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Separated at birth? The functional and molecular divergence of OLIG1 and OLIG2

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Summary

The basic helix-loop-helix transcription factors oligodendrocyte transcription factor 1 (OLIG1) and OLIG2 are structurally similar and, to a first approximation, coordinately expressed in the developing CNS and postnatal brain. Notwithstanding these similarities, it was apparent from early on after their discovery that OLIG1 and OLIG2 have non-overlapping developmental functions in patterning, neuron subtype specification and the formation of oligodendrocytes. Here, we summarize more recent insights into the separate functions of these transcription factors in the postnatal brain during repair processes and in neurological disease states, including multiple sclerosis and malignant glioma. We discuss how the unique biological functions of OLIG1 and OLIG2 may reflect their distinct genetic targets, co-regulator proteins and/or post-translational modifications.

Introduction

The appearance of myelinating oligodendrocytes represented a major step forward in the evolution of the vertebrate CNS, as these cells enabled more efficient axon insulation and conduction of action potentials and, consequently, much greater brain complexity. The essential roles of oligodendrocytes in myelin production, establishing the nodal architecture of the axon, and saltatory conductivity were recognized by the late 1960s. The biological relevance of the structures and functions enabled by oligodendrocytes came from the gradual realization that damage to the myelin sheath, subsequent demyelination and focal depletion of myelinating oligodendrocytes constitute the cellular basis of multiple sclerosis (MS)[1-4]. The typical relapsing–remitting course of this disorder highlighted the repair potential of oligodendrocytes and focused attention on two important unresolved issues in myelin development, namely the anatomical origins of oligodendrocytes and the lineage relationships between oligodendrocytes and other neural cell types.

The progress towards answering these questions was initially slow and the findings from early studies were contentious. For example, on the subject of anatomical origins, tissue explant studies suggested that oligodendrocytes arise exclusively from the ventral neural

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tube[5-8]. However, quail–chick grafting experiments suggested a more complex picture, with some oligodendrocytes originating from dorsal regions of the neural tube[9]. With respect to lineage relationships, tissue explant studies identified glial-restricted progenitor cells from the optic nerve and the spinal cord, suggesting a lineage relationship between oligodendrocytes and astrocytes[10-12]. However, grafting experiments and analysis of pattern formation in the developing spine highlighted the role of anatomy in glial specification and suggested that oligodendrocytes and astrocytes arise from separate, bipotent progenitor cells (for a review see REF. 13).

A major lacuna in the field was a lack of genetic tools. This void was filled with the cloning and characterization of the basic helix–loop–helix (bHLH) transcription factors oligodendrocyte transcription factor 1 (OLIG1) and OLIG2 — so named because early *in situ* hybridization experiments revealed that these transcription factors showed pronounced expression in myelinating oligodendrocytes and oligodendrocyte progenitors[14-16]. Gain-of-function and loss-of-function genetic assays involving *Olig1* or *Olig2*, together with Cre-lox fate-mapping experiments, resolved long-running polemics on the cellular and anatomical origins of oligodendrocytes in the developing CNS[14-18]. With respect to anatomical origins, it is now clear that oligodendrocyte progenitors arise from both the ventral and dorsal regions of the developing CNS. Most oligodendrocytes in the adult spinal cord are derived from ventral progenitors. By contrast, oligodendrocytes in the mature forebrain are derived from dorsal progenitors [19-21]. Regarding lineage relationships, the notion that oligodendrocytes and astrocytes arise from a common, bi-potent glial-restricted progenitor proved to be incompatible with genetic analysis and fate mapping studies focusing on *Olig1* and *Olig2*[17,18]. It is now evident that oligodendrocyte progenitor origins are more closely aligned to neuron subtype progenitors than to astrocytes — at least in the developing CNS.

More recent studies have revealed biological functions for *Olig1* and *Olig2* in the postnatal brain during neurogenesis[22] and reactive gliosis[23, 24], and in repair functions in experimental models of MS[25]. Preliminary, but nevertheless provocative, studies have also suggested that these genes have links to schizophrenia[26-28] and to the cognitive deficiencies associated with Down's syndrome[29]. Overshadowing these links to development and disease is a broad body of literature linking one of these genes — *OLIG2* — to a cohort of primary brain cancers known collectively as the diffuse gliomas[30-34].

Against initial expectations based on expression patterns and striking similarities in their DNA-targeting bHLH motifs, the biological functions of OLIG1 and OLIG2 are for the most part separate and non-overlapping. In this Review, we examine facets of OLIG1 and OLIG2 molecular biology that may account for their diverse functional repertoires. In the fullness of time, genetic targets, co-regulator proteins and post-translational modifications unique to OLIG1 or OLIG2 may lend themselves to development of targeted drugs for CNS injuries and for a variety of neurological disease states, including cerebral palsy, MS and malignant glioma.

OLIG structure and expression

The human genome encodes approximately 125 transcription factors that are defined by a canonical bHLH motif[35,36]. OLIG1 and OLIG2, together with a third transcription factor, OLIG3, form a recognizable subset of proteins in the bHLH family by virtue of their amino acid sequence homology — especially within the bHLH regions, which mediate transcription factor dimerization and DNA targeting (BOX 1). *Olig1* and *Olig2* are expressed exclusively within the CNS[14-16] (TABLE 1). To a first approximation, these genes are coordinately expressed in both space and time, although there are some important

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distinctions in their patterns of expression — especially at the level of subcellular localization of the two OLIG proteins (see below).

Olig2 expression is detected at very early time points in CNS development within the radial glia of the neural tube that ultimately give rise to motor neurons and oligodendrocytes[37,38]. Expression of *Olig2* in multipotent progenitor cells of developing forebrain is recapitulated *in vitro* in cultures of multipotent neurospheres, in which most mitotic cells are OLIG2-positive[39].

Anatomically refined studies of *Olig1* and *Olig2* expression have been carried out in developing spinal cord. Here, both *Olig1* and *Olig2* have been shown to be downstream targets of the ventralizing signal Sonic hedgehog[14, 15]. *Olig1* is first expressed in the dorsal portion of the 'p3' progenitor domain of the ventral neural tube and then becomes confined to the pMN (progenitors of motor neurons) domain by embryonic day (E) 10.5. *Olig2* expression is also observed at early development stages prior to neural tube closure in the ventral-most p3 domain and then becomes confined to the pMN domain by approximately E9–E9.5. By the time that *Hb9* (homeobox protein Hb9) is expressed (E9-E9.5), *Olig1* and *Olig2* expression is down-regulated in motor neuron lineage cells. Studies by Chen *et al.* show that a microRNA (*mir-17-3p*) may be the effector of this down-regulation — at least for *Olig2*[40]. Both *Olig1* and *Olig2* show sustained expression in oligodendrocyte precursor cells as these cells progress to become mature oligodendrocytes (FIG. 1)[14-16]. In contrast to *Olig1* and *Olig2*, *Olig3* is expressed in the dorsal-most domains of the developing spinal cord, patterned by bone morphogenetic proteins (BMPs), and is also expressed in multiple tissues outside of the CNS (TABLE 1)[16, 41, 42].

Roles in development

Although structurally related and, to a large extent, coordinately expressed in developing embryos, the biological functions of *OLIG1* and *OLIG2* are only partially redundant (TABLE 2). *Olig1* and *Olig2* each contribute to patterning of the spinal cord, although *Olig2* plays the dominant role (FIG. 1A). Indeed, in the spinal cord, OLIG2 excusively promotes the specification of motor neurons and early oligodendrocyte progenitors that express platelet-derived growth factor receptor α (PDGFRα) (FIG. 1B). The switch from maintaining immature pMN progenitors to the production of motor neurons involves regulation of OLIG2 levels as well as phosphorylation of this protein at a site within its bHLH domain (see below)[43, 44]. Loss-of-function studies also indicate roles for *Olig2* in neuron development within the ventral forebrain, particularly in cholinergic neuron populations[45].

Ablation of *Olig1* has no impact on the formation of motor neurons and early oligodendrocyte progenitors (FIG. 1B). However, subsequent maturation of oligodendrocyte progenitors within the spinal cord is delayed in *Olig1* knockout mice[17, 46].However, occasional foci of PDGFRα-positive oligodendrocyte progenitors are seen in the forebrain of *Olig2* knockout mouse embryos and these PDGFRα-expressing cells are even more abundant in the hindbrain[17]. Combinatorial knockout of *Olig1* and *Olig2* is required to ablate these last vestiges of oligodendrocyte formation completely[18], suggesting that the role of OLIG1 in the specification phase of oligodendrocyte formation is partially redundant in the presence of OLIG2.

