Complex Regulation of Olig2 Activity During Spinal Cord Development

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Abstract

The transcription factor Olig2 is essential for the specification of neurons and oligodendrocytes, as well as a subset of astrocytes during both neural tube and telencephalon development. Yet, how this single transcription factor is capable of generating these disparate cell types has remained largely unknown. This work elucidates multiple mechanisms, including temporal phosphorylation and dimerization partner choice, which govern the developmental action of Olig2. These mechanisms work in concert to elegantly regulate the temporal functions of Olig2. In the developing spinal cord, Olig2 is phosphorylated at three residues, S147, T151, and S265, which serve as molecular gates between cell fate commitment and terminal differentiation of Olig2-derived cell types. Initial phosphorylation of Olig2 at S147 and T151 is required for motor neuron and oligodendrocyte cell fate specification. When this motif is phosphorylated, Olig2 prefers homodimerization. Investigation into Olig2 dimerization revealed that only the Olig2 homodimer is capable of generating motor neurons. Conversely, when unphosphorylated at S147 and T151 there is an increase in Olig2 interactions with heterodimer partners Ngn2 and E47. Heterodimers with Ngn2 and E47 do not play a role in cell fate specification but may be involved in the down-regulation of Olig2. Unlike motor neuron specification, oligodendrocyte specification can be driven by both the Olig2 homodimer and the Olig2-Olig1 heterodimer. Consistent with this finding, ChIP assays with these two cell-fate specifying dimers, Olig2-Olig2 and Olig2-Olig1, demonstrate that each is recruited to distinct genomic loci that correspond with their unique roles. Together, these data suggest that phosphorylation at S147 and T151 is coupled with dimer partner choice, which determines cell fate specification through target gene binding. Additionally, phosphorylation at S147 and T151 permits subsequent phosphorylation at S265, which serves as a critical regulatory site for Olig2 function. Once Olig2 is phosphorylated at S265, the cell fate specification activity of Olig2 is halted. Considering that Olig2 expression must be down-regulated in order for motor neurons to

differentiate, this inactivating phosphorylation site is essential for maintaining the balance between proliferation and differentiation during spinal cord development. Unlike phospho-S147 and -T151 Olig2, which are localized in the nucleus, phospho-S265 is predominately perinuclear and cytoplasmic. This suggests that phosphorylation at S265 triggers nuclear export of Olig2, thereby rendering it inactive as a transcription factor. Finally, we propose that S265 is phosphorylated by CaMKIV. CaMKIV is highly expressed in motor neurons as they transition from progenitors to post-mitotic cells; therefore the temporal expression of CaMKIV expression is consistent with the timing of S265 phosphorylation. Additionally, CaMKIV increases phosphorylation of Olig2 at S265, while, conversely, inhibiting CaMKIV *in vitro* decreases S265 phosphorylation. Together, the data presented in this dissertation delineate how processive phosphorylation governs the activity of Olig2 during spinal cord development and provide a mechanistic basis for the disparate roles of Olig2.

List of Abbreviations

CNS	Central Nervous System
SC	Spinal Cord
MN	Motor Neuron
IN	Interneuron
OL	Oligodendrocyte
OPC	Oligodendrocyte Precursor Cell
VZ	Ventricular Zone
pMN	Motor Neuron Progenitor
pOL	Oligodendroctype Progenitor Domain
bHLH	Basic Helix Loop Helix
HD	Homeodomain
A	Alanine
E	Glutamic acid
S	Serine
T	Threonine
IP	Immunoprecipitation
ChIP	Chromatin Immunoprecipitation
ISH	In Situ Hybridization
IHC	Immunohistochemistry
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
GST	Glutathione-S-Transferase Fusion Proteins
dpe	Days Post Electroporation

DIF	Days in Differentiation Media
EnR	Engrailed
VP16	Transcriptional Trans-activation Domain
Ct	Carboxyl-Terminus
Nt	Amino-Terminus
Isl1	Islet-1 Transcription Factor
Lhx3	LIM Homeobox Protein 3
NLI	Nuclear Lim Interacting Protein / Lim Domain Binding Protein 1
Nkx2.2	NK2 Homeobox 2
Nkx6.1	NK6 Homeobox 1
Olig2	Oligodendrocyte Transcription Factor 2
Chx10	Visual System Homeobox 2
Sox10	SRY (Sex Determining Region Y)-Box 10
MNR2	Homeodomain Protein
Hb9	Motor Neuron and Pancreas Homeobox 1
PDGFRa	Platelet Derived Growth Factor Receptor, Alpha Polypeptide
MBP	Myelin Basic Protein
Ngn2	Neurogenin 2
Sox2	SRY (Sex Determining Region Y)-Box 2
GFAP	Glial Fibrillary Acidic Protein
GS	Glutathione Synthetase
Olig1	Oligodendrocyte Transcription Factor 1
Olig3	Oligodendrocyte Transcription Factor 3
E12	Tanscription Factor 3

E47	Tanscription Factor 3
CamKIV	Calcium/Calmodulin-Dependent Protein Kinase IV
DS	Down Syndrome

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Introduction

Overview of Spinal Cord Development

During development, cells become specified and refined through temporally and spatially orchestrated processes to create a mature organism. In chordate animals, these processes transform a relatively homogenous population of progenitors in the neural tube into the incredible array of neurons and glia that form the brain and spinal cord, which constitute the central nervous system (CNS). On the cellular level, transcription factors often guide cell fate determination and differentiation through the selective activation or repression of gene expression. A central question in developmental neuroscience is how transcriptional regulation contributes to the formation of the brain and spinal cord.

