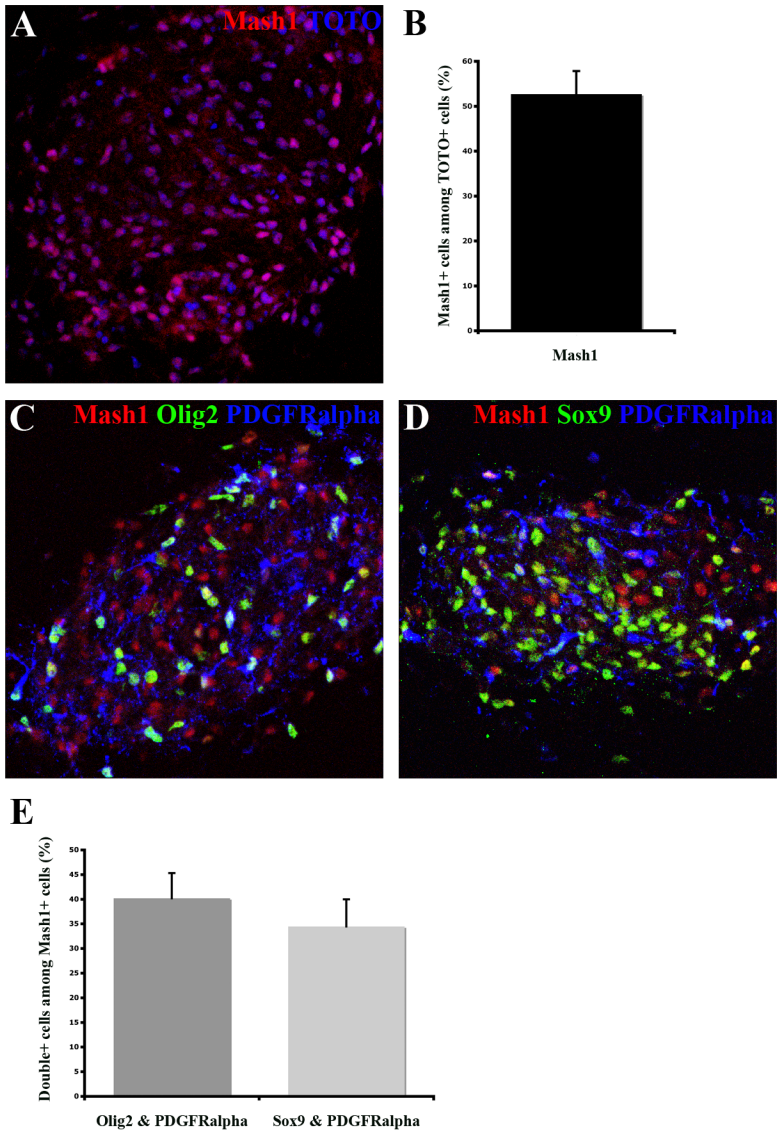


Figure 18. Oligospheres coexpress Mash1, Olig2, Sox9 and PDGFRα. ***A & B***, Cryosectioned oligospheres were immunostained with anti-Mash1, anti-Olig2, anti-Sox9 and anti-PDGFRα antibodies. Approximately 52% of all cells in the oligospheres express Mash1. Note that nuclei are counterstained with TOTO-3. ***C-E***, Among the Mash1+ population, 40% coexpress Olig2 and PDGFRα (***C & E***), and 34% coexpress Sox9 and PDGFRα (***D & E***).

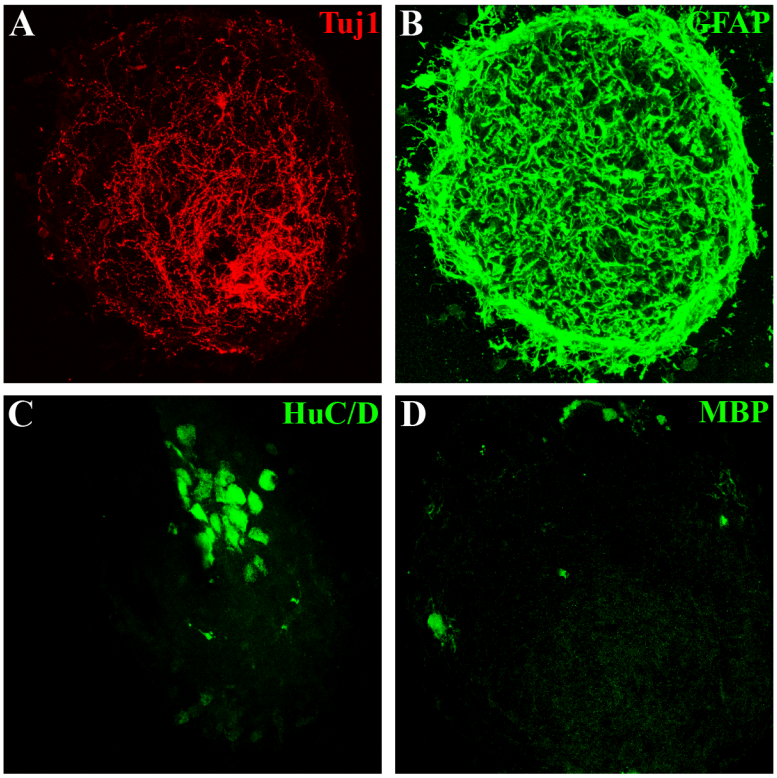


Figure 19. Oligospheres contain neurons, astrocytes and a small number of mature oligodendrocytes. ***A-D***, Immunostained cryosectioned oligospheres contain Tuj1+ (***A***) and HuC/D+ (***C***) neurons, GFAP+ astrocytes (***B***) and some MBP+ oligodendrocytes (***D***).

We conclude that the oligospheres were a good system in which to study the function of Mash1 in oligodendroglial cell fate specification. Firstly, oligospheres contained a significant proportion of Mash1+ cells that coexpressed potential cofactors required for oligodendrogenesis, namely Olig2 and Sox9, in addition to the early OPC marker, PDGFRα. Moreover by modulating the size of the starter cultures, sufficient material for subsequent ChIP-on-chip analyses can be obtained. Although oligosphere cultures contained some neurons, we propose to dissociate the neurogenic and oligodendrogenic function of Mash1 using a subtractive approach. Mash1 ChIP-on-chip and ChIP-seq data from the ventral mouse telencephalon at a time of peak neurogenesis in development (at E12.5) have been generated (Diogo Castro, unpublished data). Indeed, this data set could provide a platform on which to potentially subtract Mash1 neurogenic targets from the oligosphere data set, and thus enrich for specific oligodendroglial targets in these cultures. We therefore use the oligosphere culture method as a model to investigate the molecular mechanisms underlying Mash1 function in oligodendroglial cell fate specification.

3.4 Mash1 locational analysis in oligosphere cultures with Chip-on-chip technology

To identify promoters of genes expressed in OPCs, which are bound by Mash1 in order to uncover the mechanisms that confer oligodendrocyte lineage specific expression of Mash1 targets, we performed locational analysis using ChIP technology. A specific antibody to Mash1 that has been extensively characterised for ChIP (Diogo Castro, personal communication) was used to coprecipitate chromatin from mouse oligosphere culture material (at *in vitro* day 14 of oligosphere formation). Although no direct targets of Mash1 in the oligodendroglial lineage have been reported, we hypothesised that Mash1 shares some common targets in the specification of neuronal and oligodendroglial cell fates. Critical to the function of proneural genes is the activation of the Notch signalling pathway (Artavanis-Tsakonas et al., 1999). Indeed, activation of the Notch ligand *Delta* genes, including *Deltalike-1* (*Dll1*), by proneural factors is evolutionarily conserved in neurogenesis (Artavanis-Tsakonas et al., 1999; Casarosa et al., 1999; Fode et al., 2000). Delta1 is coexpressed with Mash1 at E13.5 in the ventral telencephalon (Castro et al., 2006), including in PDGFRα+ OPCs (Carlos Parras, unpublished data). Thus, we propose that common targets of Mash1 in neuronal and oligodendroglial cell fate acquisition, may include genes involved in the Notch signalling pathway, such as *Dll1*.

Previously, an evolutionarily conserved *cis*-regulatory region in the promoter of the mouse *Dll1* gene was identified (Beckers et al., 2000), which contains a proximal neural enhancer, referred to as *DeltaM* (Castro et al., 2006). The activity of the *DeltaM* enhancer *in vivo* is restricted to the dorsal spinal cord

and ventral telencephalon, regions of endogenous Delta1 expression that also express Mash1 (Castro et al., 2006). *DeltaM* activation is dependent on Mash1 function, indeed Mash1 protein directly associates with *DeltaM* in the developing telencephalon, as defined by ChIP analyses (Castro et al., 2006). In addition to a pair of evolutionary conserved E-boxes in the *DeltaM* sequence, responsible for the activation of this enhancer by Mash1, a proximal evolutionarily conserved consensus binding site for the POU family of homeodomain proteins, otherwise referred to as an octamer (Nishimoto et al., 2003), permit Mash1 and members of the POU II and POU III classes to bind cooperatively at the *DeltaM* sequence and subsequently synergise to activate the *Dll1* promoter (Castro et al., 2006). An *in silico* screen to identify conserved genomic regions with a similar Mash1/Brn motif (a 15 base pair consensus sequence (ATT[A/T]NCAT[A/T/G]CAG[C/G]TG) within 100kb from the most proximal gene or within introns, across divergent species using the transcription factor binding site (TFBS) cluster program (Donaldson et al., 2005) identified a number of positive hits (Castro et al., 2006). These included genes involved in Notch signalling (*Dll1*, *Dll3*, *Jagged2*, and the glycosyl transferase, *Lunatic fringe* (*Lfng*)), cell differentiation (*insulinoma-associated 1, IA-1* (*Insm1*), a zinc-finger factor essential for pancreatic and intestinal endocrine cell differentiation; Mellitzer et al., 2006; Gierl et al., 2006), and in cell cycle (*Fbxw7*, a subunit of an SCF-type ubiquitin ligase complex that targets positive regulators of the cell cycle for degradation to promote cell cycle exit; reviewed Onoyama et al., 2008). Coprecipitation of these sequences with Mash1 were validated using chromatin material prepared from E12.5 ventral telencephalon and subsequent quantitative PCR, or ChIP-PCR, using primer specific sequences to the mouse genome.

These studies found that, whilst an antibody to Mash1 coprecipitated the Mash1/Brn motif-containing sequences associated with *Delta1*, *Delta3*, *Insm1*, and *Fbxw7*, the sequences associated with *Lfng* were not deemed to be significantly coprecipitated (Castro et al., 2006).

We therefore asked whether the Mash1 proneural protein directly interacts with the *Dll1*, *Dll3*, *Dll4*, *Lfng*, *Fbxw7*, and *Jagged2* enhancers in oligosphere cultures by performing ChIP experiments. An antibody to Mash1 coprecipitated the *DeltaM*, *Fbxw7*, *Dll3*, and *Lfng* sequences in chromatin prepared from oligosphere cultures, but not the *Dll4* or *Jagged2* sequences nor the *Delta1* or *Fbxw7* coding sequence (*Dll1* ORF and *Fbxw7* ORF) used as negative controls, or *Actin B* another negative control (Figure 20). Conversely, a control IgG antibody did not coprecipitated the *DeltaM*, *Fbxw7*, *Dll3*, or *Lfng* sequences nor the *Dll1* ORF, *Fbxw7* ORF or *Actin B* (Figure 20). Therefore, in oligosphere cultures Mash1 specifically binds to the *DeltaM*, *Fbxw7*, *Dll3*, and *Lfng* genomic regions. Note that *DeltaM* and *Fbxw7* sequences were the most robust Mash1 coprecipitated segments, and therefore used to test the relative levels of enrichment and thus quality of the ChIP in subsequent experiments. It is important to state at this point that we can not be certain that these Mash1 coprecipitated genomic segments are those bound and present in an oligodendroglial rather than a neuronal population, since oligosphere cultures are inherently heterogeneous in nature.

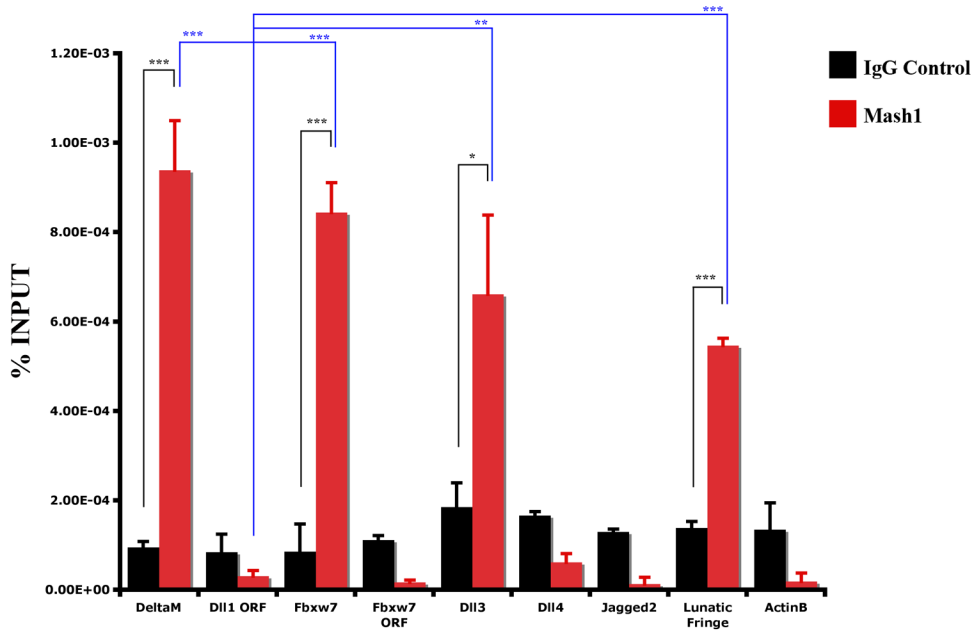


Figure 20. Mash1 binds to regulatory elements of Notch signalling genes in oligospheres. ChIP with chromatin from day 14 oligospheres and a Mash1 antibody, analysed by qPCR are significantly enriched for *DelatM*, *Fbxw7*, *Dll3* and *Lfng* segments compared to control IgG ChIP (black asterisk), and *Dll1* ORF negative region that is not bound by Mash1 (blue asterisk). * P<0.05, ** P<0.01, *** P<0.001.

3.4.1 Validation of oligosphere cultures for Chip-on-chip

To generate chromatin material for ChIP-on-chip, we cultured and characterised mouse oligospheres as described previously. Oligospheres expressed Mash1 in approximately 60% of all cells (Figure 21A & B), and within the Mash1+ population 27% coexpressed Olig2 and PDGFRα (Figure 21C & E), and 22% coexpressed Sox9 and PDGFRα (Figure 21D & E). An antibody to Mash1 coprecipitated the *DeltaM* and *Fbxw7* sequences robustly in technical chromatin replicates prepared from oligosphere cultures, compared to negative control regions that were not coprecipitated in this assay, *Dll1* ORF and *Fbxw7* ORF respectively, as defined by ChIP-PCR (Figure 22). A control IgG antibody did not coprecipitate the *DeltaM*, *Fbxw7*, *Dll1* ORF, nor the *Fbxw7* ORF sequences (Figure 22). We concluded that the robust enrichment of *DeltaM* and *Fbxw7* sequences relative to negative control regions in Mash1 coprecipitated chromatin preparations, in addition to the lack of *DeltaM* and *Fbxw7* sequences copreciptated in the IgG controls, were suitable for ChIP-on-chip.

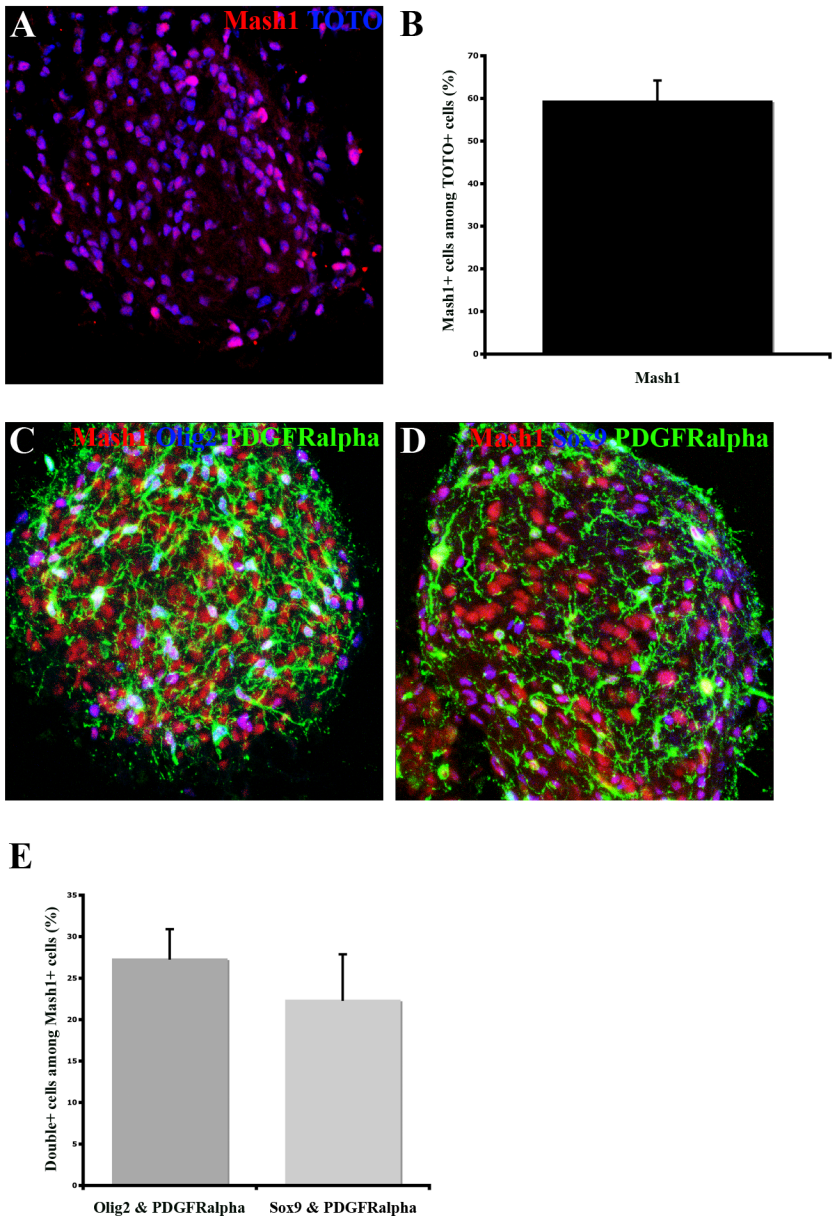


Figure 21. Characterisation of oligospheres for ChIP experiments. Immunostaining of cryosectioned oligopsheres with anti-Mash1, anti-Olig2 and anti-PDGFRα antibodies. ***A-B***, Approximately 60% of all cells in the oligospheres express Mash1. Nuclei are counterstained with TOTO-3. ***C-E*** Among the Mash1+ population, 27% coexpress Olig2 and PDGFRα (***C*** & ***E***), and 22% coexpress Sox9 and PDGFRα (***D*** & ***E***).

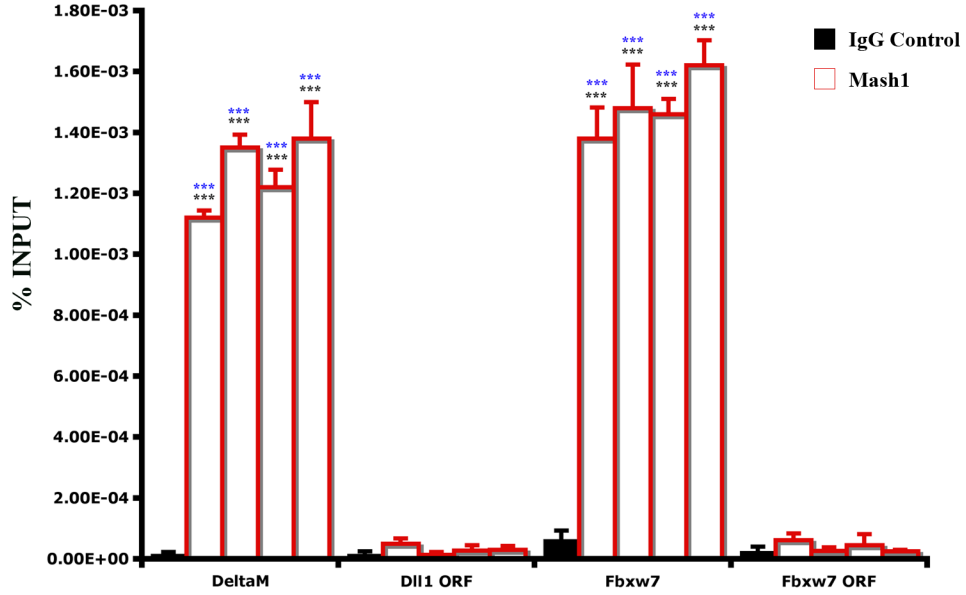


Figure 22. Validation of Mash1 ChIP. *DelatM* and *Fbxw7* regions are enriched significantly in ChIP experiments in oligosphere cultures with an anti-Mash1 antibody compared to a control ChIP experiment with an anti-IgG antibody (black asterisk) and to n egative regions *Dll1* ORF and *Fbxw7* ORF (blue asterisk), respectively. * P<0.05, ** P<0.05, *** P<0.001.

3.4.2 An oligodendrocyte specific array to identify Mash1 bound segments

To identify Mash1 binding events using a ChIP-on-chip strategy, a custom designed oligodendrocyte specific array was generated (eArray, Agilent Technologies, in collaboration with Diogo Castro). In brief, the array specifications were designed so that coverage of 16kb was attained per gene, centred on the transcription start site (TSS) (-8kb upstream to +8kb downstream of the TSS), with an average distance between probes of 120bp and repetitive regions masked. We reasoned that an initial small-scale focused experiment, centred on genes previously associated with oligodendrogenesis, would permit validation of the oligosphere material and ChIP-on-chip technique before embarking on a larger mouse promoter array screen.