In rodents and humans, expression of *OLIG2* in the parenchyma is generally considered to identify a cell of the oligodendrocyte lineage, but there are several exceptions to this characterization. First, *Olig2* expression in putative astrocyte precursor cells has been reported in the postnatal day (P) 7 neonatal rodent brain[47]. Second, Cahoy *et al.* reported that a small percentage (~3%) cells in the adult mouse brain that were positive for the

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astrocytic marker ALDH1L1, also expressed *Olig2* [48]. Third, *Olig2* expression has been reported in proliferating reactive astrocyte precursor cells [49]. However, *Olig2* expression is down-regulated upon terminal differentiation of astrocytes, an observation confirmed *in vitro* (see below).

One critical neurogenic function seems to be unique to OLIG2. Generally, the bHLH transcription factors that control neurogenesis can be classified as being either anti-neurogenic (pro-mitotic) or neurogenic (anti-mitotic). At early stages of development, expression of anti-neurogenic (pro-mitotic) transcription factors prevents cell cycle exit and thereby expands the pool of neural progenitors. At later stages of neural development, expression of neurogenic (anti-mitotic) factors promotes cell cycle exit, subtype specification and differentiation[50-52]. In this context, OLIG2 stands apart, showing functional characteristics of both sets of transcription factors in multipotent neural progenitor cells. In the embryonic spinal cord, for example, OLIG2 is required for specification and the ultimate differentiation of motor neurons and oligodendrocytes[17]. However, at early time points in development in the pMN domain, OLIG2 functions in pattern formation as an anti-neurogenic factor (pro-mitotic factor), to sustain the replication competent state of some pMN progenitors that are destined for the second wave of gliogenesis[43]. An emerging body of literature suggests that this early anti-neurogenic (pro-mitotic) function of OLIG2 may be co-opted by the stem-like 'tumor-initiating cells' of malignant glioma (see below).

The developmental functions (TABLE 1) and expression (TABLE 2) of *OLIG3* are divergent from those of *OLIG1* and *OLIG2*. Accordingly, OLIG3 will not be discussed beyond this point.

Roles in disease and neural repair

A broadening body of literature documents non-overlapping postnatal roles for OLIG1 and OLIG2 in neurological diseases and in response to injury (TABLE 2). For example, both proteins are expressed in fresh surgical isolates of human diffuse gliomas[30-34]. However, human gliomas have been reported to contain a subpopulation of highly tumorigenic cells[53] and *OLIG2*, but not *OLIG1*, is selectively expressed in such cells[39]. Beyond merely marking glioma progenitors, OLIG2 is required for intracranial tumor formation in genetically relevant murine models of human glioma[39, 54-56] and for the proliferation of authentic human glioma cells implanted into the brains of severe combined immunodeficiency (SCID) mice[57]. These findings do not rule out a role for OLIG1 in gliomas, but show that it is dispensable — unlike OLIG2 — in certain subtypes of these tumors.

As indicated in TABLE 2, a postnatal role for OLIG1 is demonstrated in the repair of white matter injury. Although Xin *et al.* have described an *Olig1* knockout mouse with a relatively severe block in myelination, leading to early postnatal lethality, the originally reported *Olig1* knockout mouse strain exhibits only a mild developmental delay in oligodendrocyte maturation, even when both copies of *Olig1* are ablated, and survives to develop fully myelinated axons in the brain and spinal cord[17,46]. The two mouse strains differ in terms of the targeting of the *Olig1* locus and genetic background, and these differences might underlie the observed differential of the strong versus mild knockout phenotypes. In any case, the relatively benign developmental phenotype of the original *Olig1* knockout strain allows scrutiny of *Olig1* functions in murine models of MS, and Arnett *et al.* have showed that the functions of *Olig1* in response to demyelinating injury are more readily apparent than the developmental functions. Indeed, *Olig1* null mice were severely limited in their ability to repair demyelinated lesions that were induced by various gliotoxins (cuprizone, lysolecithin and ethidium bromide). The loss of *Olig1* had no effect on the genesis or

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recruitment of early oligodendrocyte progenitors expressing NG2, OLIG2 or homeobox protein NKX2.2 into the lesion. Rather, the *Olig1* null progenitors were markedly impaired in their ability to differentiate into myelinating oligodendrocytes and wild-type levels of OLIG2 could not compensate for the absence of OLIG1 in this regard[25].

One distinctive feature of OLIG1 in the developing CNS is recapitulated in the repair of demyelinating insults in murine models and also in postmortem brain tissues from patients with MS. During mouse embryonic development, OLIG1 is localized to the nucleus of oligodendrocyte progenitors (FIG. 2). However, in mature oligodendrocytes in the CNS from 2 weeks after birth, OLIG1 is mostly located in the cytoplasm. Translocation of OLIG1 from the nucleus to the cytoplasm is a precise marker of the terminal differentiation of oligodendrocytes[58]. By contrast, OLIG2 is localized to the nucleus at all stages examined and in all regions of the CNS. The differential localization of OLIG1 and OLIG2 is also seen in the adult human brain. Arnett *et al.* showed that demyelinating injuries to the adult mouse CNS create an environment that recruits immature oligodendrocyte progenitors with nuclear localization of OLIG1 [25]. The relocalization of OLIG1 observed in murine models of MS is recapitulated in postmortem brain tissue from patients with MS[25]. Cells containing cytosolic OLIG1 are present in normal-appearing white matter of the human brains but nuclear OLIG1 is present at the edges of active MS lesions. Collectively, these findings fit into an emerging theme in human white matter injuries as diverse as adult MS and neonatal brain injury leading to cerebral palsy, whereby various cell-intrinsic and environmental influences may limit the repair response by arresting maturation of oligodendrocyte progenitors, resulting in fixed demyelinated lesions[59-63].

Two other facets of the response to demyelination in both rodents and humans are the proliferation of microglia and astrocytes (known as reactive gliosis; reviewed in REF. 64). Neither of these reactive responses to demyelination was impaired in the *Olig1* null mice[17, 25]. However, a series of mouse studies supports a functional role for *Olig2* in reactive gliosis. Within several days following a cortical stab-wound injury, the number of *Olig2*-expressing cells increased in the lesioned area, but there was no rise in the number of *Olig1*-expressing cells[24]. Going beyond the correlative level, Chen *et al.* showed that targeted ablation of *Olig2*$^{fl/fl}$ with a GFAP-Cre driver, reduced the number of reactive astrocytes following injury, whereas ablation of *Olig2* in neuronal cells or oligodendroglial cells had no impact on this phenotype [49].

The OLIG2-positive cell type that gives rise to reactive astrocytes is somewhat of a mystery. Early studies showed that neither *Olig1* nor *Olig2* is expressed in mature astrocytes[14, 15] and that OLIG2 developmentally acts as a repressor of astrogenesis[65-67]. Moreover, fate-mapping studies in the developing CNS failed to reveal any lineage relationship between OLIG protein-positive cells and astrocytes[17, 68]. In the postnatal brain, *Olig* expression is confined almost exclusively to transit-amplifying cells of the subventricular zone, NG2-positive glia and mature myelinating oligodendrocytes. NG2-positive glia do have progenitor-like qualities. However, fate-mapping studies indicate that these cells are probably not the progenitors of reactive astrocytes[69-71]. It is conceivable that injury scenarios trigger transient re-expression of *Olig2* in an as yet poorly characterized type of progenitor of reactive astrocytes, which appears to express excitatory amino acid transporter 1 (EAAT1; also known as GLAST), as well as other astroglial markers [72]. A transient non-lineage-restricted period of *Olig2* expression would be consistent with several reports on reactive gliosis that document the early export of OLIG2 from the nucleus of proliferating progenitors followed by apparent degradation of the cytosolic protein[45, 73-75].

The most common genetic cause of intellectual disability is triplication of chromosome 21, giving rise to Down syndrome. *OLIG1* and *OLIG2* are co-localized to chromosome 21

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within or near a region on the long (q) arm (the so-called Down syndrome critical region) that is thought to be most tightly associated with the cognitive facets of the Down syndrome phenotype. Does overexpression of the two *OLIG* genes contribute to the neurological facets of Down syndrome? Chakrabarti *et al.* have generated support for this view in their studies with a well-characterized murine model of trisomy 21, the Ts65dn mouse[29]. The parental Ts65dn mouse shows many of the cognitive and neuro-anatomical defects associated with Down syndrome. In careful anatomical studies, Chakrabarti *et al.* first noted that the Ts65dn mice have a substantial increase in the number of forebrain inhibitory neurons. This observed increase in forebrain inhibitory neurons resonated with earlier studies suggesting that a major functional defect underlying the behavioral abnormalities in Ts65dn mice is an imbalance between excitation and inhibition[76, 77]. A total of 128 genes are triplicated in the Ts65dn mouse. Chakrabarti intercrossed these mice with a heterozygous *Olig1/2* double knockout mouse ($Olig1/2^{+/-}$), so as to selectively reduce the dosage of *Olig1* and *Olig2* from three copies to two[29, 78, 79]. This genetically precise reduction in gene dosage rescued the overproduction of interneurons. Cognitive tests were not performed on the *Olig1/Olig2*-rescued animals; however, the published observations show a plausible molecular and cellular route towards the neurological facets of Down syndrome wherein OLIG1 and OLIG2 have pivotal roles.