One of the unique features of spinal cord development is the establishment of distinct progenitor domains, which then each give rise to individual classes of neurons and glia. Although many transcription factors play similar roles in both spinal cord and brain development, the discrete progenitor domains in the neural tube make it an especially tractable system for studying the molecular mechanisms that underlie CNS cell fate specification. Additionally, genetic manipulation techniques in the neural tube, in particular in ovo electroporation, make loss-of-function and gain-of-function studies remarkably fast and adaptable. For these reasons, as well as the applicability to our understanding of human disease, the studies presented here primarily utilize the developing neural tube.

Early in neural tube development, morphogen gradients induce the expression of transcription factors that pattern progenitors along the dorsal-ventral axis into discrete domains. In the dorsal spinal cord, BMPs and Wnts are the predominate signals, and, in the ventral spinal cord, the morphogen is Shh. Following exposure to these signals, progenitor cells located along the ventricle begin expressing homeodomain (HD) or basic helix loop helix (bHLH) transcription factors in a concentration dependent manner (Ericson et al. 1997; Briscoe et al. 2000). The unique combination

of bHLH and HD transcription factor expression in a group of progenitors defines a domain. Once induced, the cross-repressive interactions between these transcription factors further refines the boundaries of the progenitor domain. As development proceeds, these transcription factors then control the expression of genes required for specifying unique neuronal and glial cell types and promoting differentiation. This process creates the mature spinal cord, made up of diverse neuronal and glial cells that are each derived from discrete progenitor pools.

One of these transcription factors, Olig2, is the focus of this work. Olig2, which belongs to bHLH family of transcriptions factors, is expressed by progenitors in both the developing spinal cord and brain and is essential for specifying both neurons and glia. Olig2 is required for motor neuron generation in the spinal cord (Novitch et al. 2001; Mizuguchi et al. 2001; Park et al. 2002) and, similarly, cholinergic neuron production in the basal forebrain (Furusho et al. 2006). Additionally, Olig2 is necessary for oligodendroctye specification throughout the CNS (Zhou and Anderson 2002; Lu et al. 2002; Takebayashi et al. 2002b; Park et al. 2002; Cai et al. 2005, 2007; Vallstedt et al. 2005; Fogarty et al. 2005; Miyoshi et al. 2007). Like Olig2, a number of transcription factors are used in different spatial and temporal contexts to achieve disparate ends. For many transcription factors, the mechanisms that regulate these changes in activity are not well understood. In addition to posing an interesting developmental question, understanding what mediates the developmental activity of these factors has implications for human disease, as well. Specifically, aberrant Olig2 function is known to be causative in pediatric disorders, like Down Syndrome and diffuse gliomas (Chakrabarti et al. 2010; Ligon et al. 2004; Tabu et al. 2007; Lu et al. 2012), and is thought to play a role in degenerative disorders like ALS (Sims et al. 2009; Satoh et al. 2015). Given the importance of Olig2 in development, as well as in human disease, this work aims to elucidate the mechanisms that underlie how a single transcription factor, Olig2, is able to generate a number of diverse cell types in the developing CNS.