Initially, to generate the oligodendrocyte specific array, a list of genes to be tiled on the array was compiled. These genes were predominantly extracted from three published expression array data sets (Dugas et al., 2006; Ligon et al., 2007; Hu et al., 2004) (Figure 23). The largest contribution of genes to the list were derived from the first detailed genomic analysis of OL differentiation, which documents the progressive gene expression profile of synchronously differentiating oligodendrocytes from pure primary rat OPCs (isolated from postnatal cortices) upon mitogen withdrawal and T3 exposure (521 genes, Dugas et al., 2006). These data detail the specific cell-autonomous gene expression modifications involved in the complex transition from a committed OPC to a mature oligodendrocyte (Dugas et al., 2006). A smaller set of genes were obtained from an expression array study in a rat culture model of OPCs and NSCs to identify molecular events that occur during the transition from NSCs to

OPCs (42 genes, Hu et al., 2004). Specifically, NSCs isolated from the embryonic rat forebrain were induced to form OPCs using B104CM, and the differential expression between these two distinct populations assayed (Hu et al., 2004). Notably, these studies identify significant expression changes in OPCs when compared with NSCs, and include genes involved in OPC differentiation (Hu et al., 2004). In addition, we incorporated a subset of genes from a genome-wide microarray expression screen comparing wild-type and *Olig2* null mutant neurospheres (110 genes, Ligon et al., 2007). Notably, *Olig2* null neurospheres lack the characteristic gene expression signature of the oligodendroglial lineage, namely expression of PDGFRα, PLP, and MBP markers (Ligon et al., 2007). In addition, a literature search was performed to include genes associated with oligodendrogenesis that were absent in the aforementioned expression data sets (84 genes). Positive controls tiled on the array included elements previously shown to coprecipitate with a specific antibody to Mash1, such as *Dll1*, *Fbxw7* and *Insm1* (Castro et al., 2006). Furthermore, a distal putative enhancer of *Olig2* (approximately 82kb upstream of the Olig2 TSS), identified from an *in silico* screen to map Mash1 (CAGSTG) and Brn (ATTWNYAW) conserved binding sites using TFBS cluster, which coprecipitates with Mash1 using chromatin prepared from E12.5 ventral telencephalon tissue (a developmental time when OPCs are generated in this region) in ChIP-on-chip and ChIP-PCR experiments, was tiled on the array (Diogo Castro, unpublished data). Note that Olig2 plays a crucial role in oligodendroglial cell fate specification in the developing CNS, and thus we include this element in our screen. Housekeeping genes that function as internal or reference controls were also tiled on the array.

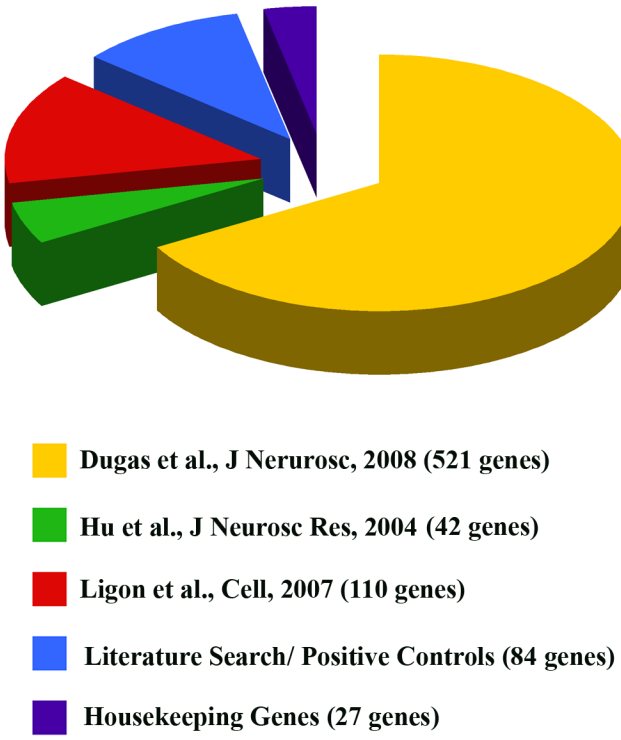


Figure 23. Schematic representation of genes tiled on the oligodendrocyte specific array.

Technical replicates of Mash1 coprecipitated chromatin and control IgG coprecipitated chromatin were fluorescently labelled with cyanine 3-dUTP dye and input chromatin with cyanine 5-dUTP dye, and subsequently hybridised onto the custom designed oligodendrocyte specific array (hybridisations were performed by Doug King, Windeyer Institute, UCL). Arrays were scanned on an Agilent DNA Microarray Scanner and the initial data extraction was performed using Agilent Feature Extractor Software, version 9.5.1 (performed by Doug King, Windeyer Institute, UCL).

3.5 Chip-on-chip analysis

To identify Mash1 specific binding events, the intensity of the signal of the Mash1 coprecipitated chromatin relative to the signal of the input chromatin, for every probe tiled on the array, were analysed using the peak-calling ChIP Analytics software (Agilent Technologies). We applied inter-array median and intra-array Lowess (intensity-dependent) normalisations. Briefly, the intra-array Lowess normalisation adjusts for intensity-dependent variation resulting from dye properties, which are caused by inconsistencies in the relative fluorescence intensity between Cy5 and Cy3 dyes. Note that the ChIP Analytics software generates a number of output reports detailing probe and sequence information, which are subsequently used to locate specific genomic sequences, and relative location of proximal genes on the UCSC Genome Browser (http://genome.ucsc.edu/, Kent et al., 2002). The quality control (QC) reports generated from the ChIP Analytics software displays a plot of the immunoprecipitated extract (IP) versus the whole cell extract (WCE) or input

chromatin, and thus permits the identification of enriched versus unenriched targets. Analyses of Mash1 coprecipitated chromatin identified 315 significant bound probes (P<0.001), corresponding to a total of 76 bound segments and 9.7% of all the regions tilled on the array (Figure 24B & Table 1, data is an average of 3 replicates). On the other hand, analyses of IgG control coprecipitated chromatin detected 49 significant bound probes (P<0.001) corresponding to 21 bound segments and 2.7% of all the regions tilled on the array (Figure 24A). Note that the proportion of genes with associated binding events in Mash1 coprecipitated chromatin, were representative of their relative contribution from the different expression array lists originally tiled on the oligodendrocyte specific array (Figure 25). We concluded that in mouse oligosphere cultures Mash1 was bound to regions proximal to genes that are expressed during the transition of an uncommitted NSC progenitor cell to an cell fate restricted OPC, and finally to a mature fully differentiated oligodendrocyte.

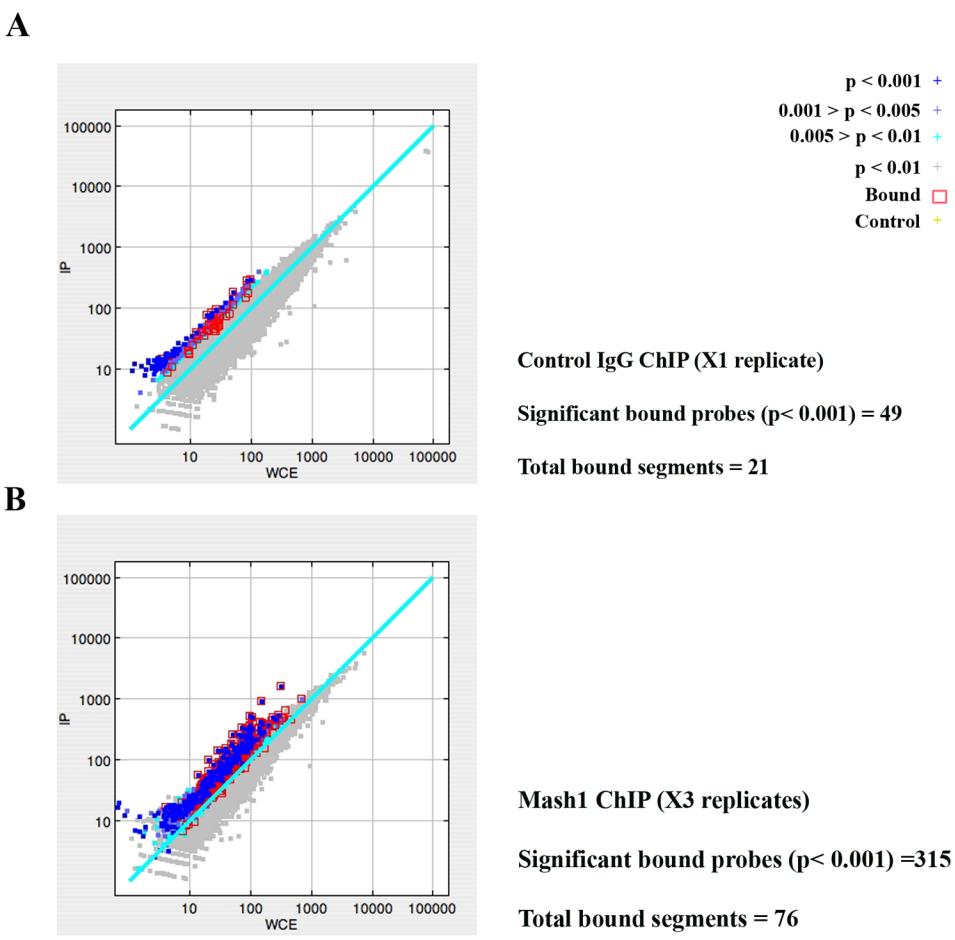


Figure 24. ChIP Analytics Quality Controls. ***A-B***, Scatter plots of immunoprecipitated chromatin versus whole cell extract (or input) with significant bound probes, for control IgG ChIP (***A***) and Mash1 ChIP (***B***, 3 technical replicates).

Chromosome Location	Proximal Gene
chr17:15080888-15083354	Dll1
chr7:28010104-28012194	Dll3
chr3:85023223-85025376	Fbxw7
chr2:145908225-145909658	Insm1
chr5:141082673-141084225	Gna12
chr2:152426056-152427584	Id1
chr6:125114481-125115888	A930037G23Rik
chr14:65962637-65964093	Bnip3l
chr3:45470575-45473084	Pcdh10
chr11:69653811-69655115	1810027O10Rik
chr3:45482127-45483571	Pcdh10
chr3:98366112-98367572	Hmgcs2
chr14:119611554-119613205	Rap2a
chr6:125180331-125182321	Vamp1
chr16:91028853-91030133	Olig2
chr5:100277849-100279215	Hnrpdl
chr13:90212699-90214282	Vcan
chr11:98559726-98561210	Thra
chr2:26321876-26323507	Notch1
chr10:21612094-21613761	Sgk1
chr16:52369743-52371742	Alcam
chr17:25302446-25304580	Sox8
chr9:62281661-62282863	Coro2b
chr17:36636368-36637722	Mog
chr9:45708438-45709998	Sidt2
chr16:34821636-34822943	Mylk
chr10:57472240-57473959	Fabp7
chr1:134475223-134476375	Nfasc
chr4:22584421-22585564	Pou3f2
chr11:81782128-81783226	Accn1
chr2:26323727-26325199	Notch1
chr15:25928382-25929537	Zfp622
chr9:50504719-50505973	Cryab
chr6:125112241-125113904	A930037G23Rik
chr16:91154582-91155746	Olig1
chr7:64174997-64176605	BB128963
chr19:4045993-4047154	Gstp2
chr1:155507669-155509571	Rgs16
chr11:86809565-86810983	Ypel2
chr1:12702869-12704030	Sulf1
chr10:86918323-86919788	Ascl1
chr18:47494285-47495706	Sema6a
chr6:136773165-136774372	H2afj
chr1:12677866-12679443	Sulf1
chr10:94258944-94260453	Plxnc1
chr5:138195930-138196966	Gje1
chr14:35960003-35961588	Rgr
chr11:76665566-76666745	Cpd
chr11:77753442-77755065	Sez6
chr11:112597173-112598308	Sox9
chr1:42635488-42636732	Pou3f3
chr8:83773849-83774976	Gab1
chr9:43487970-43489323	Pvrl1
chr9:56666280-56667556	Cspg4
chr6:54741347-54742641	Znrf2
chr3:28168688-28169784	Pld1
chr11:8562667-8564326	Tns3
chr4:71625485-71626935	Tle1
chr17:25291852-25292881	Sox8
chr14:53889555-53891009	Cmtm5
chr16:91110447-91111555	Olig2
chr7:100536847-100538144	Plekhb1
chr3:88086424-88087589	Bcan
chr2:119494486-119495710	Tyro3
chr13:28966856-28968126	Sox4
chr16:91151016-91152746	Olig1
chr17:53034658-53035960	Kat2b
chr6:137419339-137420549	Ptpro
chr6:66964888-66966655	Gadd45a
chr4:116224794-116226043	Tesk2
chr16:8586270-8587349	Carhsp1
chr13:28957764-28958896	Sox4
chr11:109427612-109429024	Wipi1
chr3:45480794-45482186	Pcdh10
chr11:79318667-79319797	Omg
chr11:102754621-102755977	C1ql1
chr7:37271933-37273072	Zfp536
chr13:64388569-64389693	Ctsl
chr16:90110013-90111170	Sod1
chr16:37732750-37733982	Fstl1
chr1:155506688-155507862	Rgs16

Table 1. List of segments bound by Mash1 in oligospheres. List of significant Mash1 bound segments with corresponding proximal gene. Segments on the list are in order of descending significance. UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

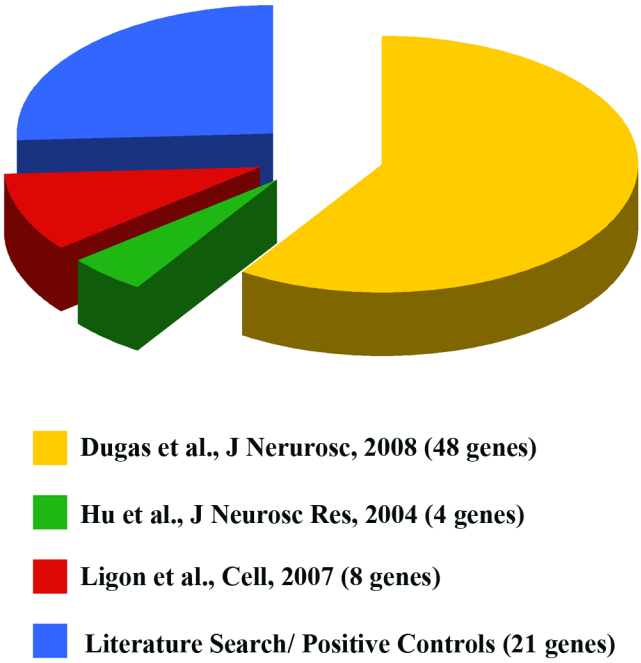


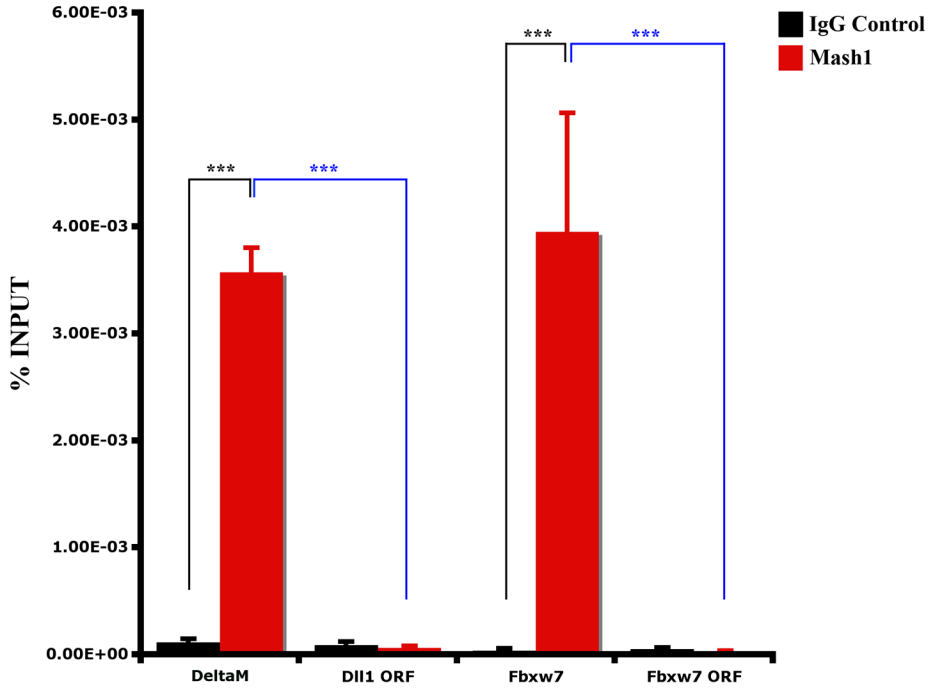
Figure 25. Schematic representation of genes bound by Mash1 on t he oligodendrocyte specific array.

3.5.1 Mash1 binds to early OPC and late oligodendrocyte genes and other genes with functions throughout oligodendrogenesis

To obtain a general overview of the quality of Mash1 binding data, and validate that Mash1 protein directly interacts with these genomic elements in the oligosphere cultures, we performed ChIP-PCR experiments and calculated the number of false rate positives. Initially, the robustness of the Mash1 ChIP was tested with specific primers for the *DeltaM* and *Fbxw7* sequences, as described previously. We found that *DeltaM* and *Fbxw7* sequences were highly enriched in oligosphere chromatin preparations using a Mash1 antibody compared to negative control regions *Dll1* ORF or *Fbxw7* ORF, respectively, or to the IgG control ChIP (Figure 26A), and therefore used this material for subsequent ChIP-PCRs.

A total of 26 putative elements were randomly selected for ChIP-PCR analysis. We found that 54% of these elements were significantly coprecipitated with Mash1 when compared to the negative region, *Dll1* ORF or to the IgG control ChIP (Figure 26B). In addition, we analysed a distal putative enhancer element of *Sox9* (approximately 500kb upstream of the *Sox9* TSS) identified from an *in silico* screen to map Mash1 (CAGSTG) and Brn (ATTWNYAW) conserved binding sites using TFBS cluster, which coprecipitates with Mash1 using chromatin prepared from E12.5 ventral telencephalon tissue in ChIP-on-chip and ChIP-PCR experiments (Diogo Castro, unpublished data). Notably, the *Sox9* distal element was significantly coprecipitated with Mash1 in the oligospheres compared to the *Dll1* ORF negative region or to the IgG control ChIP (defined as, *Sox9* (vt enhancer), Figure 26B).

A


6.00E-03
5.00E-03
4.00E-03
3.00E-03
2.00E-03
1.00E-03
0.00E+00
% INPUT
IgG Control
Mash1
***
***
***
***
DeltaM
Dll1 ORF
Fbxw7
Fbxw7 ORF

B

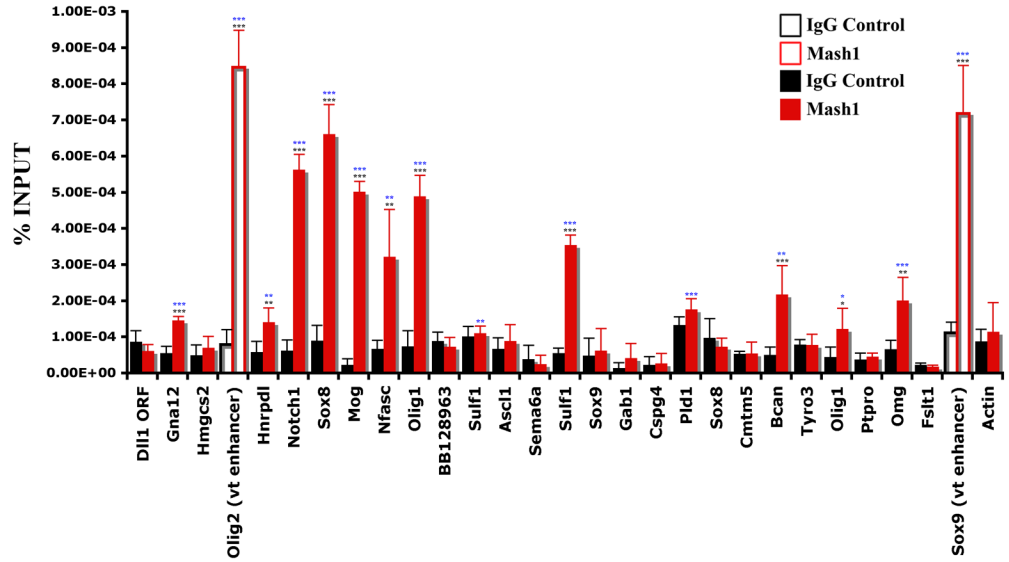

1.00E-03
9.00E-04
8.00E-04
7.00E-04
6.00E-04
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
% INPUT
IgG Control
Mash1
IgG Control
Mash1
Dll1 ORF
Gna12
Hmgcs2
Olig2 (vt enhancer)
Hnrpdl
Notch1
Sox8
Mog
Nfasc
Olig1
BB128963
Sulf1
Ascl1
Sema6a
Sulf1
Sox9
Gab1
Cspg4
Pld1
Sox8
Cmtm5
Bcan
Tyro3
Olig1
Ptpro
Omg
Fslt1
Sox9 (vt enhancer)
Actin

Figure 26. Validation of Mash1 bound segments in oligosphere cultures. *A & B*, ChIP-PCR analysis of Mash1 bound segments. ***A***, Validation of oligosphere material, *DelatM* and *Fbxw7* regions are enriched significantly in Mash1 ChIP compared to control IgG control ChIP (black asterisk) and to negative regions *Dll1* ORF and *Fbxw7* ORF (blue asterisk), respectively. ***B***, qPCR of randomly selected segments, significantly enriched regions in Mash1 ChIP compared to control IgG control ChIP (black asterisk) and to negative regions *Dll1* ORF (blue asterisk). Calculated false rate positives: 46%. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Binding profiles, as a ratio of the IP signal over the whole cell extract signal, of segments bound by Mash1 are plotted in relation to their genomic location along the x-axis. Specific genomic regions bound by Mash1, as defined by the ChIP Analytics programme, are marked with a blue asterisk (Figure 27-33). Note that background ratios of the control IgG ChIP in some regions are high, this may be a result of non-stringent washes during hybridisation. Interestingly the expression pattern of genes with proximal regions bound by Mash1 in oligosphere cultures, as analysed using Genepaint data base (http://www.genepaint.org/; Visel et al., 2004) in the developing mouse CNS at E14.5, are not solely restricted to the oligodendrocyte lineage (Figures 27-33) to suggest that their expression are regulated by a number of distinct elements. Indeed the expression of Sox10, whose function is crucial for the development of neural crest–derived and oligodendroglial populations throughout embryogenesis, is dynamically regulated. Transgenesis strategies in mouse and zebrafish have identified multiple conserved elements with overlapping functions, which regulate Sox10 spatial and temporal expression during development (Antonellis et al., 2008; Werner et al., 2007).