Reports that have yet to undergo functional validation link *OLIG2*, but not *OLIG1*, to schizophrenia and Alzheimer's disease. Genome-wide association work identified several single nucleotide polymorphisms (SNPs) in *OLIG2* that are associated with schizophrenia in a UK population[26]. One of the SNPs identified by the UK team (SNP rs762178) has been confirmed in a study of schizophrenia in a Chinese Han population[28]. Two of the above-mentioned *OLIG2* SNPs (SNP rs762237 and rs2834072) have also been linked to a cohort of Alzheimer's disease patients with psychotic symptoms[27].

Genetic targets

As discussed above and summarized in TABLE 2, data from multiple studies indicate that OLIG1 and OLIG2 have largely non-overlapping roles in development, tissue repair and disease. Paradoxically, these two genes are co-localized within 40 kb of each other on human chromosome 21 and their expression patterns are largely overlapping. Moreover, OLIG1 and OLIG2 have highly homologous DNA-targeting bHLH motifs. In this, and subsequent sections, we explore ways in which their non-overlapping biological functions may reflect separate genetic targets, co-regulator proteins and post-translational modifications. We begin with discussion of their genetic targets.

Expression profiling studies of *Olig1* and *Olig2* knockout mice versus their wild-type counterparts show that various non-overlapping sets of genes are up-regulated or down-regulated in the absence of *Olig1* or *Olig2*[39, 80] (S. Mehta, H. Liu, J. Alberta, E. Huillard, D. Rowitch, C. Stiles, unpublished observations) (FIG. 3). However, expression profiling cannot discriminate between direct genetic targets of OLIG1 and OLIG2 and downstream sequelae of their deletion. The basic domain of bHLH transcription factors mediates the interaction between these proteins and DNA sequences that contain the core hexanucleotide motif CANNTG, known as an E-box (reviewed in REF. 81). For OLIG2, specification of motor neurons following E-box binding seems to be channeled largely through its functions as a transcription repressor. In chick embryo electroporation assays, involving ectopic motor neuron formation as a biological readout, the neurogenic effect of OLIG2 is mimicked when the OLIG2 DNA-binding domain is fused to the transcription repressor domain of engrailed. Equivalent fusions to the transcription activator VP-16 lack neurogenic activity[82-84]. One important downstream effector gene of the OLIG2 repressor function seems to be *Hb9*[43]. Lee *et al.* have shown that OLIG2 binds to an E-box element in the *Hb9* promoter. The

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OLIG2–*Hb9* promoter interaction results in repression of *Hb9* transcription, preventing differentiation into post-mitotic motor neurons and sustaining the replication competent state of pMN neural progenitors during neural tube development[43]. OLIG2 also binds to promoter elements of the cell cycle repressor gene *p21* and suppresses its expression[39]. Suppression of *p21* transcription by OLIG2 may contribute in part to the growth of normal and malignant neural progenitors and to the notorious resistance of p53-positive human gliomas to radiation and genotoxic drugs[57].

All that being said, it is unclear if OLIG2 solely acts as a transcriptional repressor. Expression profiling reveals multiple genes that are down-regulated in *Olig2* null neural progenitors compared with wild-type counterparts, consistent with the view that OLIG2 might stimulate expression of such genes[39]. Recent data show that, in mice, OLIG2 binds to an enhancer site upstream of *Sox10*, thereby inducing its expression and increasing oligodendroglial activity[85]. More recently, two groups have used ChIP-seq protocols to identify new OLIG2 target genes[86, 87]. Weng *et al.* identified *Sip1* as a direct, inducible target gene of OLIG2. Specifically, they showed that OLIG2 stimulates expression of *Sip1*, which goes on to promote the maturation of oligodendrocyte progenitors via inhibition of SMAD7 signalling [87].

What about genetic targets of OLIG1? As noted in the section on disease and neural repair, the truly distinctive feature of OLIG1 is that it is localized to the cytosol in the postnatal brain where it cannot possibly have any direct genetic targets[25]. Indeed, as nuclear localization is essential for transcription factor function, it can be surmised that the critical phase of OLIG1 transcriptional activity in oligodendrocyte differentiation has been completed by the time it becomes localized to the cytoplasm. Genetic targets of OLIG1 have been inferred from gene expression profiles of wild-type versus *Olig1* null tissues. Compared with their wild-type counterparts, optic nerve, spinal cord and brain tissue from *Olig1* null mice show reduced mRNA and protein levels for several genes involved in oligodendrocyte maturation, including myelin basic protein (*Mbp*), myelin oligodendrocyte glycoprotein (*Mog*) and myelin proteolipid protein (*Plp1*) [25, 46, 80, 88, 89]. OLIG1 directly binds to the promoter region of *Mbp*, thereby inducing its expression[46, 90]. The *Mbp* promoter contains one E-box that is entirely conserved between human, mouse, rat, chick and zebrafish[90]. Mutation of this E-box reduces the binding affinity of OLIG1 for the *Mbp* promoter in luciferase promoter assays and gel mobility shift assays[90]. OLIG1 also induces expression of zinc finger protein 488 (*Zfp488*), as shown in a luciferase reporter assay. There are 20 conserved E-box sequences in the *Zfp488* promoter region, but it has not been tested whether OLIG1 directly or indirectly activates *Zfp488* expression[80].

Co-regulator proteins

All bHLH transcription factors function in a dimeric state as homodimers or as heterodimers with another bHLH protein. Once in contact with promoter or enhancer elements of a target, bHLH homodimers and heterodimers serve as scaffolding upon which a multimeric complex of transcriptional co-regulator proteins can be assembled[91-94]. Transcriptional co-regulators serve many functions, but broadly speaking they can be assigned into two groups — those that are components of the basal transcriptional machinery and those that modify the structure of chromatin[92]. Against this backdrop, the non-overlapping biological functions of OLIG1 and OLIG2 could reflect differential dimeric partners for the two transcription factors and/or differential interactions with co-regulator proteins.

As a general rule, tissue-specific bHLH transcription factors (termed class B factors) form heterodimers with ubiquitously expressed class A bHLH transcription factors such as E12, E47 (E2A immunoglobulin enhancer binding factors E12/E47, also known as transcription

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factor 3, TCF3) or TCF12 (transcription factor 12)[81]. However, OLIG2, which is highly tissue-specific, seems to favour homodimerization over heterodimerization with class A bHLH transcription factors. Indeed, although OLIG2–E12 and OLIG2–E47 heterodimers have been detected [43,95], Lee *et al.* found that OLIG2 homodimers are the preferred product when OLIG2 and E47 are co-expressed in yeast[43]. In addition, OLIG2 will form heterodimers with OLIG1 and, under certain conditions, HLH transcription factors such as DNA-binding protein inhibitor ID-2 (ID2) and ID4. These OLIG–ID interactions are detected when differentiation of progenitors towards the oligodendrocyte lineage is suppressed by BMPs [95]. Lacking a basic domain, HLH factors cannot interact with DNA. Accordingly, HLH transcription factors are thought to function as natural dominant-negative agents for bHLH transcription factors by forming heterodimers that cannot bind to E-box elements of their target genes[96].

Physical interactions between bHLH proteins and members of the homeodomain protein family regulate the development of several other tissues, including the pancreas, the pituitary gland and muscle[97-99]. OLIG2 has been shown to interact with Nkx2.2, a homeodomain protein that defines the p3 progenitor domain of developing spinal cord and is specifically required for production of V3 interneurons and maturation of oligodendrocytes[100].

Another co-regulator protein that has been linked to OLIG2 is histone acetyltransferase p300 (p300)[94, 101], which, via its associated histone acetyl transferase activity, functions to decondense the structure of chromatin and thus promotes transcription[94, 101, 102]. The presence of p300 as a co-regulator resonates with data suggesting that OLIG2 can stimulate the expression of some genes[85]. Considering the known function of OLIG2 as a transcription repressor in the genesis of motor neurons [43], it is somewhat surprising that studies to date have not identified any known members of the general transcription co-repressor complex in association with OLIG2. Conceivably, directed antibody pull down experiments and unbiased yeast-two-hybrid screens lack sensitivity or cell-type specificity to display these interactions.

Most HLH-containing proteins that have been identified as OLIG2-binding partners show some binding affinity for OLIG1 as well (the exception is cyclin-D1-binding protein 1 (CCNDBP1)). This is not surprising since the bHLH domains of OLIG1 and OLIG2 show more than 80% amino acid identity (BOX 1). CCNDBP1 is a helix–loop–helix protein that lacks a DNA-binding region. Ikushima *et al.* showed that this protein inhibits transcription of transforming growth factor β (TGF-β)-induced genes that require the SMAD complex for their activation[103]. They demonstrated in NmuMG and U373MG cells (normal murine mammary gland and glioma cell lines, respectively) that CCNDBP1 recruits OLIG1, thereby interfering with the OLIG1–SMAD interaction. A comprehensive list of all co-regulator proteins that have been associated with OLIG1 and OLIG2 can be found in Supplementary information S1.

Post-translational modifications

How are the proliferative and developmental functions of OLIG1 and OLIG2 regulated at different developmental stages and in different cell types? Most neurogenic bHLH transcription factors (for example, mammalian achaete-scute homologue 1 (MASH1), mammalian atonal homologue MATH, neurogenin 1 (NGN1) and NGN2) are only expressed transiently in progenitor cells at times when their functions are required. Notably, neither neurogenic nor anti-neurogenic bHLH transcription factors are generally expressed in fully formed, terminally differentiated neurons[50-52]. However, expression of *OLIG1* and *OLIG2*, initiated in oligodendrocyte progenitors, is sustained throughout development and

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occurs in the postnatal brain, where initial *in situ* hybridization images indicate that the two genes are expressed in white matter tracts of the corpus callosum, the optic nerve and the cerebellum[14-16]. Thus, for OLIG1 and OLIG2, post-translational modifications, rather than the timing of gene expression per se, might be the key to the developmental control of their functions. Obviously, factor-specific post-translational modifications could contribute to differential interactions with co-regulator proteins and genetic targets as discussed above.

Phosphorylation regulates OLIG2 function

Computer algorithms reveal a number of conserved potential phosphorylation sites in OLIG2 and recent studies indicate that several of these sites are functional (FIG. 4A and Supplementary information S2). Developmentally regulated phosphorylation events may account for the functional versatility of OLIG2 in cell cycle regulation and differentiation.

A striking structural feature of OLIG2 is a string of 12 contiguous serine and threonine residues at position Ser77-Ser88 in its N-terminal region (known as the ST box). Huillard *et al.* showed that a murine OLIG2 protein fragment containing the ST box, fused to glutathione S-transferase, was a substrate for the serine–threonine protein kinase CK2[104]. Tryptic peptide digests revealed phosphorylation of the OLIG2 fragment at residues Ser85 and Ser87 within the ST box. Interestingly, targeted disruption of the gene encoding CK2β (an essential regulatory subunit of CK2) results in impaired oligodendrocyte differentiation *in vivo* and *in vitro*. These observations largely resemble the differentiation phenotype of the *Olig2* knockout mice. Moreover, an *Olig2* deletion mutant that lacks the entire ST box is unable to rescue the formation of oligodendrocyte progenitor cells when it is transduced in *Olig2* null neural progenitor cells. Collectively, these observations provide circumstantial evidence that the phosphorylation state of the ST box could regulate separate functions of OLIG2 in proliferation and differentiation.

One concern, however, is that phosphorylation of the ST box has never been detected in living cells. Sun *et al.* isolated endogenous OLIG2 from mouse neural stem cells, human malignant gliomas and ectopic OLIG2 transfected into COS7 cells and screened these isolates for post-translational phosphorylation events by mass spectrometry. Phosphorylation of the ST box in the OLIG2 extracts was not detected[105]. Conceivably, phosphorylation of the ST box *in vivo* is a spatially and temporally restricted event that was not duplicated in the cell types assessed by Sun and colleagues. However, it is also possible that misfolding of the OLIG2 fragment in the *in vitro* experiment described above exposed CK2 substrates that are normally occluded in native OLIG2. It may be noteworthy that the ST box itself is not especially well conserved through phylogeny, especially when compared with several other phosphorylation motifs interesting OLIG2 (see FIG. 4A and Supplementary information S2). Thus, the biological functions of the ST region have yet to be fully resolved *in vivo*.

Cortical progenitor cultures that have been expanded by incubation with basic fibroblast growth factor (FGF) and epidermal growth factor (EGF) for two weeks contain 90% OLIG2-positive cells[39]. Following withdrawal of FGF and EGF and exposure to ciliary neurotrophic factor (CNTF), OLIG2 is exported from the nucleus to the cytosol where it is rapidly degraded. In an elegant series of studies, Setoguchi and Kondo showed that this CNTF-induced relocalization of OLIG2 coincides with activation of the serine–threonine protein kinase Akt[66]. Moreover, they showed that Ser30 in the N-terminus of OLIG2 is phosphorylated by Akt *in vitro*. A phosphomutant version of OLIG2 containing a Ser30Ala mutation, is retained in the nucleus of neural progenitor cells. This coincides with impaired CNTF-induced astrocytic differentiation after FGF and EGF withdrawal, compared to cells overexpressing vector control. Together, their observations are consistent with a model wherein the phosphorylation state of OLIG2 at Ser30 dictates a fate choice decision for

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cortical progenitor cells to differentiate into astrocytes or remain as uncommitted neuronal progenitors.

Setoguchi *et al.* did not demonstrate Ser30 phosphorylation of endogenous OLIG2 in CNTF-treated cortical progenitor cells. In addition, Sun *et al.* did not detect Ser30 phosphorylation in OLIG2 that was isolated from proliferating mouse neurospheres or glioma progenitor cells[105]. However, OLIG2 in cycling neurosphere cultures is localized strictly to the nucleus, and Sun *et al.* did detect some Ser30 phosphorylation in OLIG2 that was isolated from COS7 cells, in which an appreciable amount of the protein is found in the cytosol[105, 106]. These observations would be consistent with the nuclear export and degradation function of Ser30 phosphorylation, as was suggested by Setoguchi and Kondo[66]. One caveat regarding the functional relevance of the Ser30 site is that this site is not well conserved in OLIG2 from different animal species (FIG. 4A).

As noted above in our comments on development, one important function of OLIG2 in the early stages of development is to sustain the replication-competent state of neural progenitors[43]. At later stages of development however, OLIG2 is pivotal for specification of motor neurons and oligodendrocytes (FIG. 1B and TABLE 2). Studies by Sun *et al.* show that the proliferative functions of OLIG2 are largely controlled by developmentally regulated phosphorylation of a triple serine motif comprising Ser10, Ser13 and Ser14 [105]. When phosphorylated at these positions, OLIG2 maintains pro-mitotic functions in normal neural progenitors. Using a phosphorylation state-specific antibody to the triple serine motif, Sun *et al.* showed that endogenous OLIG2 is phosphorylated at these residues during early stages of embryonic development when oligodendrocyte progenitors are proliferating. In postnatal white matter, the same serine residues are in a non-phosphorylated state. Strikingly, cells expressing a phosphomimetic mutant version of OLIG2 (wherein the negatively charged amino acids aspartate or glutamate were substituted for Ser10, Ser13 and Ser14) were more tumorigenic in a murine model of high-grade glioma. Conversely, cells expressing a phosphonull mutant of OLIG2 (with the neutral amino acids glycine or alanine were substituted for the three serine residues) were less tumorigenic. On a final note, OLIG2 expressed in p53-positive human gliomas is phosphorylated at the triple serine motif. The data presented by Sun *et al.* and in a related paper by Mehta *et al.* suggest that phosphorylated OLIG2 inhibits the genetic and biological responses to p53 [57]. Collectively, these data suggest that phosphorylated OLIG2 may contribute to the notorious resistance of human gliomas to radiation and genotoxic drugs. This OLIG2 triple serine motif is stringently conserved throughout evolution (FIG. 4A). In fact, these serines and their flanking amino acids are nearly as well conserved as the bHLH motif itself. To date, the protein kinases and phosphatases that regulate the phosphorylation state of this triple serine motif have not been identified.

As described above (TABLE 2), OLIG2 is essential for specification of motor neurons as well as oligodendrocytes in the pMN domain of the developing spinal cord. Li *et al.* identified a phosphorylation site in the bHLH domain of OLIG2 that regulates the motor neuron to oligodendrocyte transition[44]. A bio-informatic search for predicted phosphorylation sites and conserved amino acids in OLIG2 drew the authors attention to a predicted protein kinase A (PKA)-phosphorylated serine at position 147 in the second helix of OLIG2. A phospho-specific antibody showed that Ser147 is phosphorylated in the developing mouse spinal cord during the window of time wherein OLIG2 specifies the formation of motor neurons. The phosphorylation state of Ser147 reaches a maximum at day E9.5 during the peak of neurogenesis in the pMN and then decreases over time, disappearing entirely after E12, when the switch towards oligodendrocyte production takes place. Li *et al.* also generated mice that expressed *Olig2* encoding a serine-to-alanine substitution at position 147 (Ser147Ala OLIG2) to study the biological function of Ser147 phosphorylation

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in vivo. Ser147Ala OLIG2 mice have a diminished pMN domain (revealed by *Pax6* and *Nkx*2.2 expression), as do *Olig2* null mice. Also, as noted with *Olig2* null mice, Ser147Ala OLIG2 mice die at birth, because they do not generate motor neurons.