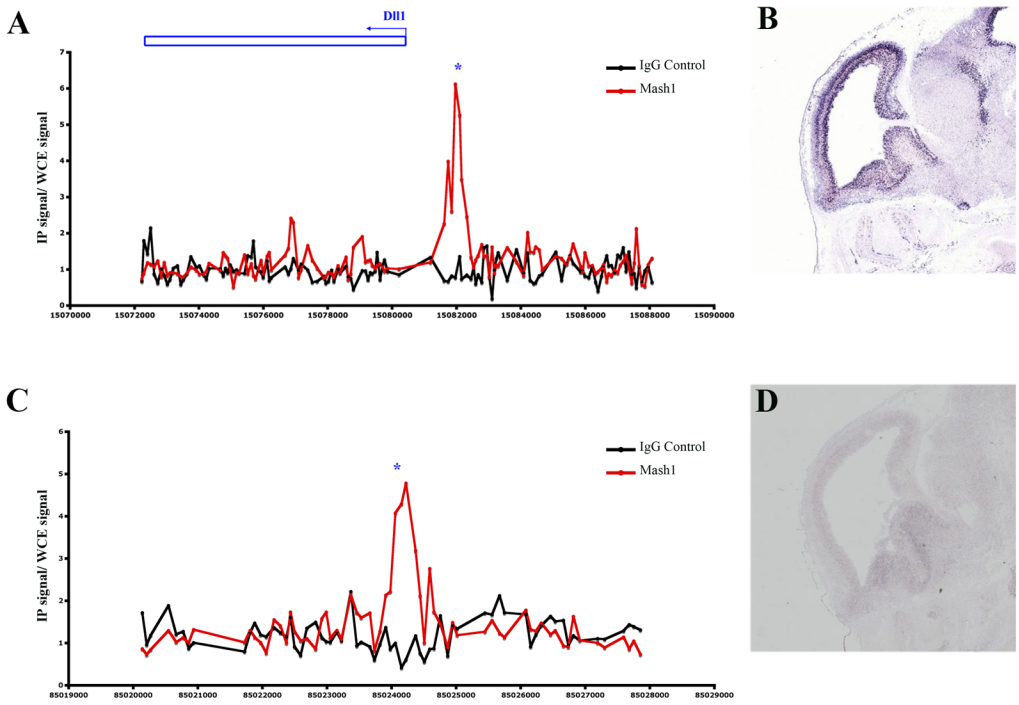


Figure 27. Mash1 binds to regulatory elements of *Dll1* and *Fbxw7* genes. ***A-D***, Mash1 and control IgG ChIP binding profiles of *Dll1* (***A***) and *Fbxw7* (***C***) positive control genomic regions (blue asterisk marks point of Mash1 binding), with Dll1 (***B***) and Fbxw7 (***D***) *in situ* hybridisation data of saggital sections through the telencephalon of WT embryos at E14.5 (Ge nepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

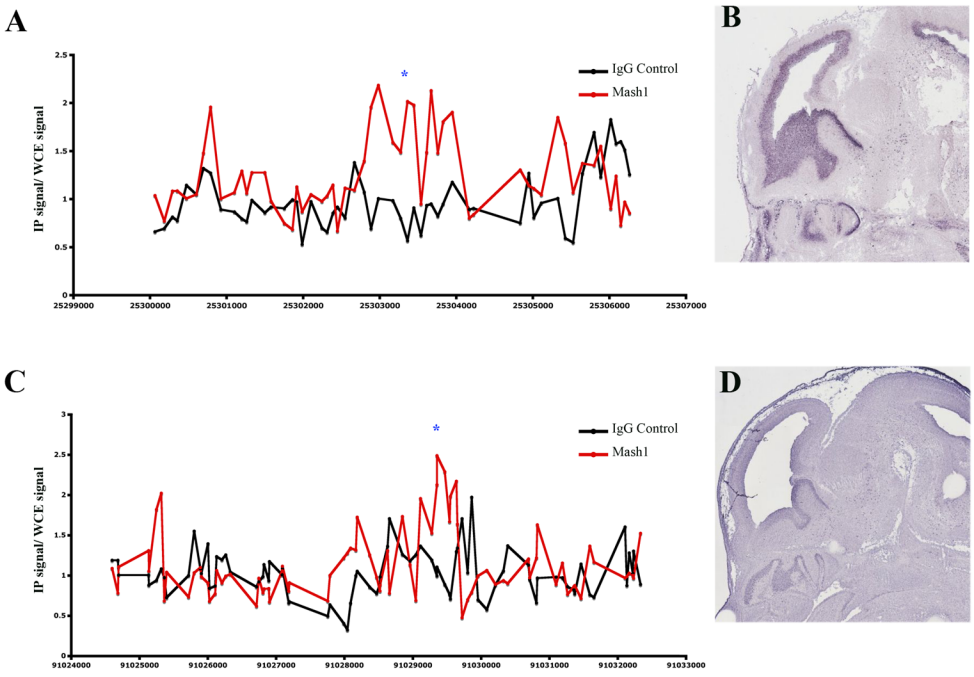


Figure 28. Mash1 binds to regulatory elements of OPC genes *Sox8* and *Olig2*. *A-D*, Mash1 and control IgG ChIP binding profile of *Sox8* (***A***) and *Olig2* (***C***) (blue asterisk marks point of Mash1 binding), with Sox8 (***B***) and Olig2 (***D***) *in situ* hybridisation data of saggital sections through the telencephalon of WT embryos at E14.5 (Ge nepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

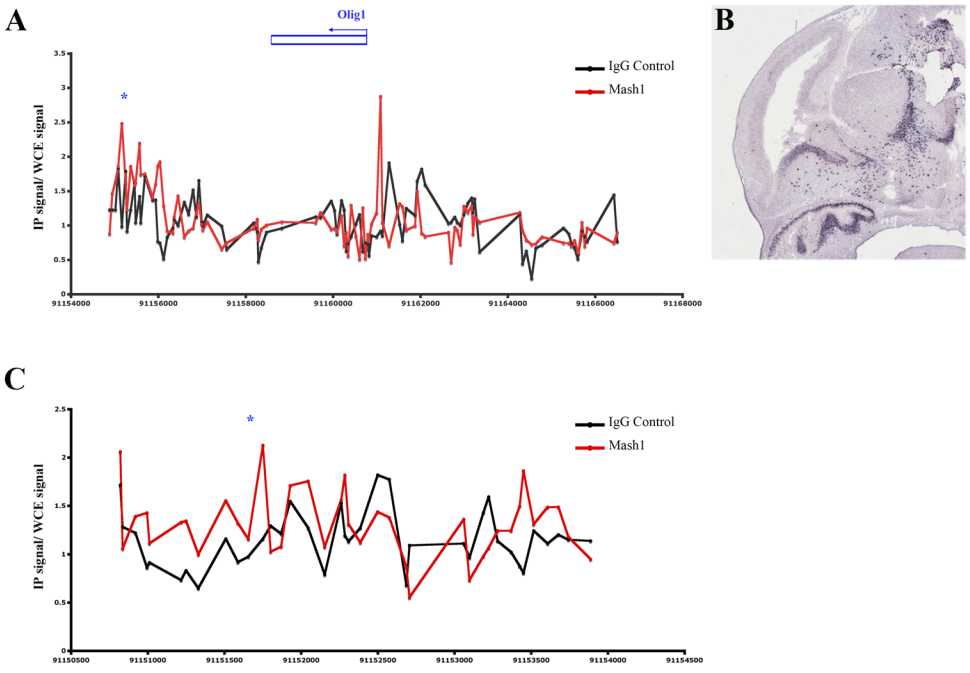


Figure 29. Mash1 binds to regulatory elements of OPC gene *Olig1*. ***A-C***, Mash1 and control IgG ChIP binding profiles of *Olig1* (***A*** **&** ***C***) (blue asterisk marks point of Mash1 binding), with Olig1 (***B***) *in situ* hybridisation data of a saggital section through the telencephalon of WT mouse embryo at E14.5 (Genepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

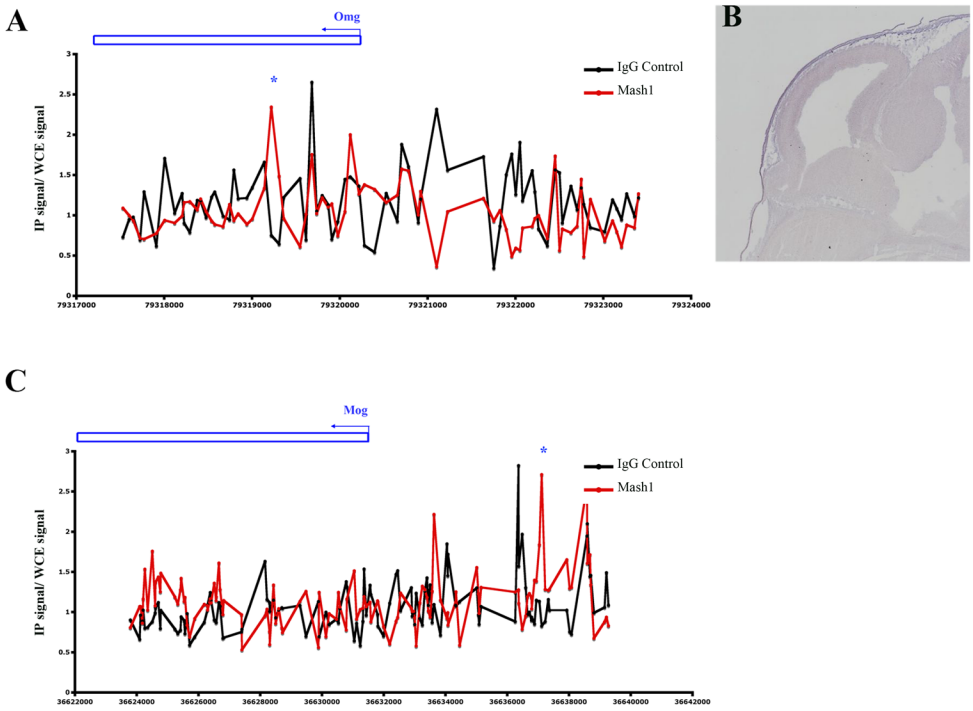


Figure 30. Mash1 binds to regulatory elements of mature oligodendrocyte genes, *Omg* and *Mog*. *A-C*, Mash1 and control IgG ChIP binding profile of *Omg* (***A***) and *Mog* (***C***) (blue asterisk marks point of Mash1 binding), with Omg (***B***) *in situ* hybridisation data of saggital sections through the telencephalon of WT embryos at E14.5 (Genepaint). Note that there is no *in situ* hybridisation data available for Mog on the Genepaint data base. Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

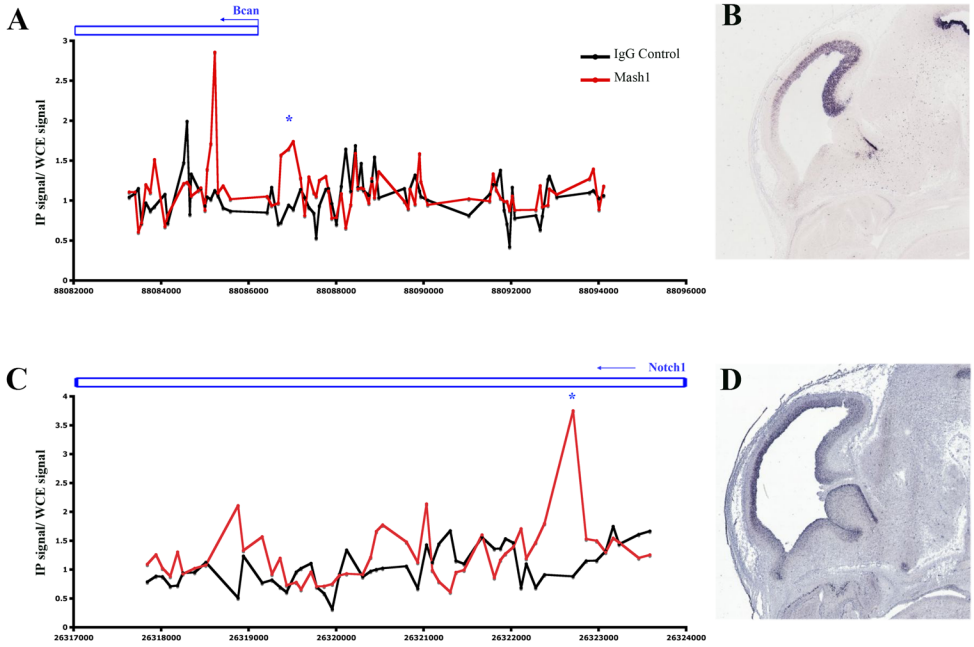


Figure 31. Mash1 binds to regulatory elements of *Bcan* and *Notch1* genes. ***A-D***, Mash1 and control IgG ChIP binding profile of *Bcan* (***A***) and *Notch1* (***C***) (blue asterisk marks point of Mash1 binding), with Bcan (***B***) and Notch1 (***D***) *in situ* hybridisation data of saggital sections through the telencephalon of WT embryos at E14.5 (Ge nepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

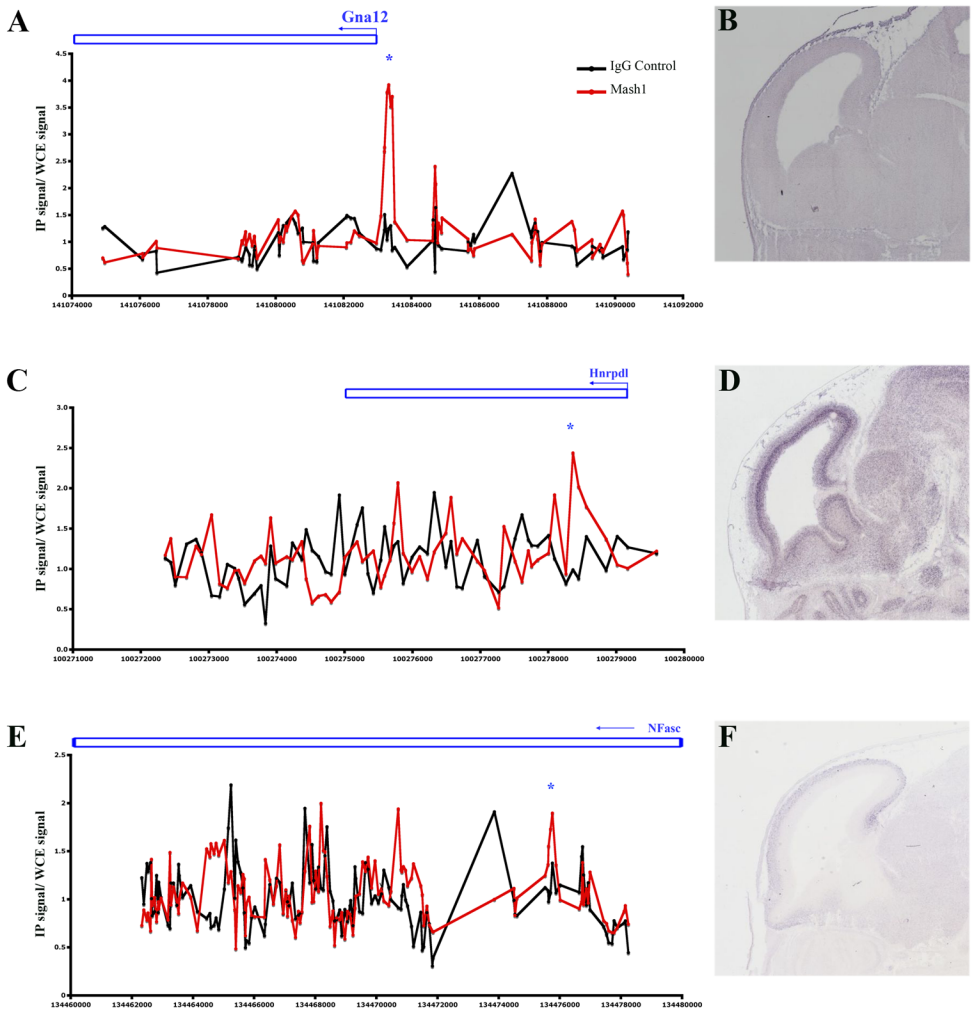


Figure 32. Mash1 binds to regulatory elements of *Gna12*, *Hnrpdl*, *Nfasc* genes. ***A-F***, Mash1 and control IgG ChIP binding profile of *Gna12* (***A***), *Hnrpdl1* (***C***) and *Nfasc* (***E***) (blue asterisk marks point of Mash1 binding), with Gna12 (***B***), Hnrpdl1 (***D***) and Nfasc (***F***) *in situ* hybridisation data of saggital sections through the telencephalon of WT embryos at E14.5 (Genepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

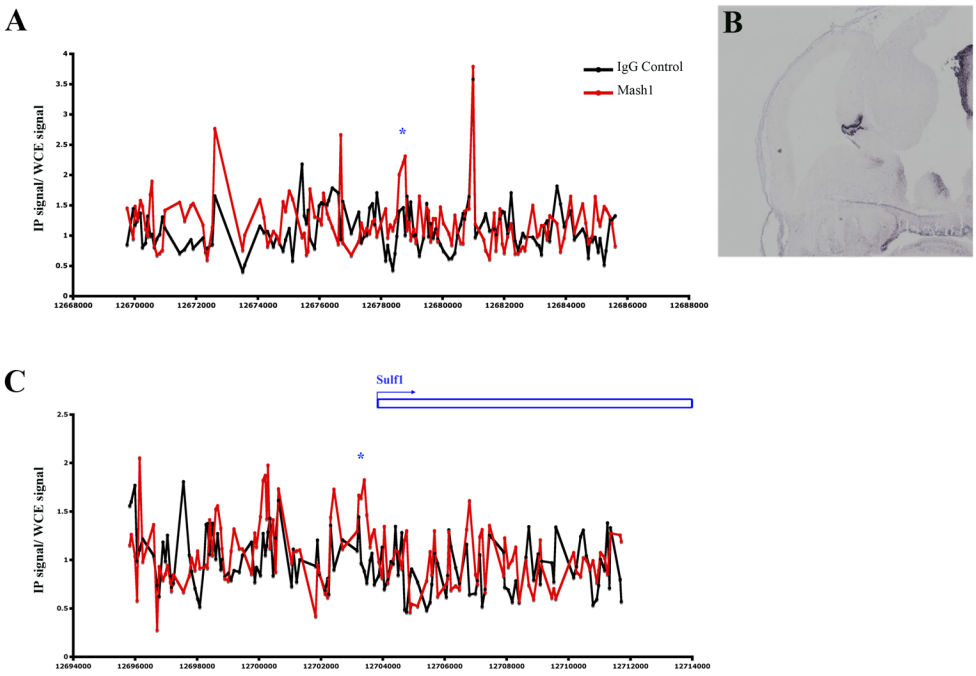


Figure 33. Mash1 binds to regulatory elements of the *Sulf1* gene. ***A-C***, Mash1 and control IgG ChIP binding profiles of *Sulf1* (***A & C***) (blue asterisk marks point of Mash1 binding), with Sulf1 (***B***) *in situ* hybridisation data of a saggital section through the telencephalon of WT mouse embryo at E14.5 (Genepaint). Genomic position is on the x-axis, UCSC Genome Browser (http://genome.ucsc.edu/), mouse assembly mm8, NCBI Build 36.

Mash1 was bound to previously described regulatory elements, *DeltaM* and *Fbxw7* sequences (Castro et al., 2006), that we use as positive control regions in oligosphere cultures (Figure 26A, Figure 27A and 27C, respectively). Furthermore, these analyses reveal that Mash1 binds to genomic segments proximal to early OPC genes such as *Sox8* and *Olig1* (Figure 26B, Figure 28A and Figure 29A/C). Notably, the *Olig2* distal element was significantly coprecipitated with Mash1 in the oligospheres compared to the *Dll1* ORF negative region or to the IgG control ChIP (defined as, *Olig2* (vt enhancer), Figure 26B and Figure 28C). In addition, Mash1 binds to genes associated with mature oligodendrocytes, including *Mog* and *Omg* (Figure 26B and Figure 30A/C). Interestingly, *Mog* and *Omg* are not transcribed early in development, but are bound by Mash1 in non-differentiated oligospheres. It is plausible to suggest that Mash1 may function to prime these regions for rapid induction of gene expression in response to the correct cues, or to recruit necessary cofactors to these sites to regulate their transcription.

Other segments bound by Mash1 that lie proximal to genes of interest include; *Brevican* (*Bcan*) (Figure 26B and Figure 31A), a neural-specific chondroitin sulfate proteoglycan (CSPG) (Yamada et al., 1994, Jaworski et al., 1994), which belongs to the lectican family of CSPGs (reviewed Yamaguchi, 2000) actively participate in the development and maturation of the nervous system (reviewed Zimmermann and Dours-Zimmermann, 2008). Bcan is expressed highly in the VZ along the length of the neuraxis during the gliogenic phase in late embryonic and early postnatal development (Jaworski et al., 1995; Milev et al., 1998). Notably, Bcan plays a role in the development of CNS fibre tracts in the postnatal hippocampal fimbria where it is expressed in

oligodendrocytes and white matter astrocytes (Ogawa et al., 2001), it also promotes glial cell motility and thus increases glioma cell invasive properties (Zhang et al., 1998; Jaworski et al., 1996; reviewed in Nutt et al., 2001); *Notch1* (Figure 26B and Figure 31C), a component of the Notch signalling pathway, which is expressed in OPCs of the developing CNS (Givogri et al., 2002). In OPCs, the Notch1 receptor functions as an inhibitor of oligodendrocyte differentiation. Notably, Notch1 receptor activation by Delta1 or Jagged1 ligands inhibits oligodendrocyte differentiation *in vitro* (Wang et al., 1998). Notch1 also functions to prevent OPCs from premature differentiation *in vivo*, such that inhibition of Notch1 signalling results in precocious differentiation of OPCs into oligodendrocytes, in addition to premature myelination in the developing CNS (Genoud et al., 2002; Givogri et al., 2002). These data suggest that Notch1 is required in the temporal regulation of differentiation of OPCs to oligodendrocytes; *Guanine nucleotide binding protein (G protein) alpha 12* (*Gna12*) (Figure 26B and Figure 32A), is a member of a super-family of signal transduction proteins that mediate a range of extracellular signals from G-protein coupled receptors to intracellular effectors, regulating cell growth, differentiation, and apoptosis (reviewed in Radhika and Dhanasekaran, 2001; Kurose, 2003). Gna12, has been associated with glioma cell motility, from differential expression studies in glioma and primary glioblastoma cells (Tatenhorst et al., 2004); *Heterogeneous nuclear ribonucleoprotein D-like1* (*Hnrpdl1*) (Figure 26B and Figure 32C), belongs to the subfamily of ubiquitously expressed RNA binding proteins, namely the heterogeneous nuclear ribonucleoproteins (hnRNPs), that complex with heterogeneous nuclear RNA (hnRNA). Hnrpdl1 is highly expressed in the CNS, and is more prominent in

neuronal than glial cells (Akagi et al., 2000). These proteins are associated with pre-mRNAs in the nucleus and functions in mRNA biogenesis and mRNA metabolism (reviewed in Dreyfuss et al., 1993, Krecic and Swanson, 1999, Weighardt et al., 1996); *Neurofascin* (*Nfasc*) (Figure 26B and Figure 32E) an ankyrin-binding, cell adhesion molecule of the L1 subgroup of the immunoglobulin G superfamily that has been implicated in a variety of processes including neurite outgrowth, fasciculation, interneuronal adhesion, and formation of functional nodes of Ranvier (Rathjen & Schachner, 1984; Grumet et al., 1991; Volkmer et al., 1992; Davis et al., 1993; Zhang et al., 1998; Sherman et al., 2005; Zonta et al., 2008). Moreover, a 155-kD isoform (NF155) of the Nfasc gene is strongly but transiently up-regulated in oligodendrocytes at the onset of myelinogenesis (Collinson et al., 1998), and is a crucial glial component of the paranodal axoglial junction (Tait et al., 2000; Charles et al., 2002; Pillai et al., 2009); and *Sulfatase 1* (*Sulf1*) (Figure 26B and Figure 33A/C), a secreted enzyme that modulates the sulfation state of heparan sulfate proteoglycans (HSPGs) (reviewed in Lamanna et al., 2007), is expressed in the oligodendrocyte lineage of the developing chick spinal cord and forebrain (Braquart-Varnier et al., 2004; Garcia-Lopez et al., 2009). Moreover, Sulf1 modulates Shh signalling in the embryonic ventral spinal cord of the chick, promoting the generation of oligodendroglial cells at the expense of neuronal cells, and is therefore thought to contribute to the neuronal/glial switch in ventral progenitors (Danesin et al., 2006).