At a biochemical level, Li *et al.* showed that Ser147Ala OLIG2 less readily forms OLIG2 homodimers and OLIG2–OLIG1 heterodimers relative to wild-type OLIG2 [44]. Conversely, Ser147Ala OLIG2 binds NGN2 with higher affinity than does wild-type OLIG2. The authors speculate that dephosphorylation of OLIG2 in the developing pMN domain sequesters NGN2, thereby preventing motor neuron development. In a perfect world, this sequestration of NGN2 would be antagonized by substitution of a negatively charged amino acid (aspartic acid or glutamic acid) at Ser147, as per rescue experiments with the phosphomimetic mutant for the triple serine motif, described by Sun and colleagues[105]. However, rescue experiments of this sort are not always successful and Ser147Asp and Ser147Glu OLIG2 seem to be phenotypically equivalent at a biochemical level to the Ser147Ala OLIG2 — at least with respect to their diminished capacity to form OLIG2 homodimers [44]. Interestingly, the Ser147 phosphorylation motif is conserved in the bHLH domain of OLIG1 (Ser138), which is not involved in motor neuron development (FIG. 4B and TABLE 2). It would be interesting to see if the bHLH domain of OLIG1 could be substituted for the OLIG2 bHLH domain in 'domain swap' experiments focusing on motor neuron formation.

Is OLIG1 protein post-translationally regulated?

Regardless of the structural similarities in OLIG1 and OLIG2, neither the OLIG2 triple serine motif nor the AKT phosphorylation site at Ser30 have equivalents in OLIG1. However, as mentioned above, the predicted PKA-phosphorylated serine at Ser147 in the bHLH domain of OLIG2 is represented at an equivalent position in OLIG1 (Ser138). As described earlier, one of the features of OLIG1 is its nuclear to cytoplasm relocalization during oligodendrocyte development[25]. Niu *et al.* transduced *Olig1* null rat oligodendrocyte progenitor cells with a OLIG2 phosphomutant construct (Ser138Ala) or with its rescue construct (Ser138Asp)[107]. They showed that Ser138Ala OLIG1 is expressed primarily in the nucleus of oligodendrocyte progenitor cells, whereas Ser138Asp OLIG2 has a cytoplasmic localization. Future work should clarify whether this serine has a functional role *in vivo*, as this study did not include any direct analysis of the phosphorylation state of this serine with either a phosphorylation state-specific antibody or mass spectrometry, or any *in vivo* mouse work.

One other intriguing protein modifier that is relevant to transcription factor biology is SUMO (small ubiquitin-like modifier). SUMOylation has been implicated in numerous biological functions within the nucleus, including transcription factor activity, protein–protein interactions, promyelocytic leukemia (PML) nuclear body integrity, DNA repair and sub-nuclear localization [108-111]. SUMOylation of NF-κB essential modulator (NEMO), which regulates NF-κB, is sufficient for nuclear import of NEMO [112,113]. By contrast, several studies show an increase in substrate SUMOylation concurrent with substrate nuclear export[114-116]. It is unclear whether SUMOylation initiates export or occurs in the cytoplasm, perhaps as a mechanism to retain proteins in the cytoplasm through masking of a nuclear localization signal or context-dependent protein–protein interactions. As shown in FIG. 4B, OLIG1 does contain a conserved SUMOylation motif, and it was predicted to be a target of SUMOylation in a genome-wide screen for this modification[117]. Accordingly, it is at least conceivable that a SUMOylation event dictates the developmentally regulated localization of OLIG1 observed in remyelinating white matter (FIG. 2) [25].

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Conclusions and future directions

This Review has highlighted disparate aspects of the biology of OLIG1 and OLIG2 during development and in human disease. Despite much progress in elucidating this biology, many important questions remain regarding OLIG protein functions and activity. Indeed, given their multiple stage-specific roles, we have argued that insight into the post-translational regulation of OLIG1 and OLIG2 activity is crucial for understanding the functional roles of these proteins.

The phenotype of *Olig2* knockout mice is severe and the biological functions of the OLIG2 are readily apparent (TABLE 2). By contrast, the phenotype of *Olig1* null mice is relatively nuanced [17]. Investigators naturally gravitate to systems that are ‘black and white’ rather than ‘shades of gray’ and an OLIG2 bias is readily apparent in the literature. Indeed, the PubMed database at the National Library of Medicine (USA) currently shows a four to one ratio of papers with OLIG2 in their title to those with OLIG1. *OLIG1* and *OLIG2* lie in close proximity to each other in humans, rodents and numerous other species in a region that is genetically well conserved[118]. In our view, the biological functions of OLIG1 in development and disease are understudied and deserving of more attention. Indeed, many questions regarding its function remain. For example, are there early roles for OLIG1 in forebrain neurogenesis? Can OLIG1 compensate in part for OLIG2 during gliomagenesis? What are the important factors regulating OLIG1 at the post-translational level?

Overshadowing these technical challenges and underserved areas of inquiry are a series of therapeutic opportunities. Small molecule activators of OLIG1 and OLIG2 could have practical applications in MS and spinal cord injury respectively. Conversely, recent data suggest that small molecule antagonists of OLIG2 might serve as highly targeted therapeutics for malignant glioma. Against these therapeutic opportunities lies one formidable challenge. Transcription factors are generally considered to be unattractive targets for drug development because their interactions with DNA and heterodimeric partner proteins involve large and complex surface area contacts. Surrogate targets for OLIG drug development may be embedded within enzymatically active gene targets, partner proteins or post-translational modifying enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Cre-lox fate-mapping — This procedure installs a stable marker protein (usually colorimetric, such as LacZ) into a genetically defined cell type and all of its daughter cells.

Tumor-initiating cells — In tumors with a heterogeneous cell population (such as glioblastoma multiforme) these undifferentiated “stemlike” cells are thought to be

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	responsible for propagating the tumors in serial animal transplantation protocols.
SCID mice	This "severe combined immunodeficiency" mouse strain is used as a host animal for transplantation experiments with human tumors.
Transit-amplifying cells	Also known as "type C" cells, these rapidly dividing neural progenitor cells in the subventricular zone of postnatal brain are the immediate progeny of the more slowly replicating multipotent adult neural stem cells.
SNP	Single nucleotide polymorphisms ("SNPs") are DNA sequence variations that differ between individual members of a biological species or between paired chromosomes in a single individual.
Expression profiling	This procedure identifies the gene types that are expressed in a particular cell type by processing mRNA into cDNA and then annealing the cDNA to gene sequences arrayed on a solid surface.
Sumoylation	This post-translational modification event involves covalent ligation of Small Ubiquitin-like Modifier (SUMO) proteins to regulate various cellular processes.

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Box 1 | The oligodendrocyte transcription factor family: a closer look at structure

In humans, the genes encoding oligodendrocyte transcription factor 1 (OLIG1) and OLIG2 are localized within 40 kb of each other on chromosome 21 (syntenic to mouse chromosome 16). Co-localization of these genes is also observed in numerous other species and the chromosomal region in question is well conserved[118]. By contrast, *OLIG3* maps to human chromosome 6 (mouse chromosome 10)[16].

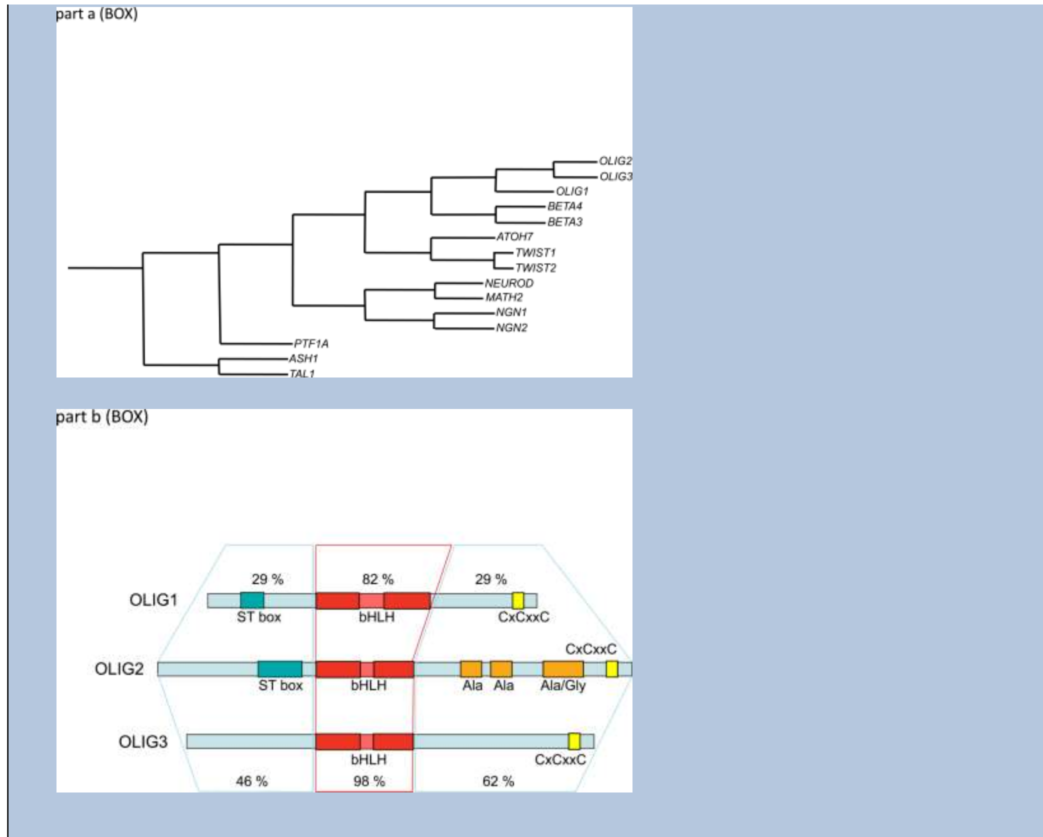
OLIG1, OLIG2 and *OLIG3* are recognizable as a core subset of genes in the family of basic helix–loop–helix (bHLH) transcription factors by nucleotide sequence homologies in regions encoding to the bHLH domain and amino and carboxy termini of the corresponding proteins (see part **a**; the phylogenetic tree was generated utilizing ClustalW2).

Standard amino acid sequence alignment algorithms utilizing Clustal Omega and further adjusted to “best-fit” by eye, indicate that at the protein level, OLIG2 is more closely related to OLIG3 than to OLIG1. Minimal conservation of domains outside the bHLH domains of OLIG1 and OLIG2 is suggested (see FIG. B, percentages reflect conserved substitution weighting following ClustalW2, Gonnet Pam250). However, the close relationship between OLIG1 and OLIG2 becomes more compelling when the alignment is adjusted to highlight short regions of homology, multiple insertions and deletions, and to utilize the conservation of the spacing of presumed conserved structural elements, such as proline (Supplementary information S2).

The amino terminal domain of OLIG1 is smaller than that of OLIG2 (89 amino acids versus 108 amino acids in humans). Also, the overall amino acid identity between OLIG1 and OLIG2 in this region is low, despite the presence of a distinctive serine–threonine-rich ‘ST box’ in both proteins (see part **b**). The OLIG3 amino terminus is even smaller (83 amino acids in humans) than that of OLIG1 and lacks the ST box; however, the amino termini of OLIG2 and OLIG3 are rather similar. Importantly, both contain a critical triple serine motif (see main text). It should be noted that the ST box common to OLIG1 and OLIG2 in humans and rodents is actually not well-conserved in OLIG1 and OLIG2 in other species (see Supplementary information S2).

The bHLH region is very highly conserved across all homologues in the OLIG family. However, the bHLH domain of OLIG1 is distinctive even within the broader family of bHLH transcription factors in several respects. In particular, the loop region of this domain has several unusual features in OLIG1. First, it is nearly twice the length of the loops from the other members of the OLIG family. Second, it has an extremely rare shift in the position of the helix 1-breaking proline. Third, the proline that precedes helix 2 is displaced. Last, it lacks the serine that directly precedes helix 2 in OLIG2 and which is a predicted candidate for phosphorylation.

Identity of the carboxy terminal domain in OLIG1 and OLIG2 is very low. By contrast, OLIG2 and OLIG3 are homologous in this region even though OLIG3 lacks a set of distinctive alanine and glycine-rich domains that is seen in OLIG2. The OLIG2–OLIG3 similarities extend well past the end of helix 2 of the bHLH domain and include a distinctive multi-proline motif directly next to the cysteine domain. The only domain common to all three OLIGs and conserved in all orthologues is a cysteine motif (CxCxxC) close to the C terminus. Cysteine residues and domains are implicated in multiple roles, including disulphide-bond formation, post-translational modifications such as S-palmitoylation, and recruitment of histone-modifying activities to chromatin[119-121].



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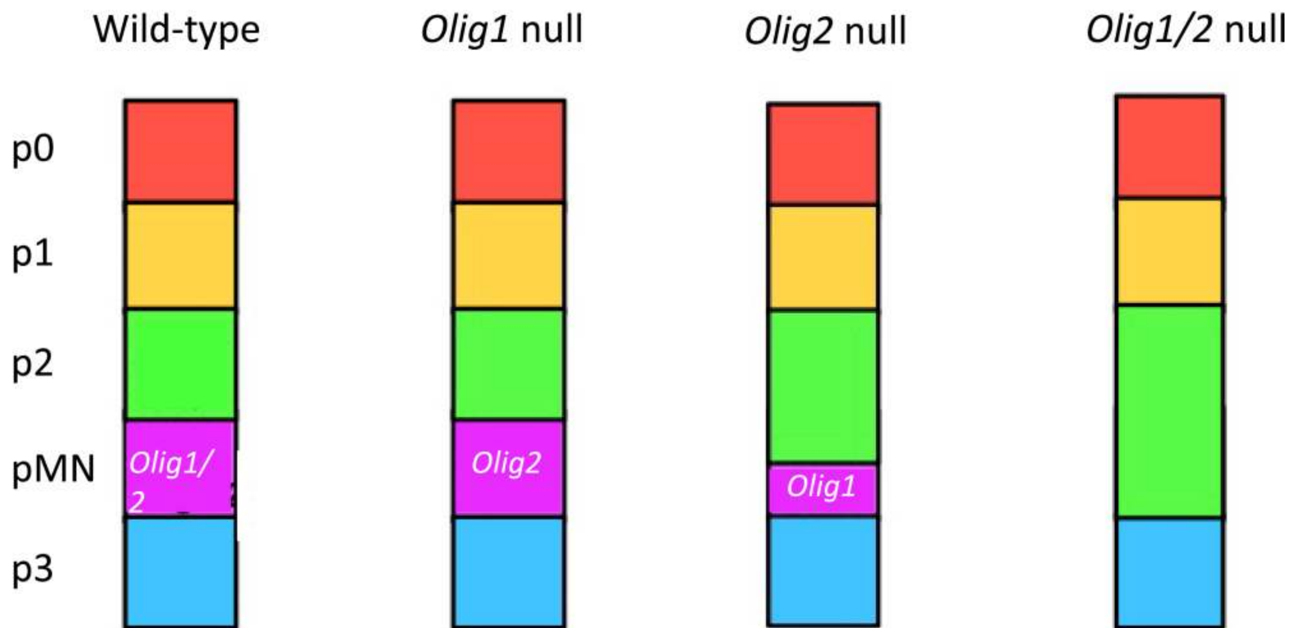

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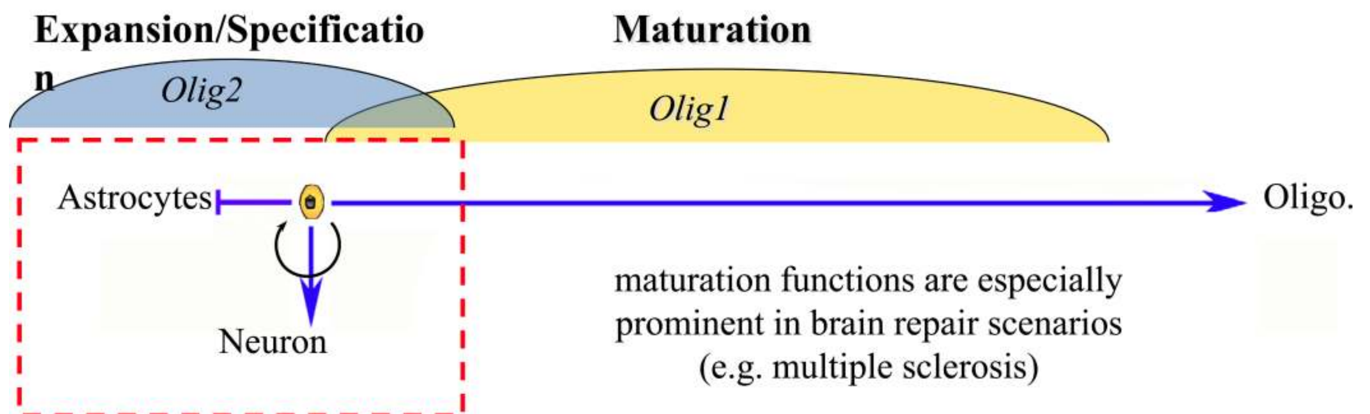