Interestingly, when genes with segments bound by Mash1 from ChIP-on-chip are cross referenced to gene expression data from *Mash1* null embryos and

an oligodendroglial specific gene list, we found that 46% of the genes are indeed regulated by Mash1 and expressed in the oligodendroglial cell lineage. The *Mash1* null expression data set were generated from the MGE/AEP region of E13.5 embryos, where the first wave of OPCs is produced in the ventral telencephalon and ventral thalamus (Carlos Parras, unpublished data). Affymetrix MOE430.2 whole genome microarrays were normalised by GeneChip Robust Multiarray Averaging (GCRMA; Wu et al., 2004) and Significance Analysis of Microarray (SAM; Tusher et al., 2001), these were further analysed using BRB-Array tool (www.linus.nci.nih.gov) and gene set analysis (GSA) software (Efron and Tibshirani, 2007). Oligodendroglial specific gene lists were compiled from: (i) a detailed transcriptome analysis of OPCs and differentiated/ myelinating oligodendrocytes from the postnatal mouse forebrain (Cahoy et al., 2008); (ii) a comparative analysis of gene expression profiles of rat OPCs and differentiated oligodendrocytes (Nielsen et al., 2006); (iii) in addition to expression arrays using *PDGFRα*-GFP and *Olig2*-GFP mouse lines (Nathaniel Heintz, unpublished data).

In conclusion, this experiment provides the first set of candidate target genes of Mash1 in the oligodendroglial lineage. To determine if these genes are indeed specifically regulated by Mash1 in the oligodendroglial lineage rather than in neuronal precursors or multipotent progenitors will require further analysis.

3.6 Validation of putative enhancer elements bound by Mash1

To investigate the regulatory potential of genomic elements bound by Mash1 in oligosphere cultures, we assayed these segments *in vitro* using transcription assays in the NS5 cell line, and asked whether Mash1 protein, alone or in combination with other oligodendroglial promoting TFs, including Olig2 and Sox9, could interact with these elements and activate luciferase reporter gene expression. The NS5 cells were chosen for this assay primarily because of the ease of obtaining sufficient numbers of homogenous cultures that were reproducible from one experiment to the next. Note that the NS5 cells do not need to generate OPCs efficiently to be an appropriate model to test the transcriptional regulation of OPC genes, and endogenously express oligodendrocyte promoting TFs, Sox9 and Olig2 as well as Mash1 (Figure 12B-G).

The proneural protein Mash1, heterodimerises with ubiquitously expressed bHLH proteins, including *E2A* splice variants E12 and E47, and binds to an E-box (a six base pair motif (CANNTG)) in promoters of target genes to regulate gene expression (Massari & Murre, 2000). bHLH proteins bind to specific subsets of E-boxes with different preferences (Bertrand et al., 2002; Powell et al., 2004), indeed Mash1 has a tendency to bind to a (CAG[C/G]TG)-type E-box (Castro et al., 2006). Nevertheless, it is important to note that the specificity to bind subsets of E-boxes alone is not sufficient to explain the recognition of target promoters by proneural proteins. A TF with a stringent six base pair recognition sequence would identify 740,000 sites in a genome of a size of 3×10^9 base pairs, which is many more times the number of total genes

contained in the mammalian genome (Kondoh et al., 2004). Evidently, other mechanisms must contribute to the specificity of target gene recognition by proneural proteins, and may include interactions between different DNA-binding proteins.

Mash1 and Brn (which bind to octamer sequences (Nishimoto et al., 2003) proteins functionally synergise to regulate a number of target genes that control multiple aspects of the neurogenic program, and include the activation of the mouse *Dll1* gene through cooperative binding to a proximal evolutionarily conserved motif (Castro et al., 2006). More recently, it was demonstrated that Mash1 cooperates with the Olig2 bHLH TF, in the specification of an early population of telencephalic oligodendrocytes in the embryo (Parras et al., 2007). However, the molecular mechanism underlying the functional synergy between Mash1 and Olig2 in the oligodendrogenic programme, has not yet been elucidated. Similar to Mash1, Olig2 binds degenerate E-boxes, however whether Olig2 recognises a specific type of E-box, is yet to be described. A yeast-two hybrid screen using mouse Sox9 (101-338 amino acids) as bait against a mouse embryo brain library identified Mash1 as a very high confidence interacting partner, suggesting that Mash1 and Sox9 may interact at a protein level (James Briscoe, personal communication). Together with the fact that Mash1 and Sox9 are coexpressed in OPCs as soon as they emerge from the VZ of the developing ventral telencephalon (Figure 10), we propose that Mash1 and Sox9 have the potential to cooperate in the specification of early born OPCs. Indeed, this combinatorial code of TFs may also contribute to the molecular mechanisms underlying Mash1 function in the oligodendroglial programme. Sox9 binds DNA through an SRY-like consensus sequences (WWACAAT, where W, (A/T)),

(Sekido & Lovell-Badge, 2008). Note that other SoxE group proteins, such as Sox10 are capable of binding DNA as monomers and dimers that bind to two SRY-like binding sites oriented in a head-to-head fashion (Peirano et al., 2000b).

To confirm that genes with associated genomic elements bound by Mash1 were indeed regulated by this proneural factor, we examined their expression in the ventral telencephalon of *Mash1* mutant embryos. The proneural protein Mash1 functions as a transcriptional activator, and therefore genes under the control of Mash1 are down regulated in *Mash1* null animals (Castro et al., 2006; Gohlke et al., 2008). We also tested the ability of the genomic elements to regulate gene expression *in vivo* with a *lacZ* reporter gene using mouse transient transgenesis, in order to define their spatial and temporal activity, and therefore identify those elements with regulatory capabilities in the oligodendroglial lineage in the CNS.

3.6.1 Validation of distal *Olig2* enhancer activity

To identify additional TF binding sites within the non-coding putative *Olig2* distal enhancer element, we used the orthologous sequence in different species to perform phylogenetic footprinting by comparative analysis. Notably, the putative *Olig2* distal element was found to be evolutionary conserved across a diverse number of distantly related species including human, rat, dog, chicken, cow and in some regions zebrafish (Figure 34A-C). Detailed analysis of the sequence revealed evolutionary conserved putative consensus binding sites for a bHLH Mash1 type E-box (Figure 34C, red box), and an octamer motif, for the

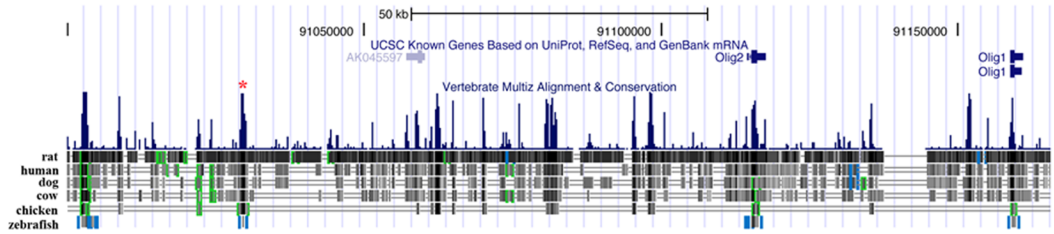
POU family of homeodomain proteins (Figure 34C blue box), as expected since this element was isolated from an *in silico* screen on the basis of the presence of proximal Mash1 (CAGSTG, where S, (C/G)) and Brn (ATTWNYAW, where W, (A/T) and Y, (C/T)) conserved binding sites. In addition we identified a putative SRY-like consensus sequences, in close proximity (Figure 34C green box).

To investigate the regulatory potential of the putative *Olig2* distal enhancer *in vitro*, we used a luciferase based transcription assay to test the ability of this genomic element to direct reporter gene expression in the NS5 cell line. Note that Olig2 is endogenously expressed in the NS5 cell line (Figure 12C). In brief, the luciferase assay is a sensitive method for determining the level of luciferase expression in cells transfected with a luciferase reporter vector. The reaction catalyzed by luciferase results in the production of light, which can be quantitated using a luminometer. Importantly, in mammalian cells luciferase has a short half-life of approximately 3 hours and does not accumulate in cells, therefore changes in promoter activity are rapidly reflected in luciferase activity.

Briefly, the mouse *Olig2* distal enhancer was cloned upstream of a minimal promoter (β-globin) directing basal luciferase expression, and luciferase activity was assayed 24 hours post-transfection. We used the previously described bicistronic vector (pCAGGS-IRES-nls-GFP), which encoded Mash1, Sox9, Olig2, Mash1EngR or Sox9EngR, to attain high levels of expression of these factors. Using this assay we examined whether Mash1, Sox9 or Olig2 alone could interact with this genomic segment and activate luciferase reporter gene expression. Furthermore, we asked whether a potential synergy between Mash1 and other oligodendroglial promoting TFs namely, Olig2 or Sox9, exist on this genomic region. The *Olig2* segment showed a high basal level of enhancer

activity without addition of any exogenous TFs (Figure 34D). Addition of Mash1 or Sox9 alone resulted in a small but significant increased enhancer activity compared to control. Moreover Mash1 enhancer activity was reduced in the presence of Sox9EngR, suggesting that perhaps Mash1 and Sox9 synergise at this genomic locus. Addition of Olig2 did not have a pronounced effect in this assay. Note that although binding of a factor to an enhancer region can be necessary, it may not be sufficient, for high levels of promoter activity. We propose that the high basal activity, the relatively small increases in enhancer activity following the exogenous addition of TFs, and the significant repression of basal activity with Mash1EngR or Sox9EngR constructs, can be accounted for by the fact that Mash1, Olig2 and Sox9 are already endogenously expressed by the NS5 cells. Indeed Olig2 and Sox9 expression levels may have reached a saturation point, above which the NS5 cells are not responsive. In conclusion, these data demonstrate that Mash1 and Sox9 proteins interact with the *Olig2* element.

A



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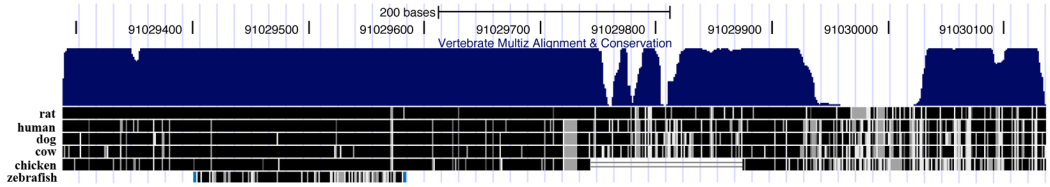

C

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97    ACATCAAATCAGTA-AGCCCTGTTGCCTGTCAGTGAATACAATGTCAGGC   mouse
97    ACATCAAATCAGTA-AGCCCTGTTGCCTGTCAGTGAATACAATGTCAGGC   rat
97    ACATCAAATCAGTA-AGCCCTGTTGCCTGTCAGAGAATACAATGTCAGGC   human
99    ACATCAAATCAGTA-AGCCCTGTTGCCTGTCAGAGAATACAATGTCAGGC   dog
101   ACATCAAATCAGTA-AGCCCTGTTGCCTGTCAGAGAATACAATGTCAGGC   cow
96    ACATCAAATCAGTA-AACCCTGTTGCCTGTCAGAGAATACAATGTCAGGC   chicken
1     ATTTGTAGCCTGCACAGTCACATTGTTTGTTGAGTCTTGCAGTG-CAGGC   zebrafish

146   CATTAGTATGTTAATAAGTCCGGAGGGCGACAGGTGGCCTACTAGACAGC   mouse
146   CATTAGTATGTTAATAAGTCCGGAGGGCGACAGGTGGCCTACTAGACAGC   rat
146   CATTAGTATGTTAATAAGTCCGGAGGGCGACAGGTGGCCTACTAGACAGC   human
148   CATTAGTATGTTAATAAGTCCGGAGGGCGACAGGTGGCCTCCTAGACAGC   dog
150   CATTAGTATGTTAATAAGTCCGGAGGGCGACAGGTGGCCTACTAGACAGC   cow
145   CATTAGTATGTTAATAAGTCCGGAGGGTGACAGGTGGCCTAGTAGACAGC   chicken
50    CGCT-------TCGTTAGCTTGGCCTTTAACGAGCTGCTTAATTGAGCTG   zebrafish
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D

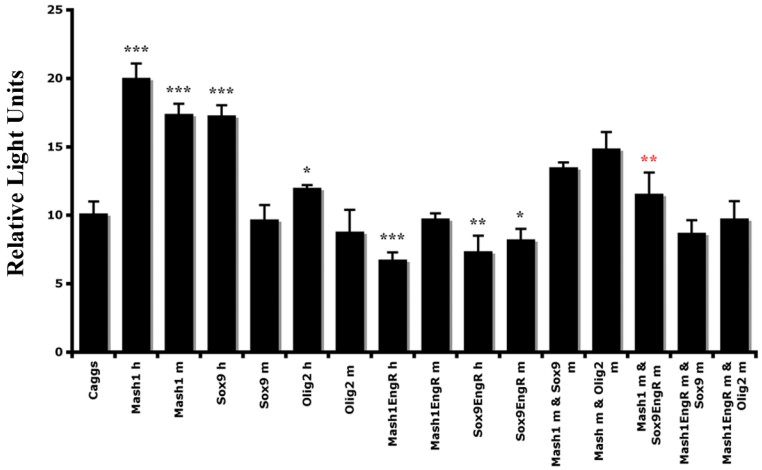


Figure 34. Activation of the *Olig2* enhancer requires Mash1 and Sox9 in NS5 cells. ***A***, *Olig2* enhancer (red asterisk) lies approximately 82kb upstream of the *Olig2* locus, from the UCSC Genome Browser (http://genome.ucsc.edu/ [coordinates are from the February 2006, mm8 NCBI Build 36 UCSC Genome Browser Mouse assembly]). ***B***, Enlargement of the *Olig2* enhancer, an 848bp interval. ***C***, Alignment of the *Olig2* enhancer sequences from the mouse, human, rat, cow, dog, chicken, and zebrafish genomes. Red box delineates the sequences of a conserved E-boxes, blue box delineates the sequence of a conserved octamer, and a green box delineates the sequence of a conserved SRY-like putative consensus sequence. ***D***, Transcriptional assay in NS5 cells cotransfected with a Mash1, Sox9, Olig2, Mash1 Engrailed Repressor, or Sox9 Engrailed Repressor plasmid and a reporter construct expressing luciferase under the control of the *Olig2* enhancer. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

To verify whether Mash1 could play a role in the regulation of *Olig2 in vivo*, Olig2 expression was analysed in *Mash1* null animals, during a period where Mash1 function is required for oligodendrogenesis in the ventral telencephalon. Notably, Olig2 expression was markedly reduced in the VZ of the MGE, a region where PDGFRα+ OPCs are specified and Mash1 is normally expressed, in *Mash1* null mutant embryos compared to wild-type aged-matched control at E12.5 (Figure 35). These data suggest that *Olig2* gene expression in the developing ventral telencephalon is likely regulated by Mash1.

The expression of Olig2 in the mammalian CNS, including in the oligodendroglial lineage has been well documented (Lu et al., 2000). To determine whether the putative *Olig2* enhancer could recapitulate part of this expression we tested its ability to regulate gene expression *in vivo* with a *lacZ* reporter gene using mouse transient transgenesis. Briefly, the *Olig2* genomic segment was cloned upstream of a minimal promoter (β-globin) and *lacZ* coding sequence, and injected into mouse pronuclei. Reporter gene expression was analysed by X-Gal staining of tissue sections at specific embryonic time points, E12.5 and E14.5, respectively. The distal *Olig2* element did not show independent enhancer activity, and failed to direct reporter gene expression in all 20+ embryos that were analysed (data not shown). We therefore concluded, that the described *Olig2* genomic segment contained no regulatory sequences able to recapitulate the temporal or spatial pattern of Olig2 expression in mouse transient transgenic analyses. It is possible that this element may function as a general enhancer that modulates an already specified expression.

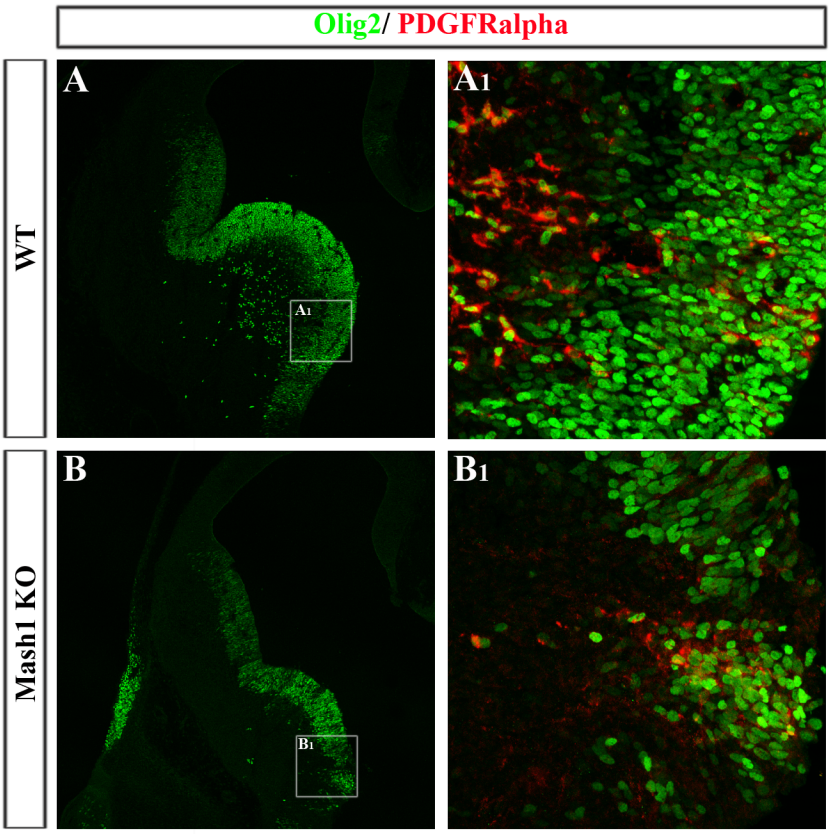


Figure 35. Olig2 expression is down regulated in the VZ in the ventral telencephalon of *Mash1* null embryos at E12.5. ***A***, Wild-type E12.5 mouse coronal section through the telencephalon, Olig2 is expressed highly in the VZ of the MGE. Inset ***A1*** is an enlargement of the area outlined by the square in ***A***, where OPCs, stained with anti-PDGFRα antibody, are specified and migrate away from the VZ. ***B***, *Mash1* null E12.5 mouse coronal section through the telencephalon, Olig2 expression is reduced in the VZ of the MGE. Inset ***B1*** is an enlargement of the area outlined by the square in ***B***. ***B1***, a reduced number of OPCs are stained with anti-PDGFRα antibody.

It is important to note at this point the number of disadvantages of the transient transgenesis system, namely the inability to visualise dynamic enhancer activity. Indeed, to get a complete overview of the activity of an enhancer multiple developmental and postnatal time points would have to be analysed. Moreover this strategy is focused towards the identification of regulatory elements that confer positive activity on gene expression, and do not lend to the discovery of regions that are involved in fine-tuning or indeed repressing gene regulation. Enhancer screening strategies would therefore benefit from co-injections with a well-characterised enhancer, such as the limb enhancer (Lettice et al., 2003), to drive expression of a different reporter gene as a positive control. Furthermore, this strategy is based on the assumption that the putative enhancer of interest is sufficient to drive reporter gene expression in a context independent manner with regards to the original locus and surrounding genomic regions from where it was extracted. Mammalian genomes are organized into high-level three-dimensional structures; therefore it is not surprising that chromatin interactions constitute a primary mechanism for regulating transcription. Indeed, distal binding sites have been shown to regulate transcription through the formation of functional long-range chromatin interactions (Fullwood et al., 2009; West and Fraser, 2005; Woodcock, 2006). In addition, some *cis*-regulatory elements may require the native basal promoter of the particular gene it regulates to confer appropriate cell-specific transcription *in vivo*. However, cloning individual gene specific basal promoters for a large enhancer screen is impractical, and it is more convenient to use a heterologous basal promoter such as the β-globin minimal promoter.

Interestingly, a sequence referred to as *ULTRA*, for ultraconserved, (mm8_chr16: 91,029,26-91,029,835) residing within the *Olig2* genomic region tested has been isolated (Chen et al., 2008). Briefly, ultraconserved elements were first defined as at least 200 base pair long sequences that show perfect conservation in alignments of the human, mouse and rat reference genomes, and are thought to represent sequences under selection for specific functions (Bejerano et al., 2004; Chen et al., 2007). *ULTRA* contains 106 base pair and 45 base pair sequences of perfect human, mouse and rat conservation, which are separated by only a 1 base mismatch in the human genome and is significantly enriched for putative TF binding motifs (Chen et al., 2008).