FIG. 1. The developmental roles of OLIG1 and OLIG2

a | Impact of *Olig1* and/or *Olig2* ablation on spinal cord patterning in mice. The combinatorial interactions of homeodomain proteins organize the ventral spinal cord of developing mouse embryos into five distinct regions, namely the p0, p1, p2, pMN and p3 domains. Different classes of ventral interneurons arise from the p0–p3 domains, whereas

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motor neurons and oligodendrocytes are derived from progenitors in the pMN domain (see REF. 13 for a review). Knockout of *Olig1* has little affect on maintenance of the pMN domain, whereas knockout of *Olig2* results in ventral expansion of the p2 domain. Combinatorial knockout leads to the complete disappearance of the pMN domain (see REF. 122 for a review). **b |** Non-overlapping roles for *Olig1* and *Olig2* in proliferation and differentiation of neural progenitors. OLIG1 promotes the differentiation of committed oligodendrocyte progenitors, a function that may be even more readily apparent in repair scenarios than in development. By contrast, OLIG2 functions at earlier developmental stages. Initially, OLIG2 acts to oppose cell differentiation and sustains the replication competent state so as to expand the pool of progenitors. At later stages of development, OLIG2 promotes the fate choice decision to form early oligodendrocyte progenitors and certain types of neurons, notably motor neurons in the developing spinal cord. Generally speaking, OLIG2 suppresses the formation of astrocytes, although there may be regional exceptions to this rule and it has been suggested that OLIG2 has a role in reactive gliosis.

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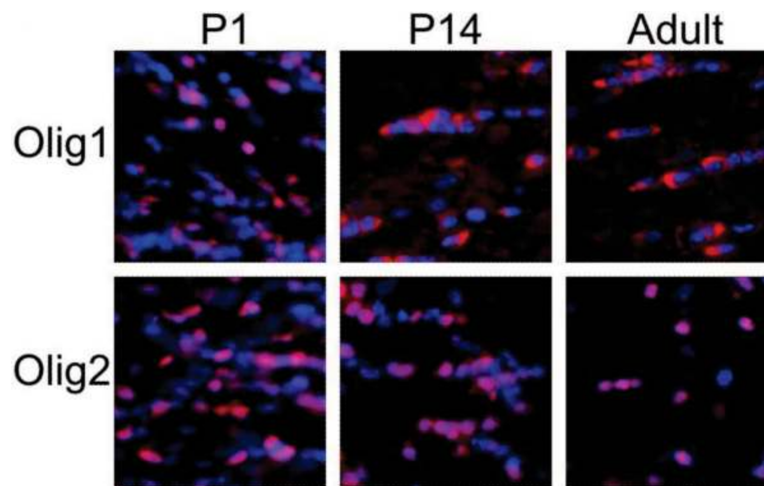

Figure 2. OLIG1 and OLIG2 localization

OLIG1 and OLIG2 (both visualized in red) are both present in the nuclei (blue) of oligodendrocytes and their progenitors at postnatal day (P) 1 in the mouse brain. OLIG2 continues to have a nuclear localization at all developmental stages, whereas OLIG1 is found almost completely in the cytoplasm in the adult mouse brain.

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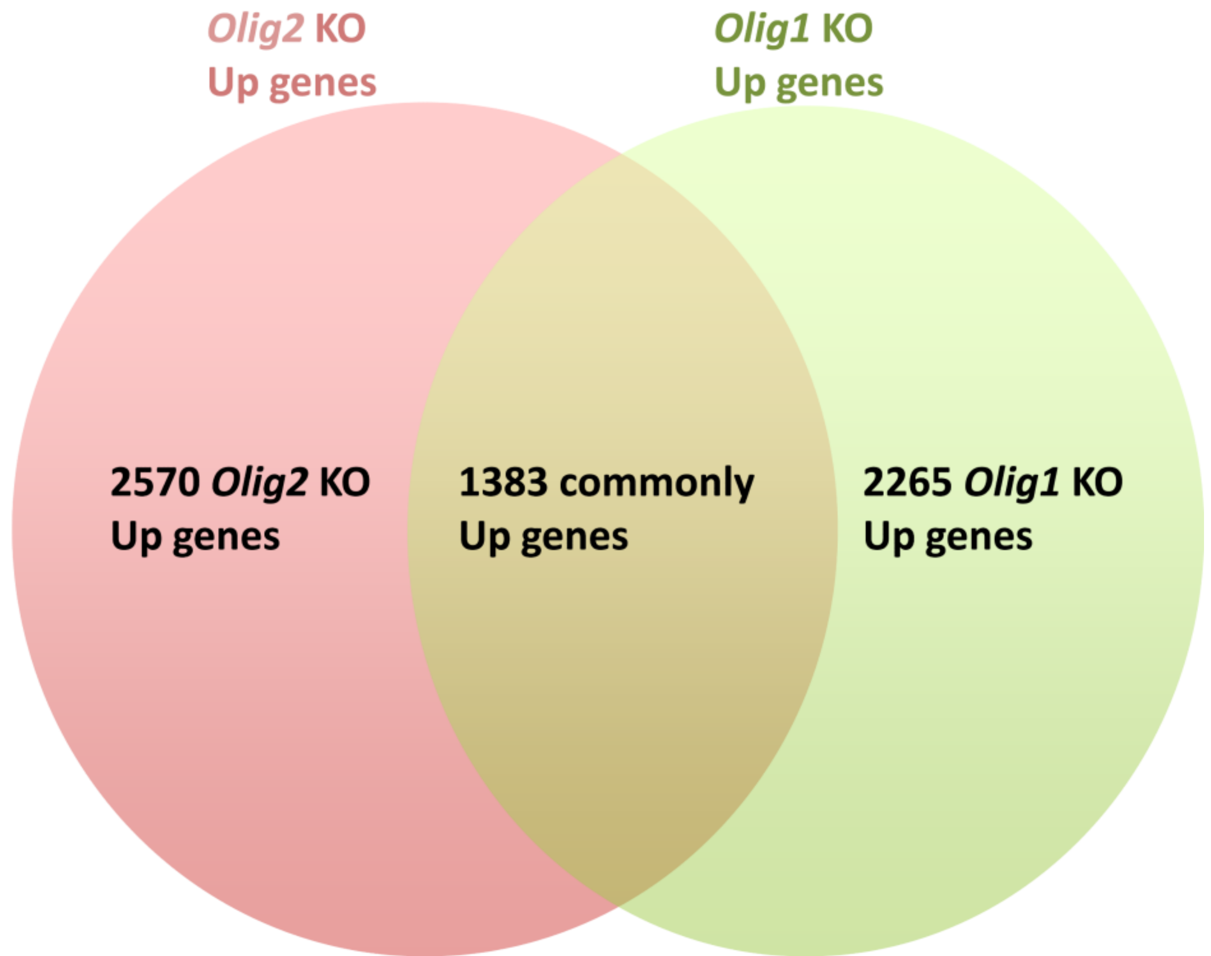