The activity of *ULTRA* in mouse undifferentiated ES cells and neural precursor cells has been characterised using the native basal promoter from the *Olig2* locus which is constitutively active in both cell types (Zhang et al., 2008; Xian et al., 2005). These analyses show that *ULTRA* contains sequences that repress Olig2 expression in undifferentiated ES cells, but not in neural precursor cells and therefore *ULTRA* may play a significant part in maintaining Olig2 expression off in the former (Chen et al., 2008). It is interesting with this information to think about the possible biological significance of Mash1 and Sox9 binding to this element. We predict a model in which Mash1 and Sox9 proteins functionally synergise to relieve the repression of Olig2 by interactions on the *ULTRA* segment as cells develop along the neural lineage, and thus permitting Olig2 expression.

Indeed we provide genetic evidence to support the fact that Mash1 regulates Olig2 expression *in vivo* (Figure 35) that corroborates with *Mash1* null

embryonic expression array data, which demonstrate a significant reduction in Olig2 transcripts (p-value, $1.79x10^{-7}$; Carlos Parras, unpublished data). However, we are yet to define the regulatory modules that confer Mash1 activity on this gene. Indeed, Olig2 regulatory elements are complex and scattered over a large genomic distance, which renders the analysis of its regulation difficult (Sun et al., 2006; Xian et al., 2005). We propose that by adopting a more gene centric strategy, using a comparative approach to identify evolutionary conserved Mash1 type E-boxes (CAG[C/G]TG) surrounding the *Olig2* locus, validating Mash1 binding using ChIP-PCR, and defining their *in vivo* spatial and temporal activity using mouse transient transgenics, will help to identify *Olig2* regulatory regions modulated by Mash1 activity in the oligodendroglial lineage of the CNS.

3.6.2 Validation of distal *Sox9* enhancer activity

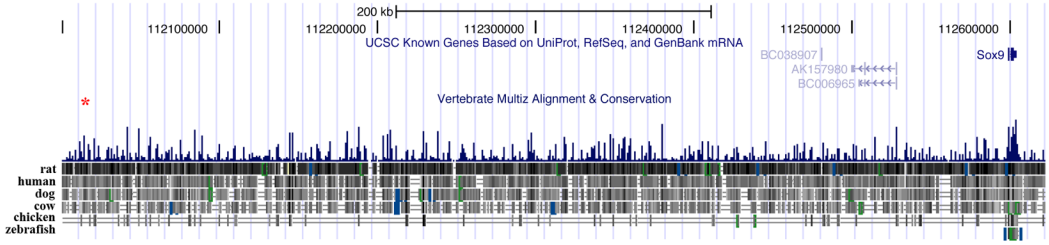
Distal *cis*-regulatory elements in vertebrates can be located far from the gene (Vavouri et al., 2006). The regulatory domain responsible for directing tissue specific expression of *Sox9* spans a vast genomic distance, and involves more than 1 Mb of upstream and downstream sequence from SOX9 (Jakobsen et al., 2007; Benko et al., 2009; Velagaleti et al., 2005; Gordon et al., 2009), to suggest that the total genomic domain regulating SOX9 expression may extend over 3 Mb. Notably, these studies highlight the complexity of *Sox9* gene regulation, and the need to identify still elusive enhancers such as those responsible for *Sox9* expression during oligodendrogenesis.

To identify additional TF binding sites within the putative *Sox9* distal enhancer element, we performed phylogenetic footprinting (as described before for the Olig2 element). The putative *Sox9* distal element was found to be evolutionary conserved across a number of mammalian species including human, rat, dog, chicken and cow (Figure 36A-C). Detailed analysis of the sequence revealed evolutionary conserved putative consensus binding sites including two proximal bHLH Mash1 type E-boxes (Figure 36C, red boxes), and two overlapping octamer motifs for the POU family of homeodomain proteins (Figure 36C blue boxes), as expected since this element was isolated from an *in silico* screen on the basis of the presence of proximal Mash1 (CAGSTG, where S, (C/G)) and Brn (ATTWNYAW, where W, (A/T) and Y, (C/T)) conserved binding sites. In addition we identified an SRY-like putative consensus sequence (Figure 36C green box).

To investigate the regulatory potential of the putative *Sox9* distal enhancer *in vitro*, we used a luciferase based transcription assay to test the ability of this genomic element to direct reporter gene expression in the NS5 cell line (as described before for the Olig2 element). Note that Sox9 is endogenously expressed in the NS5 cell line (Figure 12F). We examined whether Mash1, Sox9 or Olig2 alone could interact with this genomic segment and activate luciferase reporter gene expression. Moreover, we asked whether a potential synergy between Mash1 and Olig2 or Sox9, exist on this genomic region. The Sox9 segment showed a high basal level of enhancer activity without addition of any exogenous TFs (Figure 36D). Addition of Mash1 or Sox9 alone resulted in a moderate but significant increased enhancer activity compared to control.

Moreover the activity of Mash1 or Sox9 on this element was reduced with Sox9EngR or Mash1EngR respectively, suggesting that these TFs synergise at this genomic region. Note that addition of Olig2 to this system did not have a pronounced effect in this assay. Similar to the *Olig2* element, we propose that the high basal activity, the relatively small increases in enhancer activity following the exogenous addition of TFs, and the significant repression of basal activity with Mash1EngR or Sox9EngR constructs, can be accounted for by the fact that Mash1, Olig2 and Sox9 are already endogenously expressed by the NS5 cells. In conclusion, these data demonstrate that Mash1 and Sox9 proteins interact with the *Sox9* element.

A



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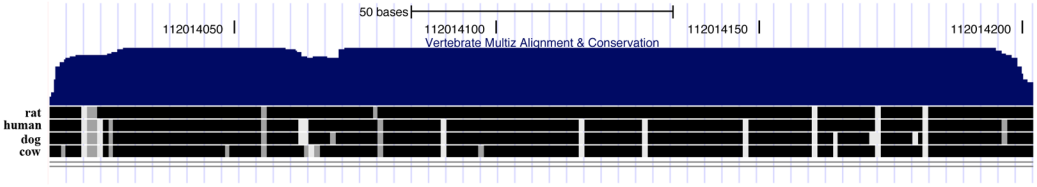

C

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1    TAAACCACCGGGAACATTCACATGGAAATGAGGTTAATTACGCAGCATCC  mouse
1    TAAACCA---GGAACATTCACATGGAAATGAGGTTAATTACACAGCATCC  rat
1    TAAACCAT---GGACATTCACATGGAAATGAGGTTAATTACACAGCATAG  human
1    TAAACCAC---GGACATTCACATGGAAATGAGGTTAATTACACAGCATAG  dog
1    TAAGCCAC---GGACATTCACATGGAAATGAGGTCAATTACACAGCATCT  cow

51   ATGCATCTAATGACTGCACCTGCAGGGCCACAACAACCTCCCTTTAACTC  mouse
48   ATGCATCTAATGGCTGCACCTGCAGGGCCACAACAACCTCCCTTTAACTC  rat
48   ATGCATCTAATGATTGCACCTGCAGTGCCACAACAACCTCCCTTTAACTC  human
48   ATGCGTCTAATGATTGCACCTGCAGTGCCACAACAACCTCCCTTTAACTC  dog
48   CCGCATCTAATGATTGCACCTGCAGTGCCACAGCAACCTCCCTTTAACTC  cow

101  ACTTTCACA----ATTAGAAAGAGCAGCTGCATTCGATTCCACAGTTAGC  mouse
98   ACTTTCACA----ATTAGAAAGAGCAGCTGCATTCGATTCCACAGTTAGA  rat
98   AGTTTCACAAACAATTACAAAGAGCAGCTGCATTCGTTTCCACAGTTAGA  human
98   AGTTTCACAAACAATTACAAAGAGCAGCTGCATTCGCTTCCACAGTTAGA  dog
98   AGTTTCACAAACAATTACAAAGAGCAGCTGCATTCGTTTCCACAGTTAGA  cow
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D

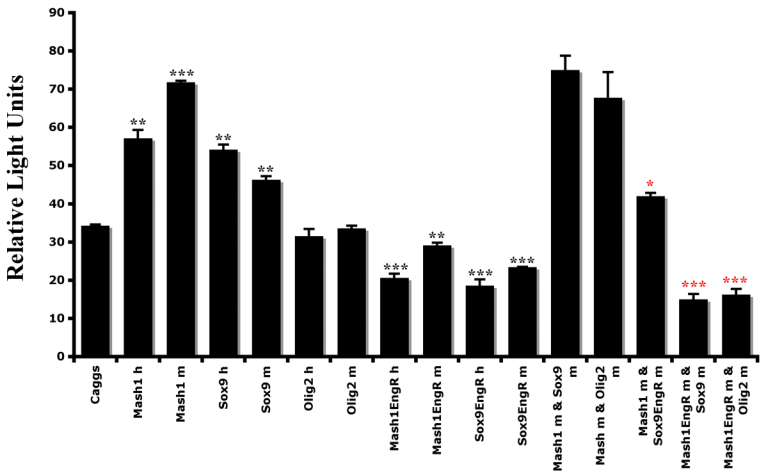


Figure 36. Activation of the *Sox9* enhancer requires Mash1 and Sox9 in NS5 cells. ***A***, *Sox9* enhancer (red asterisk) lies approximately 500kb upstream of the *Sox9* locus, from the UCSC Genome Browser (http://genome.ucsc.edu/ [coordinates are from the February 2006, mm8 NCBI Build 36UCSC Genome Browser Mouse assembly]). ***B***, Enlargement of the *Sox9* enhancer, a 137bp interval. ***C***, Alignment of the *Sox9* enhancer sequences from the mouse, human, rat, cow and dog genomes. Red boxes delineate the sequences of two conserved E-boxes, blue boxes delineate the sequence of two conserved octamer, and a green box delineates the sequence of a conserved SRY-like putative consensus sequence. ***D***, Transcriptional assay in NS5 cells cotransfected with a Mash1, Sox9, Olig2, Mash1 Engrailed Repressor, or Sox9 Engrailed Repress or plasmid and a reporter construct expressing luciferase under the control of the *Sox9* enhancer. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

To verify whether Mash1 could play a role in the regulation of *Sox9 in vivo*, Sox9 expression was analysed in *Mash1* null embryos, during a period where Mash1 function is required for oligodendrogenesis in the ventral telencephalon. Notably, Sox9 expression was strongly reduced in the VZ of the MGE, a region where PDGFRα+ OPCs are specified and Mash1 is normally expressed, in *Mash1* null mutant embryos compared to wild-type aged-matched control at E12.5 (Figure 37). In contrast, Sox9 expression remained unperturbed in the VZ of the dorsal telencephalon, a region where Mash1 is expressed albeit at low levels (Britz et al., 2006), in *Mash1* null mutant embryos compared to wild-type control at E12.5 (Figure 37). These data suggest that Sox9 expression is likely to be regulated by Mash1 in the ventral telencephalon, whilst different mechanisms may operate to regulate *Sox9* in the dorsal telencephalon.

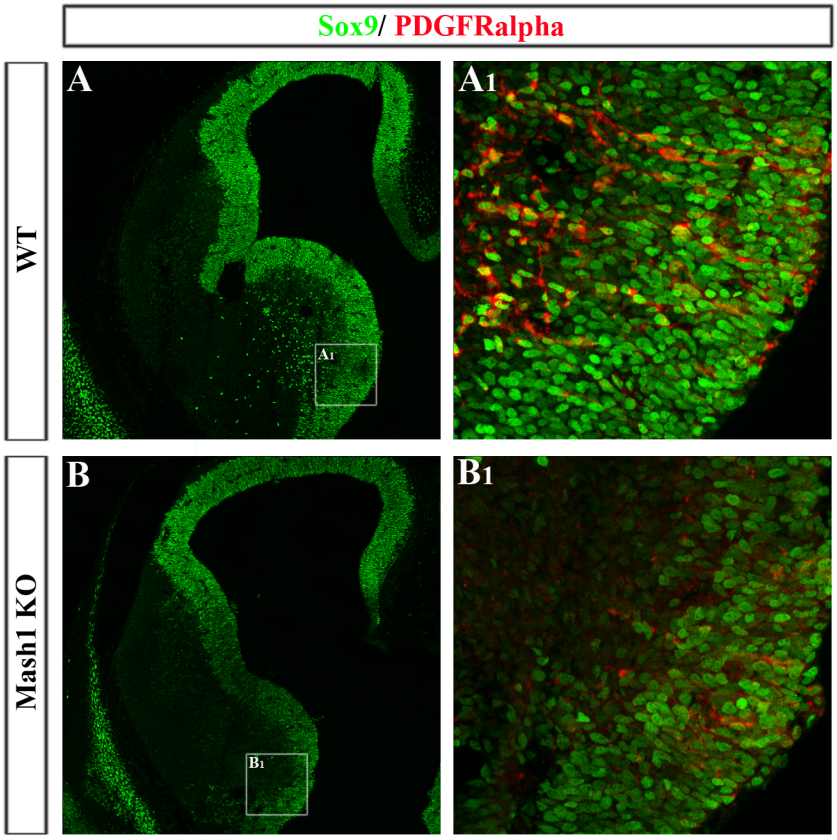


Figure 37. So x9 expression is down regulated in t he VZ in the ventral telencephalon of *Mash1* null embryos at E12.5. ***A***, Wild-type E12.5 mouse coronal section through the telencephalon, Sox9 is expressed highly in the VZ of the dorsal and ventral telencephalon. Inset ***A1*** is an enlargement of the area outlined by the square in ***A***, where OPCs, stained with anti-PDGFRα antibody, are specified and migrate away from the VZ. ***B***, *Mash1* null E12.5 mouse coronal section through the telencephalon, Sox9 expression is reduced in the VZ of the MGE in the ventral telencephalon, and is not affected in the VZ of the dorsal telencephalon. Inset ***B1*** is an enlargement of the area outlined by the square in ***B***. ***B1***, a reduced number of OPCs are stained with anti-PDGFRα antibody.

Sox9 is expressed in the VZ in the developing spinal cord and telencephalon, and is maintained in proliferating and migrating OPCs (Stolt et al., 2003; Figure 10 and data not shown). To determine whether the putative *Sox9* enhancer could recapitulate part of this expression we tested its ability to regulate gene expression *in vivo* with a *lacZ* reporter gene using mouse transient transgenesis (as previously described). Reporter gene expression was analysed by X-Gal staining of tissue sections at E12.5 of embryonic development. We found that the *Sox9* enhancer directed reporter gene expression in the dorsal and more prominently in the ventral telencephalon at E12.5 (n=1, Figure 38A). Specifically X-gal staining was visualised in a salt-and pepper fashion in the VZ of the ventral telencephalon (Figure 38A1), similar to the patterns observed in characterised Mash1 targets (Castro et al., 2006; Gohlke et al., 2008). We detected intense X-gal staining in the mantle zone of the ventral telencephalon, where post-mitotic neurons differentiate. Interestingly, we also identified two streams of X-gal positive cells migrating tangentially from the ventral ganglionic eminences to the developing cortex. Note that these tangentially migrating cells are a significant source of cortical interneurons and other cell types, including oligodendrocytes (Corbin et al., 2001). To determine whether the *Sox9* enhancer is active in the oligodendroglial lineage we used an antibody to PDGFRα in order to identify OPCs, and an antibody to β-galactosidase (β-gal) to identify cells that have experienced reporter activity. We identified a subset of PDGFRα+ cells that coepxressed β-gal close to the VZ in the ventral telencephalon (Figure 38B and C, white arrows). We propose that the *Sox9* enhancer is active in neural progenitors that reside in the VZ of the developing ventral telencephalon, that subsequently give rise to neuronal and oligodendroglial cells. From these

transient transgenic analysis we conclude that the *Sox9* genomic sequences described contained regulatory sequences able to recapitulate some temporal and spatial pattern of the endogenous Sox9 expression in the developing mouse telencephalon, which include the oligodendroglial lineage.

Indeed we provide genetic evidence to support the fact that Mash1 regulates Sox9 expression *in vivo* (Figure 37) that corroborates with *Mash1* null embryonic expression array data, which demonstrate a significant reduction in Sox9 transcripts (p-value, $5.98x10^{-5}$; Carlos Parras, unpublished data). To determine whether this distal enhancer mediates the regulation of *Sox9* by Mash1, stable transgenic mouse lines would be established, and bred with *Mash1* null mutant mice (Guillemot et al., 1993). Indeed these analyses would detail the requirement of Mash1 function for the activation of the distal *Sox9* enhancer.

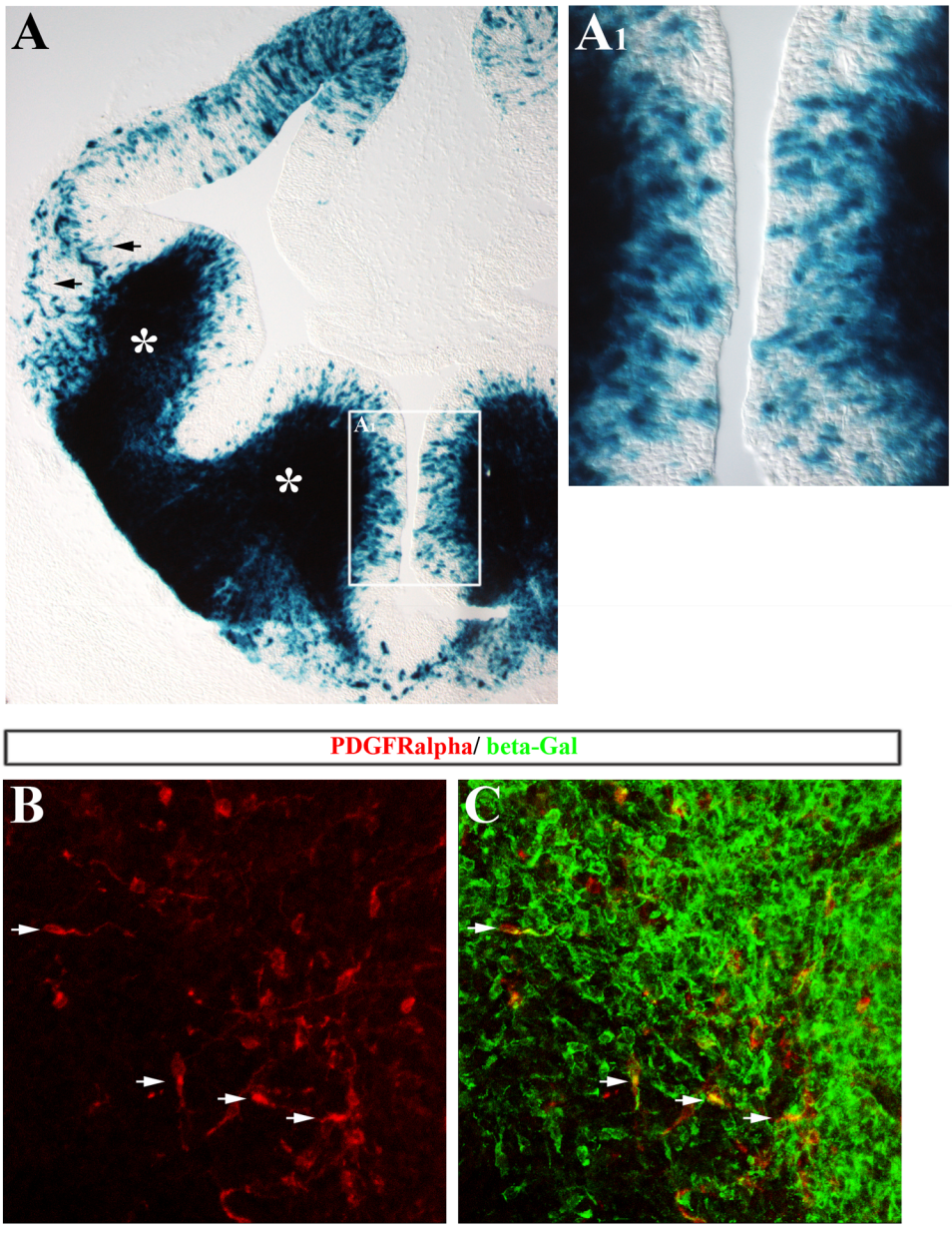

A
A1
PDGFRalpha/ beta-Gal
B
C

Figure 38. Distal *Sox9* enhancer is able to recapitulate part of *Sox9* gene expression in the ventral telencephalon, including the oligodendroglial lineage at E12.5. ***A***, Coronal section through the telencephalon of an X-gal stained transgenic embryo for the *Sox9* enhancer at E12.5. Note the two streams of dorsally migrating cells into the cortex from the ventral ganglionic eminences (black arrows), and high intensity of X-gal staining in the mantle zone of the ventral telencephalon, where neurons differentiate (white asterisks). Inset $\boldsymbol{A_1}$ is an enlargement of the area outlined by the square in ***A***, where X-gal staining in the VZ demonstrates a salt and pepper pattern. ***B & C***, A subset of PDGFRα+ OPCs close to the VZ in the ventral telencephalon co-express β-gal (white arrows).

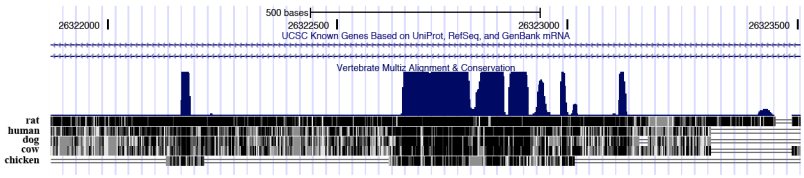
3.6.3 A mouse transient transgenic screen to isolate proximal genomic elements bound by Mash1 with activity in the oligodendroglial lineage

To establish a robust screening strategy, to identify genomic segments with regulatory activity in the oligodendroglial lineage from the list of Mash1 bound genomic elements, we performed mouse transient transgenesis (as described previously). We concluded that luciferase assays were not a reliable system to identify *de novo* enhancers, namely because enhancer activity in this assay is highly dependent on the cellular context. Moreover, this strategy fails to identify enhancer activity at the level of the cell, which can be addressed by transient transgenics in combination with X-gal staining and immunostaining for specific oligodendroglial lineage markers such as PDGFRα, to identify OPCs.