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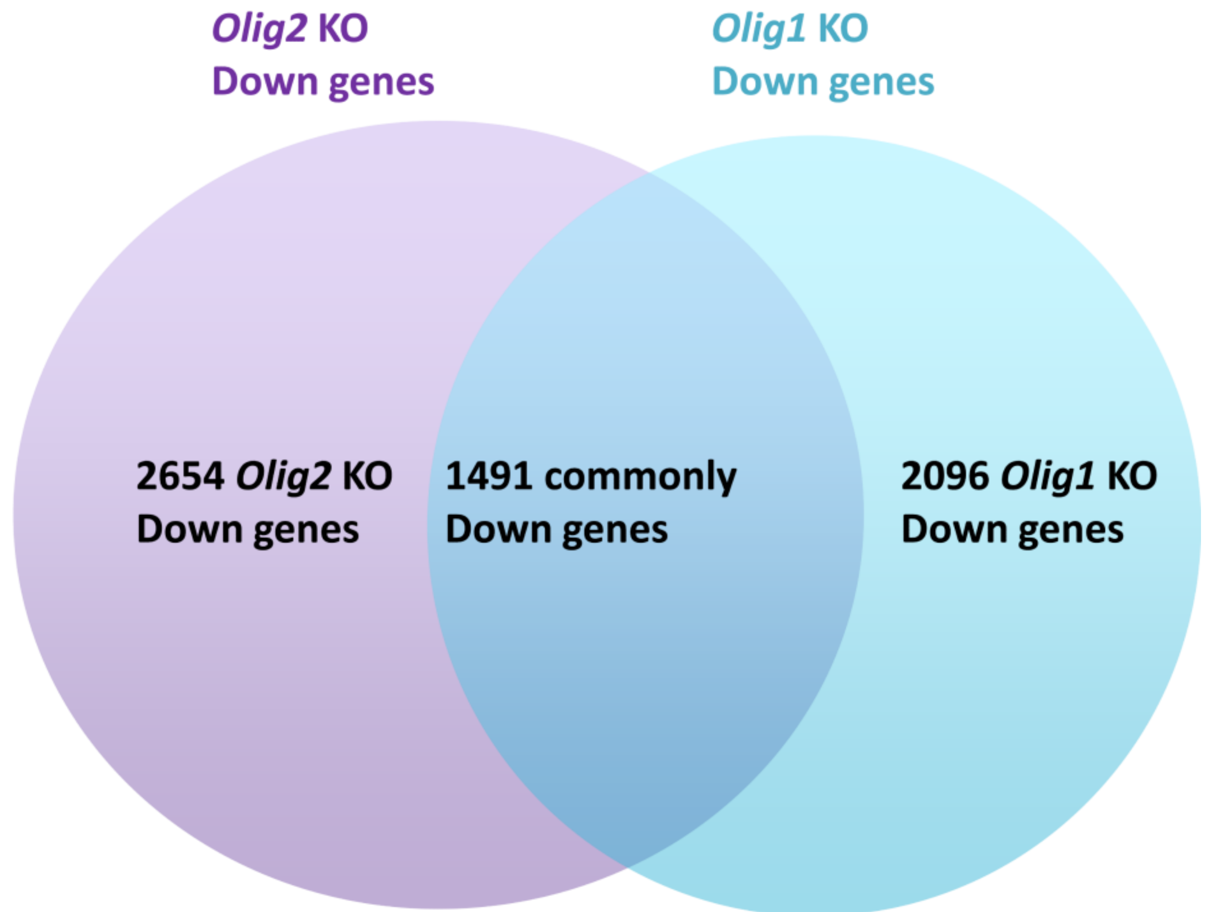

Figure 3. OLIG1 and OLIG2 downstream gene targets

The gene sets regulated by OLIG1 and OLIG2 only partially overlap. **a** | The number of genes that show upregulated expression in *Olig1* and/or *Olig2* null neural progenitor cells compared with wild-type neural progenitor cells. **b** | The number of genes that show down-regulated expression in *Olig1* and/or *Olig2* null neural progenitor cells compared with wild-type counterparts. The *Olig2* data set is taken from REF. 39, whereas the *Olig1* expression profiling data sets (S. Mehta, H. Liu, J. Alberta, E. Huillard, D. Rowitch, C. Stiles, unpublished observations) are available from the NCBI GEO database (GSE39706).

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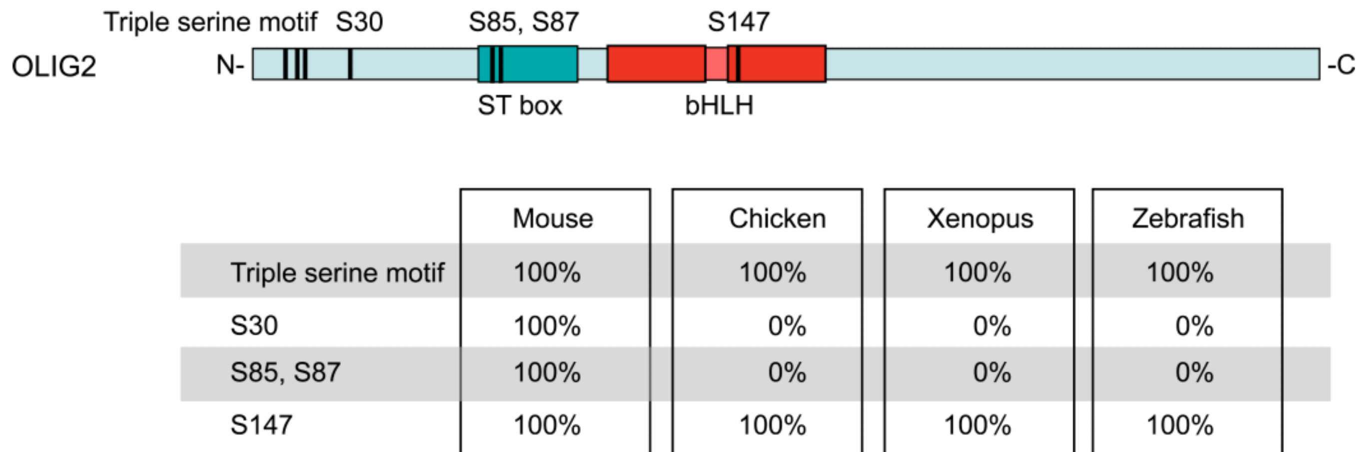

	Mouse	Chicken	Xenopus	Zebrafish
Triple serine motif	100%	100%	100%	100%
S30	100%	0%	0%	0%
S85, S87	100%	0%	0%	0%
S147	100%	100%	100%	100%

B

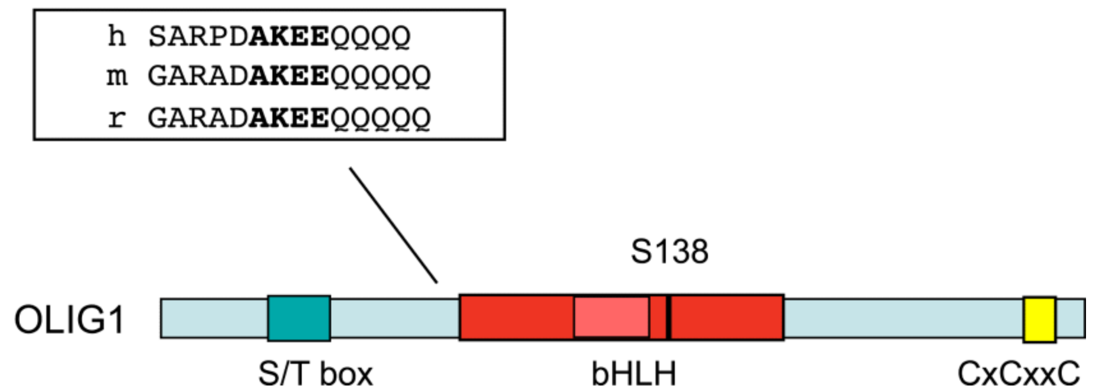

Figure 4. Post-translational modification motifs in OLIG1 and OLIG2

a | Human OLIG2 contains a number of serine phosphorylation sites, which are conserved in mouse OLIG2. The triple serine motif in N terminus of human OLIG2 and Ser147 are entirely conserved in chicken, *Xenopus laevis* and zebrafish. b | Human OLIG1 contains a serine residue at position 138 that seems to correspond to Ser147 in OLIG2. Protein

alignment of OLIG1 orthologues from human, mouse and rat reveals a conserved putative SUMOylation motif close to the basic helix–loop–helix (bHLH) domain (inset).

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Table 1

Developmental and postnatal expression of *Olig* genes

	Olig1	*Olig2*	*Olig3*	References
Developmental				
Ventral spinal cord (pMN domain)	++	+++	–	14, 15
Dorsal spinal cord	–	–	+++	42, 123
Early oligodendrocyte progenitors	+++	+++	–	14, 15
Postnatal				
SVZ transit-amplifying cells	+++	+++	NT	22
NG2-positive glia	+++	+++	NT	124
Myelinating oligodendrocytes	+++	+++	NT	14, 15
External to CNS	–	–	++	16

Expression levels (arbitrary units) ranging from no expression (–) to strong expression (+++). NT not tested.

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Table 2

Functions of OLIG proteins

	OLIG1	OLIG2	OLIG1 + 2	OLIG3	References
Developmental					
Spinal cord patterning	−	+	+++	−	14, 15
Expansion of progenitor pool	−	+++	+++	−	39
Specification of motor neurons	−	+++	+++	−	14, 15
Specification of other neurons	−	+	+	+++	29, 42, 123, 125
Specification of NG2-positive glia	−	+++	NT	NT	124
Specification of oligodendrocytes	+/−	++	+++	−	14, 15
Maturation of oligodendrocytes	+	NT	NT	−	14
Postnatal					
Malignant glioma	+/−	+++	+++	NT	39, 57
Myelin repair	+++	NT	NT	NT	25
Reactive gliosis	−	+++	NT	NT	49
Down syndrome	NT	NT	+++	NT	29
Alzheimer's / Schizophrenia*	NT	+	NT	NT	26-28
Rheumatoid arthritis*	NT	NT	NT	+	126, 127

Function (arbitrary units) ranging from no involvement (−) to essential for the specific process (+++). NT not tested.

*Genome wide association studies correlate certain OLIG2 single nucleotide polymorphisms to these disorders. These observations have not progressed beyond the correlative level.