3.6.4 Validation of proximal *Notch1* enhancer activity

To identify TF binding sites within the putative *Notch1* enhancer element, we performed phylogenetic footprinting (as described previously). The putative *Notch1* element was found to be evolutionary conserved across a number of mammalian species including human, rat, dog, chicken and cow (Figure 39). Detailed analysis of the sequence revealed evolutionary conserved putative consensus binding sites including a bHLH Mash1 type E-box (Figure 39B, red box) and a SRY-like putative consensus sequence (Figure 39B green box).

A



B

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803   ATTAATC-GCCTCCC--AACAATAGCTGCTGCCCTTCTACTGAATCCCAG  mouse
829   ATTAATC-GTCTCCC--AACAATAGCTGCTGCCCTTCTACTGAATCCCAG  rat
850   ATTAATCCGCCTCCCCCAACAATAGCTGCTGCACTTCCCCTGGATCCCAG  human
865   TCTG-CCACCGAGGAGGAGCAGGGACAGCCCAGCCCCAGGGGACACTGGG  dog
763   ATTAATCCGCCTCCCC-AACAATAGCCCCTGCACTTCCACTGAATCCCAG  cow
658   ATTAATCTGCCTCCCTTAACAATAGCTGCCGAATTTCGACCGAATCCCAG  chicken

850   CTGTC---GGCCTCTGAATGGAAGGAAATAAGAT--TTAGGGCATCAAGC  mouse
876   CTGTC---GGCCTCTGAATGGAAGGAAATAAGAT--TTAGGGCATCAAGC  rat
900   CTGTC---GGTCTCTGAATGAAAGGAAACAAGAT--TTAGGGCATCAAGC  human
914   GTGTCTGGGGGCTGAGCCTGGGCTACAAGGGGATGCCTTGGGGTGCAGGC  dog
812   CTGTC---GGTCTCTCAATGAAAGGAAACAAGAT--TTAGGGCATCAAGC  cow
708   CTGTC---GCTCTTTGAATGAAAGAAAACAAGAT--TTAGAGCGTCAAGC  chicken
```

Figure 39. Putative *Notch1* enhancer is e volutionary conserved across mammalian species. ***A***, *Notch1* putative enhancer, from the UCSC Genome Browser (http://genome.ucsc.edu/ [coordinates are from the February 2006, mm8 NCBI Build 36UCSC Genome Browser Mouse assembly]). ***B***, Alignment of the *Notch1* enhancer sequences from the mouse, human, rat, cow, dog and chicken genomes. Red box delineate the sequences of a conserved E-box, and a green box delineates the sequence of a conserved SRY-like putative consensus sequence.

Notch1 is expressed in the VZ of the developing CNS, and is more prominent in the ventral rather than dorsal telencephalon during development (Tokunaga et al., 2004; Guillemot & Joyner, 1993; Lindsell et al., 1996). To determine whether the putative *Notch1* enhancer could recapitulate part of this expression we tested its ability to regulate gene expression *in vivo* with a *lacZ* reporter gene using mouse transient transgenesis (as previously described). Reporter gene expression was analysed by X-Gal staining of tissue sections at E13.5 of embryonic development. We found that the *Notch1* enhancer directed reporter gene expression in the ventral telencephalon (n=1, Figure 40A). To determine whether the *Notch1* enhancer is active in the oligodendroglial lineage we identify OPCs with an antibody to PDGFRα, and we used an antibody to β-gal to identify cells that have seen reporter activity. We found that PDGFRα+ cells did not coexpressed β-gal, indeed their pattern of expression were mutually exclusive (Figure 40B-E). From these transient transgenic analysis we concluded that the *Notch1* genomic sequences described contained regulatory sequences able to recapitulate some temporal and spatial pattern of the endogenous Notch1 expression in the developing mouse telencephalon, which does not include the oligodendroglial lineage. Note that *Mash1* null embryonic expression array data demonstrate a significant reduction in Notch1 transcripts (p-value, 2.41×10^{-7}; Carlos Parras, unpublished data). A gene centric strategy, similar to that described for the *Olig2* locus, could also be implemented for the *Notch1* locus in order to identify regulatory elements bound by Mash1 that are active in the oligodendroglial lineage.

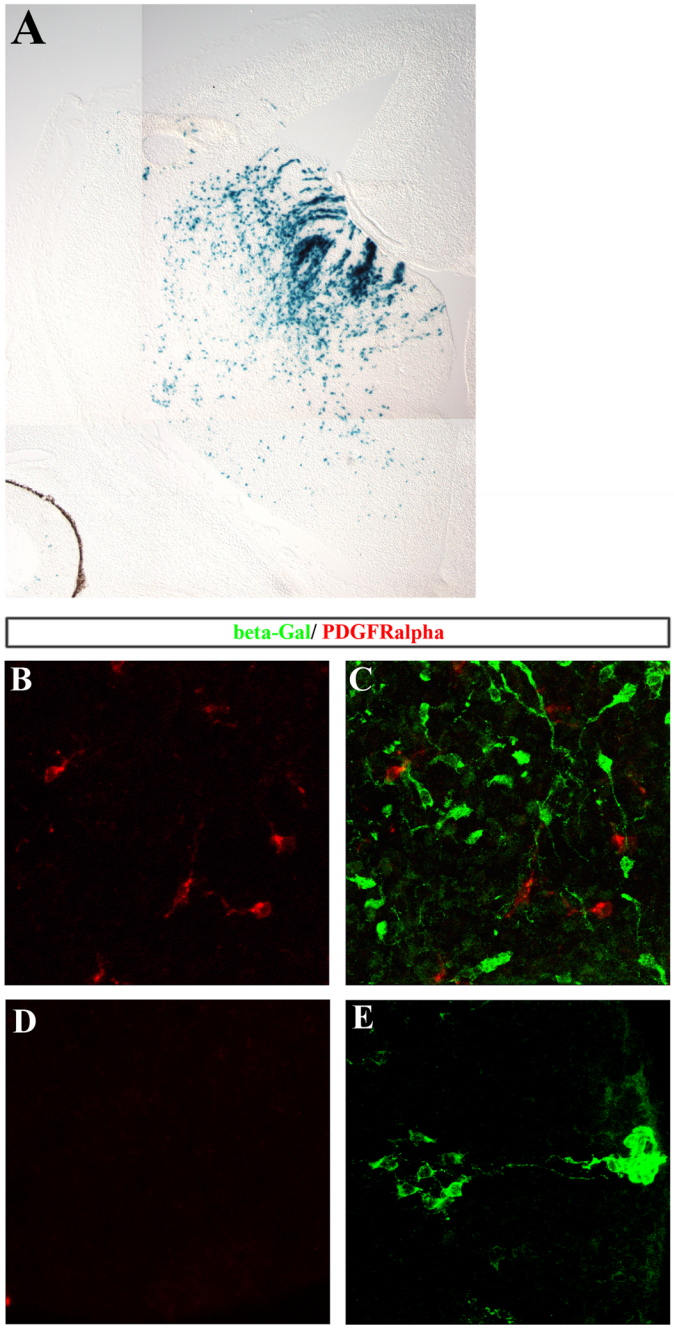


Figure 40. *Notch1* enhancer has no activity in the oligodendroglial lineage at E13.5. ***A***, Coronal section through the telencephalon of an X-gal stained transgenic embryo for the *Notch1* enhancer at E12.5. ***B-E***, PDGFRα+ OPCs in the ventral telencephalon do not coexpress β-gal.

3.6.5 Validation of proximal *Brevican* enhancer activity

To identify TF binding sites within the putative *Bcan* enhancer element, we performed phylogenetic footprinting (as described previously). The putative *Bcan* element was not strongly conserved throughout mammalian evolution (Figure 41A). Detailed analysis of the sequence revealed that although specific Mash1 type E-boxes were present, none were evolutionary conserved (data not shown). A number of mechanisms have been proposed to account for the recruitment of a particular factor in the absence of a consensus motif and may include, binding at a distal site that contains a consensus motif and looping to the site in question through protein-protein interactions; or assisted binding to a site that is similar to the consensus site, which is enhanced by protein–protein interaction with another site-specific DNA binding factor or with a specifically modified histone.

Bcan expression is restricted to zones of active proliferation in the CNS during development, and commences after the peak of neurogenesis at the onset of gliogenesis (Jaworski et al., 1994; Jaworski et al., 1995). To determine whether the putative *Bcan* enhancer could recapitulate part of this expression we tested its ability to regulate gene expression *in vivo* with a *LacZ* reporter gene using mouse transient transgenesis (as previously described). Reporter gene expression was analysed by X-Gal staining of tissue sections at E13.5 of embryonic development. We found that the *Bcan* enhancer directed reporter gene expression in scattered cells located in the dorsal and ventral telencephalon (n=1, Figure 41B). To determine whether the *Bcan* enhancer is active in the

oligodendroglial lineage we used an antibody to PDGFRα, to identify OPCs, and an antibody to β-gal to identify cells with reporter activity. We found that PDGFRα+ cells did not coexpressed β-gal (Figure 41C and D). From these transient transgenic analysis we concluded that the *Bcan* genomic sequences described contained regulatory sequences able to drive *lacZ* expression, however we cannot be sure whether the X-gal pattern observed recapitulate some temporal and spatial pattern of the endogenous Bcan expression in the developing mouse telencephalon.

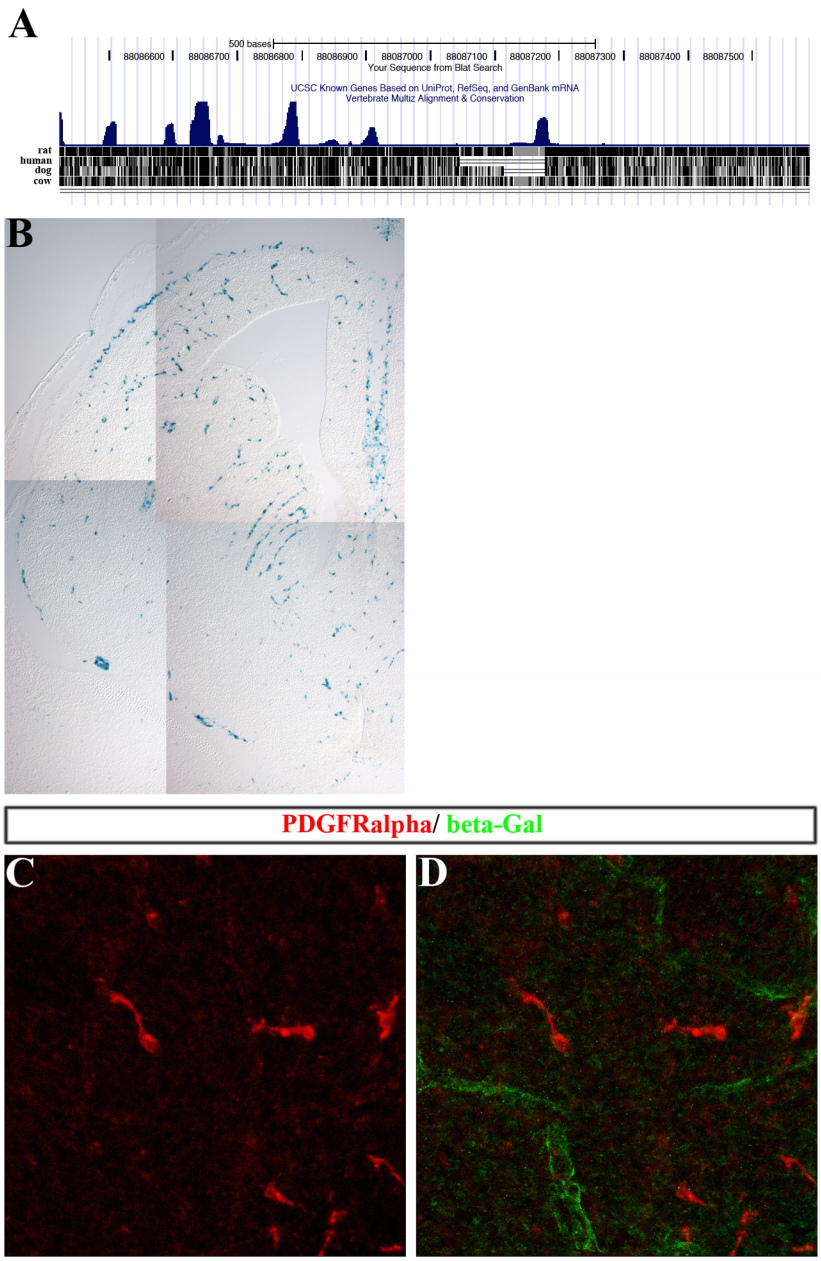


Figure 41. *Bcan* enhancer has no activity in the oligodendroglial lineage at E13.5. ***A***, *Bcan* enhancer, from the UCSC Genome Browser (http://genome.ucsc.edu/ [coordinates are from the February 2006, mm8 NCBI Build 36UCSC Genome Browser Mouse assembly]). Alignment of the *Bcan* enhancer sequences from the mouse, human, rat, cow and dog genomes, demonstrate very poor global sequence conservation in this region. ***B***, Coronal section through the telencephalon of an X-gal stained transgenic embryo for the *Bcan* enhancer at E13.5. ***C & D***, PDGFRα+ OPCs in the ventral telencephalon do not coexpress β-gal.

In conclusion, we have generated transient transgenics with 4 genomic regions that were bound by Mash1 in the oligosphere cultures, one of which had no independent enhancer activity, whilst the others contained regulatory sequences that conferred enhancer activity. Moreover, we have isolated a distal *Sox9* enhancer element that clearly recapitulates some temporal and spatial pattern of the endogenous *Sox9* gene expression in mouse telencephalon, which includes the oligodendroglial lineage. It is evident that not all genomic elements bound by Mash1 in the oligosphere cultures, represent enhancers with activity in the oligodendroglial lineage. Therefore, whilst this strategy is immensely powerful in defining the activity of enhancers with a cellular resolution, transient transgenic analysis in a faster developmental model, such in *Danerio rerio* or zebrafish, would increase the speed of the screen and thus identification of regulatory regions active in oligodendroglial cells. Notably these studies would further allow the functional categorisation of those targets with specific activity in different and overlapping cell lineages, and facilitate the identification of putative signature motifs of Mash1, which may vary between neuronal and oligodendroglial specific enhancers. Indeed, these studies would further elucidate potential co-regulators of Mash1 in oligodendroglial cell fate specification.

3.7 Combinatorial regulation in oligodendrogenesis

The combinatorial interaction of TFs and their binding to specific motifs in DNA are critical for gene regulation. Models of combinatorial regulation by several TFs at a particular genomic region can be inferred from multiple data sources, including evolutionary conservation, DNase hypersensitive sites and ChIP-on-chip binding data. Note that the average length of DNA fragments in ChIP experiments are approximately 300-500 base pairs, and thus the spatial resolution of this technique permits co-regulator analysis. Notably, the proneural protein Mash1 is likely to function in combination with other TFs in the specification of OPCs (Parras et al., 2007; Sugimori et al., 2007). To identify potential co-regulators, we asked whether genomic elements enriched in the Mash1 ChIP-on-chip study, were also bound by other oligodendrocyte promoting TFs, namely Sox9 and Olig2. Notably both Sox9 and Olig2 are coexpressed with Mash1, and a subset of these also coexpress the early OPC marker PDGFRα, both in oligosphere cultures (Figure 18), and during oligodendrogenesis in the developing mouse ventral telencephalon (Figure 10, Parras et al., 2007) and therefore have the potential to cooperate in oligodendroglial cell fate specification. Specific antibodies to Sox9 and Olig2 proteins, which have been extensively characterised for ChIP (Ben Martynoga, personal communication), were used to coprecipitate chromatin from mouse oligosphere culture material, and ChIP-PCR performed with specific primer sequences to Mash1 bound segments.

Previously experiments were performed at day 14 of oligosphere formation based on the published protocol (Chen et al., 2007), but the timing of oligodendrogenesis *in vitro* was re-examined to optimise the ChIP protocol. Briefly, oligospheres were generated over a period of 21 days, and time points were taken at 3, 5, 7, 14, and 21 days of oligosphere formation and expression levels were assayed. Note that expression levels are normalised to neurosphere cultures (at day 4 of neurosphere formation). We found that both early OPC markers, PDGFRα and Sox10, peaked at day 5 of oligosphere formation, after which they declined and were drastically reduced by day 14 and day 21 of oligosphere formation (Figure 42). All subsequent ChIP experiments were performed with chromatin prepared from day 5 oligospheres.

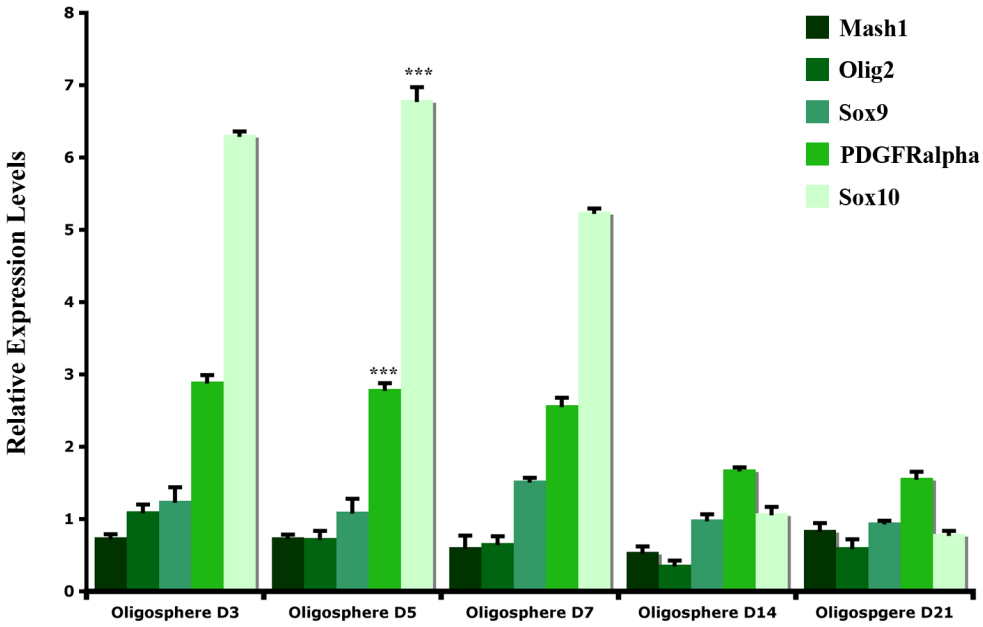
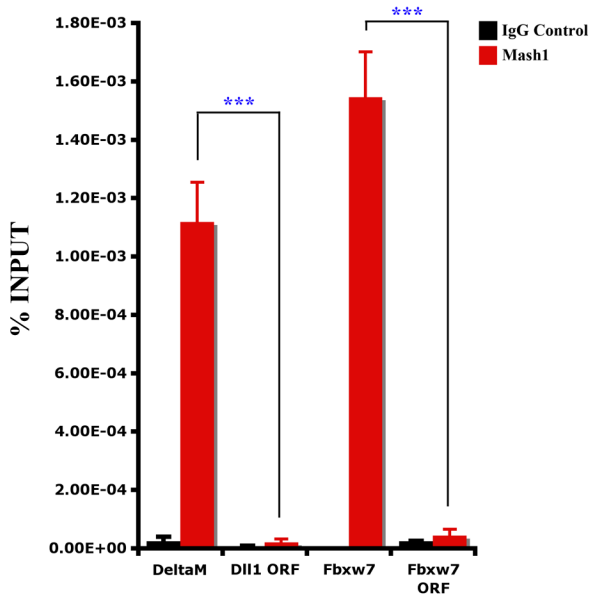


Figure 42. Sox10 and PDGFRα expression peaks at day 5 of oligosphere formation. Quantitative reverse transcriptase-PCR experiment showing the time course of expression of *Mash1*, *Sox9*, *Olig2*, *PDGFRalpha*, and *Sox10* transcripts in oligosphere cultures at 3, 5, 7, 14 a nd 21 days of oligosphere formation. All the data are normalised to neurosphere cultures (at day 4 of neurosphere formation). * P<0.05, ** P<0.01, *** P<0.001.

3.7.1 Mash1 ChIP

We asked whether the Mash1 proneural protein directly interacts with the *DeltaM*, *Fbxw7*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan* and *Omg* enhancers in addition to *Tubb3* and *MAP2*, Mash1 neurogenic enhancers (Diogo Castro, unpublished data) in oligosphere cultures material at day 5 of oligosphere formation, in order to confirm our previous analysis of Mash1 binding in day 14 oligospheres, by performing ChIP-PCR. An antibody to Mash1 coprecipitated the *DeltaM*, *Fbxw7*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* sequences in chromatin prepared from oligosphere cultures, but not the *Dll1* ORF nor *Fbxw7* ORF negative controls (Figure 43A and B). Conversely, a control IgG antibody did not coprecipitated the *DeltaM*, *Fbxw7*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* sequences nor the *Dll1* ORF, *Fbxw7* ORF (Figure 43A and B). Therefore, in oligosphere cultures Mash1 specifically binds to the *DeltaM*, *Fbxw7*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* genomic regions. We therefore confirmed that elements bound by Mash1 at day 14 were also bound at day 5 of oligosphere formation, and thus concluded that this material was suitable for ChIP analyses. Note that the fact that Mash1 was bound to neurogenic targets, *Tubb3* and *MAP2*, likely reflects the fact that oligospheres contain a fraction of neuronal precursors and neurons.

A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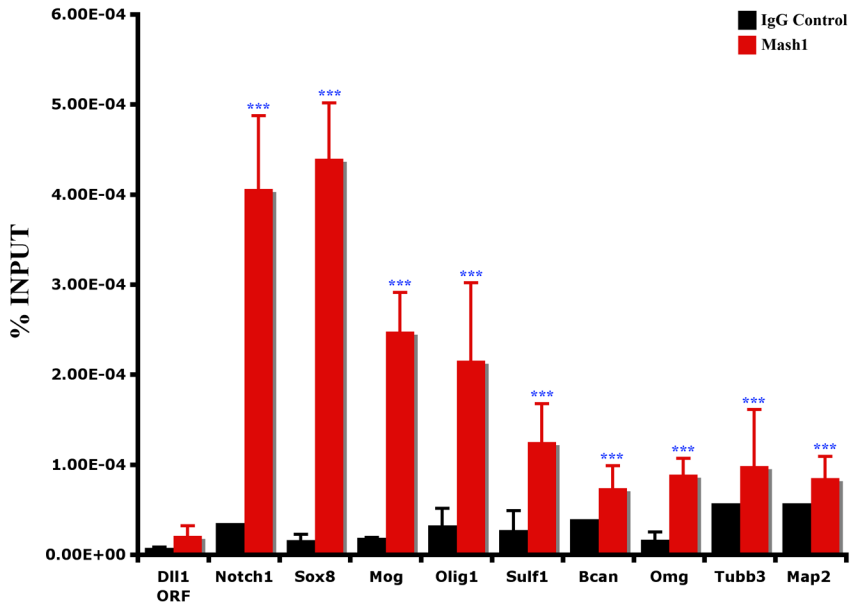
Fbxw7 ORF

B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Mash1
Dll1 ORF
Notch1
Sox8
Mog
Olig1
Sulf1
Bcan
Omg
Tubb3
Map2

Figure 43. Mash1 enriched elements in day 5 oligospheres. ***A***, *DelatM* and *Fbxw7* regions are enriched in ChIP, using an antibody to Mash1 and chromatin from oligosphere day 5 material, compared to negative regions *Dll1* ORF and *Fbxw7* ORF (blue asterisk), respectively. ***B***, Elements enriched in the Mash1 ChIP using chromatin prepared from day 14 oligospheres are also enriched in chromatin from day 5 oligospheres. Mash1 binds to neurogenic targets including *Tubb3* and *MAP2*. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

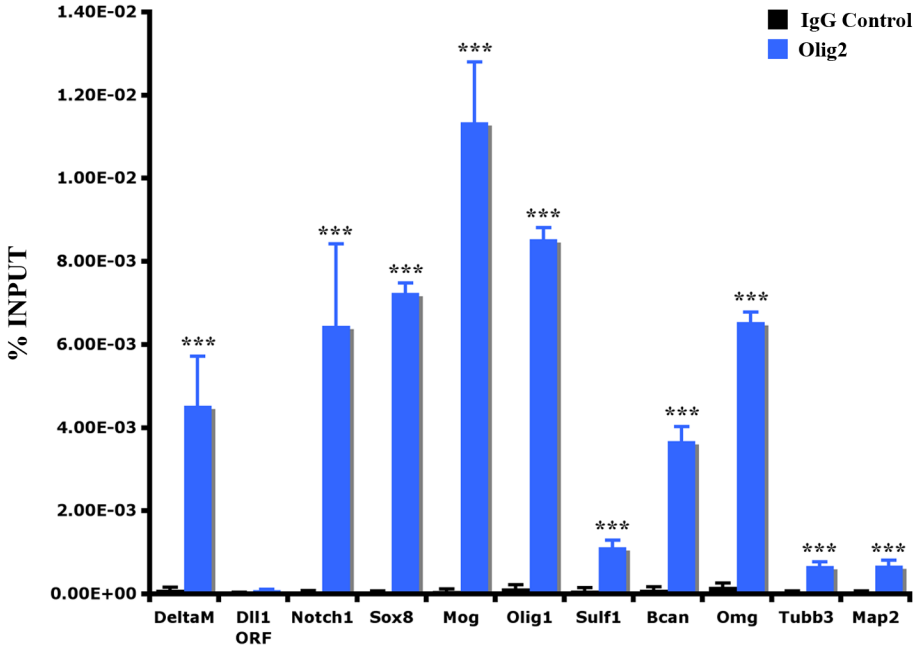
3.7.2 Olig2 ChIP

To determine whether Olig2 was a potential co-regulator of Mash1 in oligosphere cultures, we asked whether *DeltaM*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* genomic elements enriched in the Mash1 ChIP, were also bound by the Olig2 protein in oligosphere cultures material (at day 5 of oligosphere formation) by performing ChIP-PCR. An antibody to Olig2 coprecipitated the *DeltaM*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* sequences in chromatin prepared from oligosphere cultures, but not the *Dll1* ORF negative control (Figure 44A). Conversely, a control IgG antibody did not coprecipitated the *DeltaM*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* or *MAP2* sequences nor the *Dll1* ORF (Figure 44A). Therefore, in oligosphere cultures Olig2 specifically binds to the *DeltaM*, *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, *Tubb3* and *MAP2* genomic regions. Thus, all elements bound by Mash1, were also bound by Olig2 (Figure 43 and 44A). The bHLH TF Olig2 like Mash1 binds degenerate E-boxes, however whether a specific Olig2 type E-box exists is yet to be described. It is plausible that Olig2 and Mash1 bind the same sites, or that these TFs heterodimerise and bind together, analogous to the behaviour of Olig2 in the spinal cord, which not only recognises the same E-box elements that are bound by Neurogenin2 but is also capable of heterodimersation with this TF (Lee et al., 2005). Nevertheless the molecular mechanisms underlying the transcriptional synergy of Mash1 and Olig2 have not been detailed, and are likely to be complex since Mash1 and Olig2 have been shown so far to act as a transcriptional activator and repressor, respectively.

3.7.3 Sox9 ChIP

To determine whether Sox9 was a potential co-regulator of Mash1 in oligosphere cultures, we asked whether *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, and *Omg* genomic elements enriched in the Mash1 ChIP, were also bound by the Sox9 protein in oligosphere cultures material (at day 5 of oligosphere formation) by performing ChIP-PCR. In addition, we included two genomic segments that are located upstream of the mouse *PDGFRα* gene as controls, defined as *C1* (evolutionary conserved, proximal 5' flanking region) and *N2* (non evolutionary conserved, distal 5' flanking region), which are bound and not bound, respectively by Sox9 in the developing embryonic spinal cord (Finzsch et al., 2008). An antibody to Sox9 coprecipitated the *Notch1*, *Sox8*, *Mog*, *Olig1*, and *Omg* sequences in chromatin prepared from oligosphere cultures, but not *C1*, *Sulf1* nor *Bcan* sequences nor the *N2* negative control (Figure 44B). Conversely, a control IgG antibody did not coprecipitated the *Notch1*, *Sox8*, *Mog*, *Olig1*, *Sulf1*, *Bcan*, *Omg*, nor *C1* sequences nor the *N2* element (Figure 44B). Therefore, in oligosphere cultures Sox9 specifically binds to the *Notch1*, *Sox8*, *Mog*, *Olig1*, and *Omg* genomic regions. Thus, Sox9 binds to a subset of elements bound by Mash1 (Figure 43 and 44B). Note that Sox9 does not bind the *C1* element in oligosphere cultures, which is bound in embryonic spinal cord chromatin preparations, such inconsistencies may result from inherent regional differences. Assuming that oligosphere cultures retain regional identity, it is possible that different elements for PDGFRα expression exist in the spinal cord and the telencephalon, respectively.

A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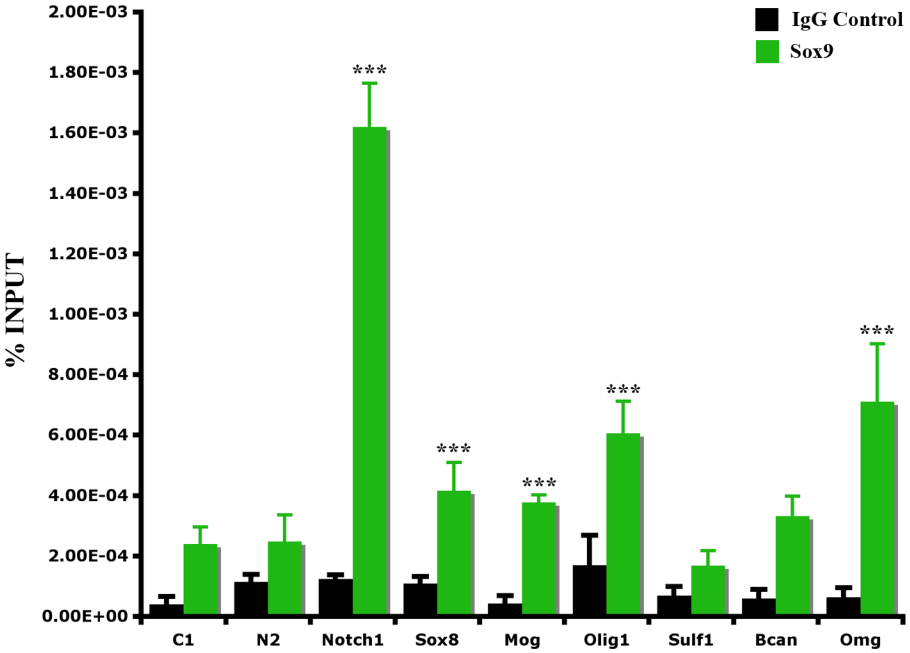
Tubb3
Map2

B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6.00E-04
4.00E-04
2.00E-04
0.00E+00
***
C1
N2
Notch1
Sox8
Mog
Olig1
Sulf1
Bcan
Omg

Figure 44. Olig2 and Sox9 bind to Mash1 enriched elements in day 5 oligospheres. ***A***, ChIP, using an antibody to Olig2 and chromatin from oligosphere day 5 material, is enriched for all elements bound by Mash1 compared to negative control region *Dll1* ORF (black asterisk). ***B***, ChIP, using and antibody to Sox9 and chromatin from oligosphere day 5 material, is enriched for *Notch1*, *Sox8*, *Olig1*, *Omg* and *Mog* elements compared to negative region *N2* (black asterisk). * P<0.05, ** P<0.01, *** P<0.001.

Gene expression regulation is a multipart process that requires the collaborative action of numerous proteins, including sequence-specific TFs, cofactors, and chromatin proteins. However, the interactions between these factors and the genome are still poorly understood. It is widely accepted that TFs and their cofactors show strong overlapping localization patterns on the genome. Indeed the fact that Mash1, Sox9 and Olig2 show a high degree of overlap in binding to specific genomic elements in oligosphere cultures enforces the notion that these regions are likely functional enhancers. Moreover, these data support the hypothesis that Mash1 interacts with other oligodendrogenic TFs, such as Sox9 and Olig2 to promote oligo-specific gene expression and specify OPCs. However there are clear limitations to this approach, namely that it does not directly address combinatorial binding or indeed regulation by these TFs. To determine whether these TFs are bound to the same segments rather than alternatively bound in different cells, sequential ChIP assays should be employed (Medeiros et al., 2009). A more complete picture of Mash1 function in oligodendroglial cell fate specification are yet to be described, undoubtedly this would be aided by adopting an integrated approach with ChIP and microarray expression data, in addition to combinatorial TF-motif analysis.

Chapter 4

Discussion

The cellular functions of the proneural gene Mash1 are well characterised (Bertrand et al., 2002), and more recently significant efforts to understand the molecular mechanisms underlying its activity in neurogenesis have been made (Castro et al., 2006; Gohlke et al., 2008; Henke et al., 2009; Long et al., 2009; Del Barrio et al., 2007). However, very little is known about the molecular mechanisms underpinning Mash1 activity in oligodendroglial cell fate specification, including target genes and interacting partners (Parras et al., 2004, 2007; Sugimori et al., 2007). This project has made an initial step towards the discovery of the gene regulatory network downstream of Mash1 in the oligodendrogenic program.

There are three main components to this work. Firstly, the identification of a suitable cellular system in which to study the role of Mash1 in oligodendrogenesis. Secondly, a promoter occupancy analysis using ChIP-on-chip technology to locate genomic regions bound by Mash1, in addition to a ChIP approach with oligodendrocyte promoting TFs, Sox9 and Olig2, to identify Mash1 co-regulators in the oligodendroglial lineage. Finally, an *in vivo* enhancer screen to assay the spatial and temporal activity of enhancers using mouse transient transgenics, in order to isolate genomic elements with regulatory capabilities in the oligodendroglial lineage of the CNS.

4.1 Mash1 is coexpressed with early OPC marker PDGFRα and oligodendrocyte promoting TFs Sox9 and Olig2 in oligosphere cultures

We describe an *in vitro* cellular system in which to study the gene regulatory network downstream of Mash1 in oligodendrogenesis, using oligosphere cultures derived from E14.5 mouse cortex (Chen et al., 2007). Mash1 protein was expressed in a significant proportion of cells in oligospheres (Figure 18A and B). Analogous to the patterns of coexpression in the developing ventral telencephalon (Figure 10; Parras et al., 2007), Mash1 was coexpressed with oligodendrocyte promoting TFs, Olig2 and Sox9 (Figure 18C and D). Moreover, a subset of Mash1+/Olig2+ and Mash1+/Sox9+ cells also coexpressed the early OPC marker, PDGFRα (Figure 18C-E). Contrary to the previously published protocol we found that: (i) oligosphere cultures were most optimal at day 5 of oligosphere formation, as defined by gene expression profiling with early OPC markers, PDGFRα and Sox10 (Figure 42); (ii) only a small fraction of cells in the oligospheres were in fact OPCs (Figure 18C-E). We propose that perhaps a difference in B104CM batch preparations may account for these discrepancies. Nevertheless, the capacity to generate significant quantities of oligosphere culture material, compared to the difficulties in isolating sufficient numbers of oligodendroglial cells from the ventral telencephalon of Sox10Cre/Rosa26YFP transgenic embryos using a cell sorting approach (Figure 11B), makes this *in vitro* cellular system ideal for use in genomic strategies.

The requirement for Mash1 function in the specification of OPCs in oligosphere cultures is yet to be defined. To address this question, oligosphere

cultures from Mash1 conditional knockout mice (Mash1CDKO, Guillemot unpublished) crossed with a tamoxifen inducible Cre recombinase under the control of the Nestin promoter (Nestin-CreER, Burns et al., 2007) would be established. Nestin is a Class VI intermediate filament, which is expressed in early embryonic neuroepithelial stem cells of the developing CNS, and is used as a marker for stem/progenitor cells. Addition of tamoxifen to fully formed neurospheres, and therefore removal of Mash1 function prior to oligosphere formation, would define the requirement of Mash1 function for the specification of OPC in oligosphere cultures. On the basis of the requirement for Mash1 in the generation of OPCs *in vivo* (Parras et al., 2004, 2007; Sugimori et al., 2007), we hypothesise that a subset of OPCs in the oligospheres would require Mash1 function for their specification.

Mash1 plays multiple roles throughout the oligodendrocyte lineage, early in the generation of OPCs (Parras et al., 2004, 2007; Sugimori et al., 2007), and in terminal differentiation of oligodendrocytes later in development (Sugimori et al., 2008). A synchronously differentiating population of mouse cells; from a NSC to an early OPC, and finally to a mature myelinating oligodendrocyte, would present an ideal system in which to study the temporal dynamics of gene regulatory networks orchestrated by Mash1 throughout oligodendrogenesis.

4.2 Mash1 binds to proximal genomic regions of early OPC and late oligodendrocyte genes

Mash1 locational analysis was performed in mouse oligosphere cultures, using a ChIP-on-chip strategy with a custom designed oligodendrocyte-specific array (Figure 23). We identified genomic segments proximal to early OPC genes, *Olig1* and *Sox8,* which were bound by Mash1 protein in oligosphere cultures (Figure 27B). In the developing mouse embryo, *Olig1* and *Sox8* gene expressions are not entirely restricted to the oligodendroglial lineage, and extends to non-differentiated neural progenitors in the VZ of the ventral telencephalon (Figure 29B and 28B, respectively). We hypothesise that Mash1 is likely first expressed in uncommitted VZ stem cells in the developing telencephalon, as is the case in a fraction of neural stem cells or *type B cells* in the adult SVZ (Pastrana et al., 2009, and Melanie Lebel, personal communication), and in the multipotent NS5 neural stem cell line (Conti et al., 2005). However, without documentation of the *in vivo* activity of the *Olig1* and *Sox8* putative enhancers, we cannot be certain whether these Mash1 bound elements are involved in regulating the expression of *Olig1* or *Sox8* TFs respectively, in cell fate restricted oligodendroglial cells or uncommitted progenitors cells in the VZ.

We also identified genomic segments proximal to late/mature oligodendrocyte genes, *Mog* and *Omg,* which were bound by Mash1 protein in oligosphere cultures (Figure 26B). *Mog* and *Omg* are not expressed early in the oligodendrocyte lineage, however Mash1 protein was capable of binding to proximal genomic regions of these genes in non-differentiated oligosphere

cultures (that present minimal MBP+ mature oligodendrocytes, Figure 19D). We propose a number of possible scenarios to explain for Mash1 binding activity at these elements. Firstly, that Mash1 functions to prime these promoters for rapid induction of gene expression in response to specific cues that promote oligodendrocyte differentiation and maturation later in development. Notably, the engagement of target genes by enhancer binding factors may facilitate a more rapid and homogeneous activation of a specific genetic program in a field of progenitor cells, in response to inductive signals. Indeed, FoxA1 binds the liver-specific *Alb1* enhancer, in the endoderm and facilitates chromatin remodelling and transcriptional activation upon liver specification (Gualdi et al. 1996; Cirillo et al. 2002).

Secondly, that Mash1 may serve to facilitate the recruitment of essential cofactors necessary for transcriptional initiation of these genes later in development. In this hypothetical situation, Mash1 would function as a pioneer factor binding to chromatin domains early in development to help establish competence for expression states. FoxA proteins function as pioneer TFs, that are among the first to bind chromatin domains prior to target gene activation during development and enable gene activity (Zaret 1999, 2002; Zaret et al., 2008). Specifically, binding of FoxA1 to nucleosomal templates *in vitro* enables binding by GATA-4 and NF1 factors at neighbouring sites (Cirillo and Zaret 1999; Cirillo et al. 2002). Moreover, FoxA1 binding to chromatin *in vivo* creates hypersensitive sites to facilitate estrogen receptor binding (Carroll et al. 2005). It is possible that the first two hypotheses are correct and that Mash1 binding may facilitate the recruitment of co-factors with chromatin remodelling activity.

Finally it is also possible that Mash1 activity at these sites is repressed during OPC specification, and is derepressed at the onset of oligodendrocyte differentiation. In this model, we propose that repression and activation activities of Mash1 at specific genomic loci are likely to be regulated by the formation of complexes with other factors. The myogenic bHLH transcription factor MyoD, plays a key role in establishing the myogenic lineage during embryogenesis and regulating the myogenic program in satellite cells of adult skeletal muscles (Puri and Sartorelli, 2000; Sabourin and Rudnicki, 2000). Notably, MyoD is a decisive transcriptional activator, however in complex with the gene repressor histone deacetylase1 (HDAC1) (Mal et al., 2001) at the myogenin promoter, MyoD behaves as a transcriptional repressor in proliferating myoblasts (Mal and Harter, 2003). Following the induction of myoblast differentiation, the myogenin promoter is replaced with a functional acetyltransferase P/CAF and MyoD complex (Mal et al., 2001), in this context MyoD behaves as a transcriptional activator and myogenin expression is induced (Mal and Harter, 2003).

4.3 Mash1 binds to proximal genomic regions of genes involved in different aspects of the oligodendroglial lineage

We identified genomic segments proximal to other genes of interest including *Sulf1*, *Notch1* and *Bcan*, which were bound by Mash1 protein in oligosphere cultures (Figure 26B). Sulf1, a secreted enzyme that modulates the sulfation state of heparan sulfate proteoglycans, is expressed in the ventral neuroepithelium prior to OPC specification (Danesin et al., 2006; García-López et al., 2009). Elevated Shh concentrations are sufficient to induce premature OPC

specification at the expense of neurogenesis, in ventral progenitors of the developing spinal cord (Danesin et al., 2006). Notably, over expression of Sulf1 protein in ventral neural progenitors of the spinal cord prior to OPC specification, leads to the apical concentration of Shh on neuroepithelial cells, a process that is critical for the neuronal to oligodendroglial switch (Danesin et al., 2006). We propose a model in which Mash1 induces *Sulf1* gene expression in ventral progenitors of the CNS, which could contribute to the mechanisms involved in cell fate switch towards the oligodendroglial cell lineage. Notably this model would provide a means through which intrinsic and extrinsic cues are integrated in progenitors at the VZ, in order to promote oligodendroglial cell fate specification.

Interestingly, we found that genomic segments proximal to a number of Notch signalling genes, including *Notch1* were bound by Mash1 protein in oligosphere cultures (Figure 20 and 26B). Canonical Notch signalling, mediated via binding of ligands, including Jagged 1-2 and Delta 1-4 to Notch1 and Notch2 receptors, restricts OPC maturation in the developing CNS and is permissive for proliferation (Louvi and Artavanis-Tsakonas, 2006). Notch activation inhibits oligodendrocyte differentiation, as demonstrated *in vitro* in the developing rat optic nerve (Wang et al., 1998), and confirmed *in vivo* with *Notch1* heterozygous mice, which show premature myelination (Givogri et al., 2002), and by specific ablation of *Notch1* in oligodendrocytes, which ultimately affects the differentiation of precursors into immature oligodendrocyte cells (Genoud et al., 2002). In this context Mash1 may function, in a cell autonomous manner, to prevent premature differentiation of newly born OPCs into oligodendrocytes, and

allow OPCs to propagate. Note that regulation of Notch signalling pathway components are not specific to OPCs but also occur in neuronal precursors (Castro et al., 2006).

Finally, we also identified a genomic segment proximal to *Bcan* that was bound by Mash1 protein in oligosphere cultures (Figure 26B). Bcan is a neural specific CSPG from the lectin family, and is a major component of the extracellular matrix (Yamaguchi, 2000; Jaworski et al., 1994; Yamada et al., 1994). Bcan is expressed in the VZ along the neuraxis of the developing embryo, and coincides with the onset of gliogenesis (Jaworski et al., 1995). Notably, Bcan expression is up-regulated in primary tumours and has been implicated in glioma cell invasion and thus glial cell motility (Jaworski et al., 1996; Zhang et al., 1998). Furthermore, Bcan expression is up-regulated in response to acute brain injury, to suggest a role in reactive gliosis (Jaworski et al., 1999). Indeed, glial process extension, a central feature in the glial response to injury, may require the re-expression of both cytoskeletal and matrix elements that are normally expressed during glial motility in the developing brain. OPCs specified in the developing ventral telencephalon display a strong inherent migratory behaviour, we propose a model in which Mash1 regulates *Bcan* expression early in the oligodendroglial lineage, and therefore may contribute to the molecular mechanisms that confer glial cell motility. To determine the requirement of Mash1 function in the migration of OPCs in the ventral telencephalon, Sox10Cre/ Mash1CDKO mice would be utilised, in order to remove Mash1 function following OPC specification.

Although locational analysis using Chip-on-chip generated detailed binding data in proximal promoter regions, no information on gene regulation in terms of expression can be extrapolated. Indeed, integration of gene expression data profiling the transition from neurosphere to oligosphere, with Mash1 binding data from ChIP-on-chip experiments using chromatin prepared from neurospheres and oligospheres respectively, would allow the identification and categorisation of genomic regions bound by Mash1 that are proximal to either active or repressed genes, in two distinct cell states. To identify genes specifically expressed in OPCs, oligosphere cultures established from Sox10Cre/R26YFP transgenic mice, would be used to sort for GFP+ oligodendroglial lineage cells. We hypothesise that this type of analysis would display overlapping and mutually exclusive Mash1 binding events in neurosphere and oligosphere cultures, and reflect a bias towards oligodendroglial cell fate specification in the latter. In conjunction these data would facilitate the identification of genomic regions bound and regulated by Mash1 in OPCs.

4.4 Olig2 binds to Mash1 enriched genomic regions

Olig2 locational analysis was performed in mouse oligosphere cultures, using a ChIP-PCR strategy, with primer specific sequences to genomic regions bound by Mash1 (Figure 44A). All genomic regions tested, including segments proximal to early OPC genes, *Olig1* and *Sox8*; segments proximal to late/mature oligodendrocyte genes, *Mog* and *Omg*; segments proximal to genes of interest *Notch1*, *Sulf1*, and *Bcan*; and segments proximal to Mash1 neurogenic target

genes *Tubb3* and *MAP2*, were also bound by Olig2 protein in oligosphere cultures (Figure 44A).

Mash1 and Olig2 are coexpressed in progenitor cells and in OPCs at the time of their specification in the VZ of the developing ventral telencephalon (Parras et al., 2007). *In vitro* studies have indicated that Mash1 can cooperate with Olig2 to activate the MBP promoter (Gokhan et al., 2005). In addition, Olig2 was shown to selectively promote the oligodendrogenic activities of Mash1 in gain of function studies in rat spinal cord neurosphere cultures (Sugimori et al., 2007). More recently, the requirement for Mash1 function for the specification of an early population of OPCs that involves a genetic interaction with *Olig2* in the ventral telencepahlon was demonstrated (Parras et al., 2007). Nevertheless, whilst it is clear that Mash1 and Olig2 cooperate in oligodendroglial cell fate specification, the molecular mechanisms that underlie this synergy are yet to be detailed.

Currently, the interaction between activators and repressors on the same gene promoter is not well-understood. We hypothesise that two distinct types of interactions exist between Mash1 and Olig2 TFs on different regulatory elements, either synergistic or antagonistic on oligodendroglial or neuronal enhancers respectively (Figure 45). Olig2 functions as a transcriptional repressor to specify motor neuron and oligodendrocyte cell fate in the ventral spinal cord (Novitch et al, 2001; Mizuguchi et al 2001; Zhou 2001). Indeed these data have led to the proposal that Olig2 functions by repressing a repressor of oligodendrocyte development (Zhou et al., 2001). However, the possibility that Olig2 may also function as an activator to promote the oligodendrocyte fate can

not be entirely excluded (Figure 45). We propose that Olig2 may repress Mash1 neurogenic targets, either through competition to bind to E-box motifs or by heterodimersation with E47 and/or Mash1. In the developing ventral spinal cord, specifically within the pMN domain, Olig2 prevents premature expression of post-mitotic motor neuron genes (Lee et al., 2005). In this system gene specific repression is achieved through a combination of mechanisms, which include interactions with bifunctional activator/suppressor E-boxes in the promoter of the *Hb9* gene, and formation of weak heterointeractions between Olig2 and E47 or Ngn2 (Lee et al., 2005). To determine whether this hypothesis is true, oligodendroglial and neuronal specific enhancers identified in transgenic embryos would be tested in luciferase reporter assays, in order to examine the synergy versus antagonism theory of Mash1 and Olig2 combinatorial activity on cell lineage specific enhancers.

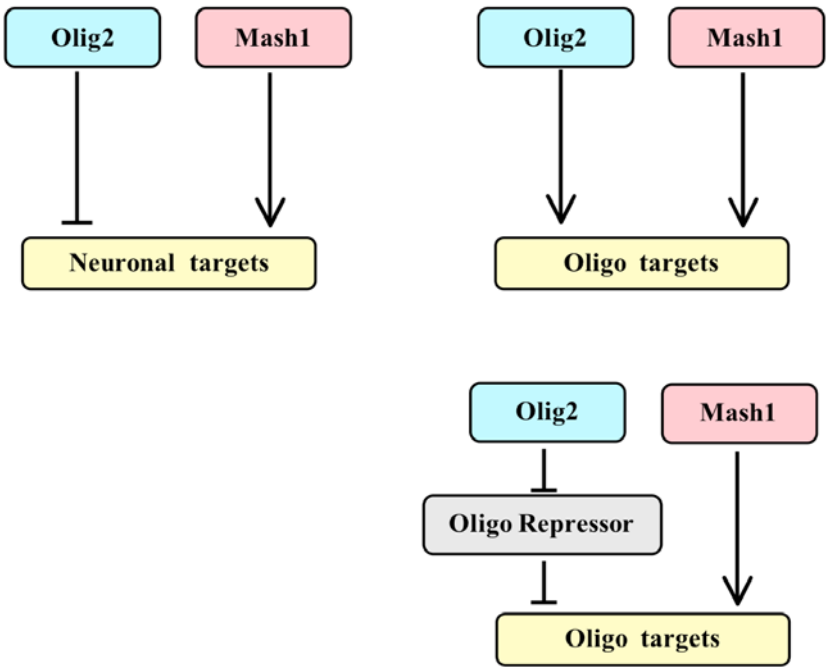

Figure 45. Regulation of neuronal and oligodendroglial target genes by Olig2 and Mash1 activities. In the scheme arrows and T-bars define positive and inhibitory interactions on target promoters.

4.5 Sox9 binds to *Notch1, Sox8, Omg* and *Mog* Mash1 enriched genomic regions

Sox9 locational analysis was performed in mouse oligosphere cultures, using a ChIP-PCR strategy, with primer specific sequences to genomic regions bound by Mash1 (Figure 44B). Genomic regions tested, including segments proximal to early OPC genes, *Olig1* and *Sox8,* segments proximal to late/mature oligodendrocyte genes, *Mog* and *Omg*, and segments proximal to a gene of interest *Notch1* were all bound by Sox9 protein in oligosphere cultures (Figure 44B). Note that unlike ChIP experiments using an antibody to Olig2, not all Mash1 bound elements were significantly enriched using an antibody to Sox9, including segments proximal to *Bcan* and *Sulf1* genes (Figure 44B and schematic Figure 46). The biological significance of this difference in co-occupancy of Mash1 bound elements, are yet to be detailed.

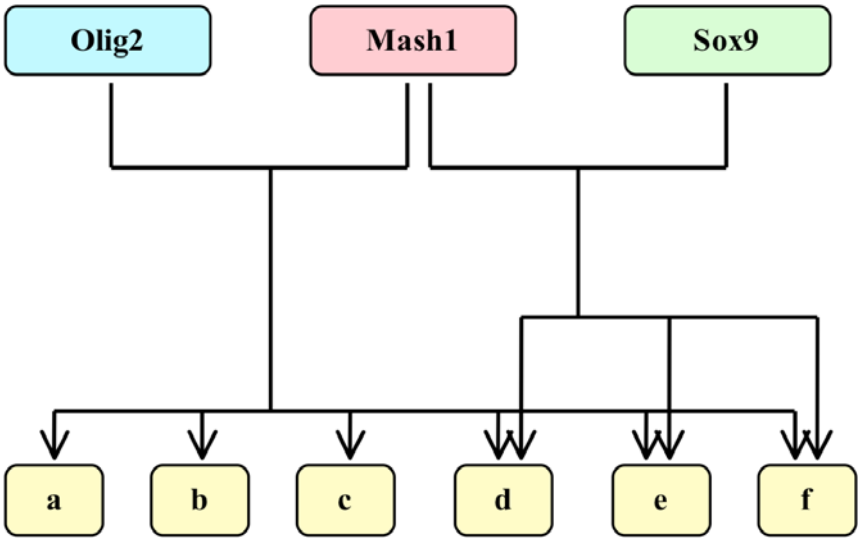

Figure 46. Mash1 binds to genomic elements that are also co-occupied by oligodendrocyte promoting TFs Olig2 and Sox9. Arrows represent occupancy. Yellow boxes (a-f) represent genomic elements.

Mash1 and Sox9 are coexpressed in progenitor cells and in OPCs at the time of their specification in the VZ of the developing ventral telencephalon (Figure 10). Early in development, Sox9 plays a key role in the generation of OPCs from the pMN domain, and has been defined as a major molecular component of the neuron-glia switch in the developing spinal cord (Stolt et al., 2003). Sox proteins bind to the minor groove of the DNA, and endorse the formation of multimeric protein complexes through modifications in DNA conformation, and thus impact on local chromatin remodelling (Wolffe, 1994; Werner and Burley, 1997). The *SoxE* group gene, *Sox10* functions both as a DNA-binding protein and as a molecular scaffold to recruit bHLH heterodimers to activate gene expression on the MBP promoter (Gokhan et al., 2005). We propose a hypothesis in which Sox9 functions to recruit Mash1 at oligodendroglial enhancers, contributing to the mechanism of discrimination between neuronal and oligodendroglial enhancers by Mash1 in neuronal versus oligodendroglial precursors respectively.

4.6 Biological significance of Mash1 co-regulators

This study has highlighted the potential function of Mash1 beyond OPC specification, oligodendrocyte differentiation and myelination. Mash1 has multiple roles throughout neurogenesis, including neuronal and sub-type specification, cell cycle regulation, Notch signalling and neuronal migration (Bertrand et al., 2002). We hypothesise that Mash1 plays a number of different roles throughout oligodendrogenesis, similar to that in neurogenesis. Notably, the functions of Mash1 are likely to overlap in the neuronal and oligodendroglial

lineages, and may include Notch signalling and migration. Recently, Sox9 and Sox10 TFs were shown to influence the survival and migration of OPCs in the developing spinal cord, through the regulation of *PDGFRα* gene expression (Finzsch et al., 2008). Similarly, the role of Mash1 in the survival and migration of OPCs could be assayed with Sox10Cre/ Mash1CDKO animals, as described previously.

We propose that Mash1 interacts with different DNA-binding cofactors to activate different subprograms of oligodendrogenesis, as has been proposed for Mash1 in neurogenesis (Castro et al., 2006; Gholke et al., 2008), and MyoD during myogenesis (Tapscott, 2005). Notably, there is a huge biological advantage in adopting such mechanisms of action. Firstly, TF interactions with distinct DNA-binding partners on different promoters allow an array of target genes to be independently regulated, a phenomenon that is well studied in the myogenic program (Tapscott, 2005). Secondly, this mode of activity permits synchronisation of independently regulated developmental programs. TF interactions may also function to prevent overlap between dissimilar programs. Olig2 and Sox9 TFs function in cell proliferation and oligodendroglial cell fate specification, as well as in neurogenesis and astrogenesis for Olig2 and Sox9 respectively (Charlie Scott, unpublished data; Lu et al., 2002: Hack et al., 2004; Stolt et al., 2003; Ligon et al., 2007). We propose that Olig2 and Sox9 TFs interact with different co-factors in order to coordinate incompatible developmental programs. We hypothesis that these TFs interact with Mash1 to promote the generation of OPCs, and that this mechanism may prevent premature oligodendroglial cell fate specification in neural precursor cells.

Mash1 functions in an instructive manner to induce oligodendroglial cell fate specification. However, although OPC numbers are drastically reduced in *Mash1* null mutant embryos, they are not completely absent, and are gradually recovered later in development (Parras et al., 2007; Sugimori et al., 2007). These data suggest loss of Mash1 in OPCs may be compensated for by other TFs in the CNS, to suggest that Mash1 independent networks in oligodendroglial cell fate specification likely exist. Similarly, early in development Sox9-deficient spinal cords demonstrate a severe reduction in the numbers of OPCs, with very few OPCs remaining (Stolt et al., 2003). However, a recovery in OPC numbers was evident at later stages of development, perhaps resulting from compensatory activities of other SoxE group members, Sox10 and Sox8, whose expressions overlap with Sox9 in the oligodendrocyte lineage (Stolt et al., 2003). Notably these data highlight the heterogeneity in the transcriptional mechanisms governing oligodendroglial cell fate specification in the CNS.

4.7 ChIP technology a general perspective for the future

In principle the ChIP-on-chip method permits the unbiased detection of DNA binding sites for proteins throughout the genome. It has the advantage of readily identifying target genes associated with bound promoters, avoiding the difficulties of identifying genes associated with bound distal elements in whole genome location analyses. However, since this approach is restricted to interrogating proximal promoter regions, it is difficult to determine whether the binding sites identified in this study were representative of the majority of the

genomic binding sites for Mash1 in oligosphere cultures. Indeed analyses of 1% of the human genome, in order to identify functional elements, performed in the ENCyclopedia Of DNA Elements (ENCODE) pilot project and by the ENCODE Consortium and others (Birney et al., 2007; Bieda et al., 2006; Kim et al., 2005; ENCODE Project Consortium, 2004; Carroll et al., 2005) have demonstrated that the incidence of TFs that bind almost exclusively at proximal promoters are likely an exception. Instead, TFs may bind to diverse regions of the genome, which include extragenic regions distant from the TSS in addition to intragenic regions (reviewed Farnham, 2009). Evidently a more unbiased method, such as the recently developed ChIP coupled with next-generation sequencing techniques, referred to as ChIP-seq, would overcome these limitations. Recently, *in vivo* mapping of p300 binding, using ChIP-seq technology has been demonstrated to be a highly accurate means for identifying enhancers (Visel et al., 2009). Note p300 is a histone acetyltransferase that is frequently located at enhancer regions (Heintzman et al., 2007; Ogryzko et al., 1996). Indeed, locational analysis with the transcriptional coactivator p300 using chromatin prepared from oligosphere cultures would further assign functionality to the Mash1 binding data set.

It is hypothesised that distal binding sites regulate transcription via long-range chromatin interactions. Indeed for these regions it is not yet possible to conclusively link a specific binding site with a specific target gene. Using methods such as chromosome conformation capture (3C), in order to identify chromosomal loops that result form long range protein–protein interactions (Dekker et al., 2002), would help to disclose a connection between an enhancer

binding protein and the promoter of a distant gene. Recently, a new unbiased technology termed ChIA-PET (Chromatin Interaction Analysis using Paired End Tag sequencing) was developed to identify *de novo* chromatin interactions on a genome-wide scale, and furthermore to interrogate the influence of these interactions on transcriptional regulation (Fullwood et al., 2009).

A clear limitation to current ChIP methods are the large quantities of material required, which consequently limits the applicability of this technology for rare cell samples. Recently, a fast microChIP (muChIP) assay using only 1,000 cells in combination with microarrays to generate a genome wide scan of histone modifications was described (Dahl et al., 2009). Notably, this new technology could be used to assay Mash1 occupancy in OPCs, following cell sorting analysis of ventral telencephalic tissue from Sox10Cre/Rosa26YFP embryos, in order to isolate GFP+ oligodendroglial cells.

We demonstrate that Mash1 binds close to TSSs, and thus likely regulates transcription at these sites by stabilizing TFs at the core promoter elements. Moreover, ChIP-seq experiments have shown that Mash1 is also capable of binding to distal regions (Diogo Castro, personal communication), at these sites Mash1 may regulate transcription by means of protein–protein interactions, perhaps by a loop formation mechanism, between distal complexes and the generic transcriptional machinery at TSSs. The discovery that regulatory regions are bound by combinations of different TFs resulted in the formation of a general concept, which states that TFs have a tendency to cluster in close proximity to cooperate in transcriptional regulatory activities (Mann & Carroll, 2002). We demonstrate that Olig2 and Sox9 TFs bind Mash1 enriched genomic elements in

oligosphere cultures. However, it is difficult to prove that these TFs bind sequences simultaneously, rather than in different cells. To investigate promoter co-occupancy using sequential ChIP technology (Medeiros et al., 2009), consecutive immunoprecipitation of chromatin for one and then a second factor would be performed in order to demonstrate that binding of these TFs occurs in the same cell and on the same DNA molecule, suggestive of molecular interactions. In conclusion, comprehensive mapping of TF-binding sites, of oligodendrocyte promoting factors, including Mash1, Olig2 and Sox9 using a whole genome ChIP approach would identify important features of the transcriptional regulatory networks that define oligodendroglial cell identity.

4.8 Transient transgenics a means to visualise enhancer activity *in vivo*

We exploit mouse transient transgenic technology using reporter constructs that include the regulatory element of interest, to visualise enhancer activity *in vivo*. Previously, the distal genomic segment to the early OPC gene *Sox9* was shown to be bound by Mash1 protein in the mouse ventral telencephalon at E12.5 (Diogo Castro, personal communication). We have validated Mash1 binding of this element in oligosphere cultures (Figure 26B). Furthermore we demonstrated that the *Sox9* distal enhancer element had the capacity to recapitulate part of *Sox9* endogenous expression *in vivo*, including that in non-differentiated neural progenitors in the VZ of the ventral telencephalon and in PDGFRα+ oligodendroglial lineage cells (Figure 38). To determine the requirement of specific binding sites within this element, including Mash1 type E-box motifs to drive reporter gene expression in transgenic

embryos, site-directed mutagenesis would be employed as has been described previously (Castro et al., 2006). Furthermore, to test whether Mash1 function is required for the regulation of this enhancer, stable transgenic lines would be bred with *Mash1* null mutant mice and reporter gene activity assayed. Note the regulation by other oligodendroglial TFs, Sox9 and Olig2, could also be similarly assayed.

To establish a large-scale functional screen of noncoding sequences, which is both efficient and rapid, we are working towards a transgenic strategy in zebrafish based on the Tol2 transposon (Fisher et al., 2006). The Tol2 transposon identified in the teleost, Medaka, is a highly mobile element. Indeed, in the presence of transposase there is a significant increase in the efficiency of genome integration mediated by the transposon. To evaluate the regulatory potential of a candidate sequence, the desired PCR-amplified intervals flanked by Tol2 transposon target sites, are injected into 1-2-cell zebrafish embryos and reporter activity analyzed at specific times throughout development. Notably, recent data demonstrates that this approach provides a high fidelity read out for the regulatory function of conserved non-coding *Sox10* mouse sequences, even in the absence of overt sequence conservation between mammals and teleosts (Antonellis et al., 2008).

Identification of differentially regulated enhancers in oligodendroglial and neuronal cell lineages *in vivo* would provide a means in which to categorise these elements and further determine whether Mash1 lineage specific signature motifs exist. To identify motifs associated with Mash1 binding sites in the

oligosphere cultures, a bioinformatics approach using validated software would be utilised. These include: Weeder, a software tool for the discovery of conserved TFBSs in sequences from co-regulated genes (Pavesi et al., 2001, 2004); Trawler, an integrated pipeline for the analysis of ChIP data, to identify over-represented motifs (Ettwiller et al., 2007); and Pscan, a software tool that scans promoter sequences from co-regulated or co-expressed genes in search for over- or under-represented motifs, which also assigns TF binding specificity (Zambelli et al., 2009). Notably the formulation of an oligodendroglial specific motif would provide a framework on which to perform a genome wide *in silco* screen, in order to identify co-regulated genes in oligodendroglial cell fate specification.

4.9 Conclusions and perspectives

Oligodendrogenesis is a complex process, and involves the generation of spatially and temporally separated populations that originate from molecularly distinct regions of the CNS (Richardson et al., 2006; Woodruff et al., 2001; Vallstedt et al., 2005; Cai et al., 2005; Ivanova et al., 2003; Spassky et al., 2001). Indeed, during development Mash1 function is specifically required for the specification of a subset of OPCs during the first wave of oligodendrogenesis in the ventral telencephalon (Parras et al., 2007), and in the spinal cord (Sugimori et al., 2007).

We have made an initial attempt to uncover the molecular mechanisms underlying Mash1 activity in oligodendrogenesis. In this study we demonstrate

that Mash1 binds directly to regulatory elements of genes expressed early in specification events and late in differentiation and maturation of the oligodendroglial programme. We propose that Mash1 functions at the top of a transcription hierarchy as a key factor, to orchestrate the expression of a wide repertoire of downstream genes in oligodendrogenesis. We hypothesise that Mash1 activity is temporally modulated by sequence specific co-regulators, which contribute to its recruitment to specific proximal promoter regions in different cell types.

In this study we demonstrated that Olig2 and Sox9 protein levels are significantly reduced in the VZ of the ventral telencephalon in *Mash1* null mutants (Figure 35 and 37, respectively). These data corroborate with gene expression array studies using ventral telencephalic tissue from *Mash1* null mutant embryos, in which *Olig2* and *Sox9* transcripts are also significant reduced (Carlos Parras, personal communication). Moreover, we have identified a distal *Sox9* enhancer with activity in the oligodendroglial lineage (Figure 38C and D), which is bound by Mash1 in the ventral telencephalon at E12.5 (Diogo Castro, personal communication) and in oligosphere cultures (Figure 26B). Sox9 overexpression in the chick spinal cord is sufficient to induce expression of the vital oligodendrocyte regulator *Olig2* (Gaber and Novitch, unpublished data). On the basis of these data, we propose a potential feed-forward loop, in which Mash1 directly regulates *Sox9* and both Mash1 and Sox9 regulate *Olig2*, either directly or indirectly through intermediates (Figure 47). Feed-forward loops confer certain advantages, indeed multiple inputs provide consistent activity that subsequently render it relatively insensitive to transient changes in individual input strength (Mangan and Alon, 2003; Mangan et al., 2003).

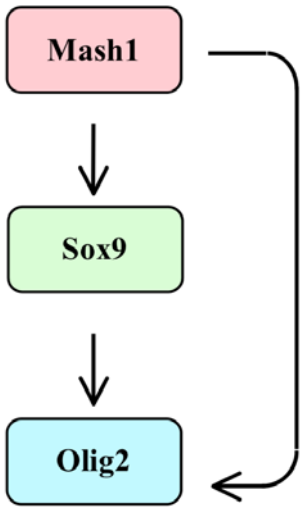

Figure 47. A feed-forward loop in which Mash1 regulates Sox9 and both Mash1 and Sox9 regulate Olig2.

In conclusion this model functions as useful framework on which to map the gene regulatory network downstream of Mash1 in oligodendrogenesis.

Understanding how key factors control gene expression in the transition from a neural stem cell to an oligodendroglial cell during development is imperative. The progression in oligodendroglial cell fate commitment requires the temporal activation of a unique transcription program. Indeed deconstructing the molecular mechanisms in this transition would provide a means to direct neural stem cells towards an oligodendroglial cell identity, a vital process for therapeutic transplantation strategies to treat demyelinating diseases. The recent surge in the identification of TF binding events on a genome wide scale has revealed the existence of complex gene regulatory networks. Understanding the mechanisms that underlie the interactions between TFs and chromatin, in particular how the epigenetic state of chromatin controls the specificity of factor recruitment and subsequently alters the permissiveness to transcription activity in addition to the requirement and function of a combinatorial code of co-regulators will prove to be the next challenging steps.

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