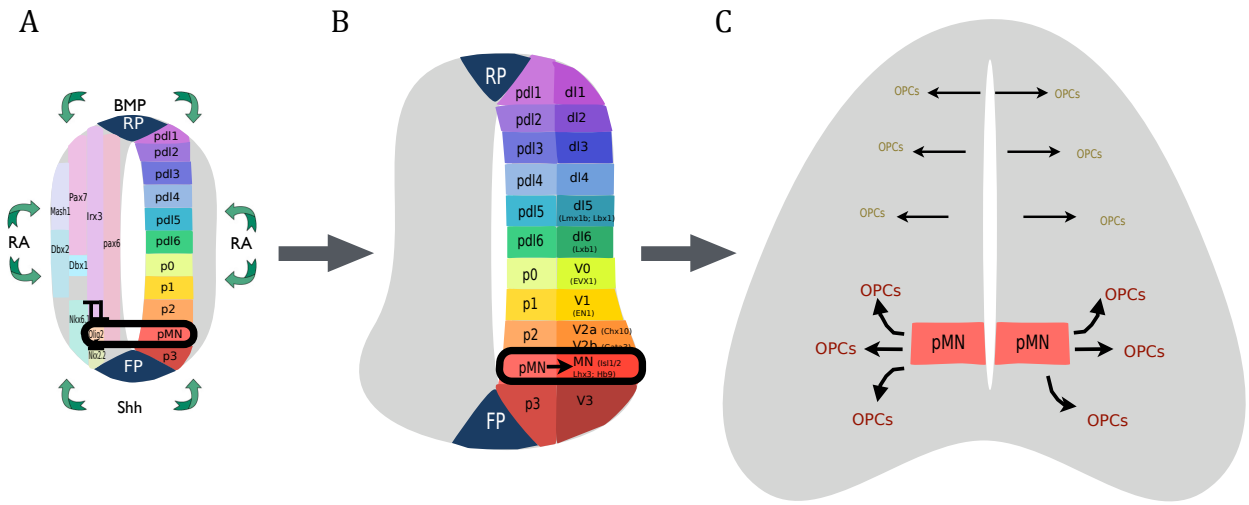


Figure 1. Spinal cord development and Olig2.
A) Progenitors residing along the ventricle of the developing neural tube are patterned by morphogens, such as BMP, RA, and Shh. Gradients of these morphogen induce the expression of transcription factors in specific groups of progenitors (shown on the left). This unique transcription factor code in particular group of progenitors defines a progenitor domain (shown on the right side). For example, Olig2 is induced by a high level of Shh and establishes the pMN through repressive interactions with neighboring transcription factors. B) Each progenitor domain later gives rise to specific neuronal subtypes, which also express unique transcription factors, some of which are listed in parentheses below the post-mitotic domain name. For example, the Olig2+ pMN domain gives rise to motor neurons (MN), which initially express Isl1/2, Lhx3, and Hb9. C) Following the motor neuron production from the pMN, this Olig2+ domain gives rise to OPCs. Some spinal oligodendrocytes are produced from pdl3-pdl5 Olig2+ progenitors as well.

The Myriad of Roles Olig2 Plays in the Developing Spinal Cord

Olig2 is induced by a relatively high concentration of Shh and is first expressed adjacent to the floor plate shortly after neural tube closure in many ventral progenitors (Ericson et al. 1997; Lu et al. 2000; Novitch et al. 2001; Dessaud et al. 2007, 2010). As development proceeds, this pattern of expression is restricted dorsally and ventrally, such that, by the onset of motor neuron generation, Olig2 is expressed in a discrete domain of progenitors in the ventral neural tube (Lu et al. 2000; Novitch et al. 2001; Dessaud et al. 2007, 2010; Chen et al. 2011). The consolidation of Olig2 expression is accomplished through repressive interactions with neighboring transcription factors, namely Irx3 dorsally and Nkx2.2 ventrally (Novitch et al. 2001; Qi et al. 2001; Chen et al. 2011).

Additionally, mir-17-3p has been shown to mediate the cross-repressive interactions between Olig2 and Irx3 (Chen et al. 2011). Thus, the first role of Olig2 in the developing spinal cord is to establish the motor neuron progenitor (pMN) domain. In Olig2 knockout mice, Irx3 is no longer repressed and expands ventrally to the p3 domain (Lu et al. 2002; Zhou and Anderson 2002; Takebayashi et al. 2002a). Without Olig2, the progenitors adjacent to the p3 domain lose their pMN identity and instead adopt p2 characteristics (Lu et al. 2002; Zhou and Anderson 2002; Takebayashi et al. 2002a). These progenitors accordingly give rise to V2 interneurons at the expense of motor neurons. Although the loss of motor neurons in Olig2 knockout mice could be explained by the absence of the pMN domain, Olig2 is also essential for promoting motor neuron identity and differentiation.

Following the establishment of the pMN, the next role that Olig2 plays in neural tube development is specifying motor neurons. Olig2 misexpression in the developing chick neural tube results in ectopic dorsal expansion of MNs at the expense of dorsal interneurons (Novitch et al. 2001; Mizuguchi et al. 2001). Olig2 is able to direct motor neuron fate specification through multiple means. Misexpression studies reveal that Olig2 induces expression of MNR2 and Lhx3, both of which promote motor neuron cell fate identity (Novitch et al. 2001). MNR2 and Hb9, a closely related transcription factor found in mammals, are indispensable for motor neuron differentiation (Arber et al. 1999). Similarly, Lhx3 forms a transcriptional complex with Isl1 and NLI, both of which are more broadly expressed in the developing neural tube, that is essential for motor neuron production (Thaler et al. 2002; Lee and Pfaff 2003; Novitch et al. 2003; Lee et al. 2004, 2005, 2008). Misexpression of Olig2 also results in expansion of the Ngn2+ domain (Novitch et al. 2001; Mizuguchi et al. 2001). In contrast to MNR2 and Lhx3, which specifically promote motor neuron identity, Ngn2 is a pro-neural bHLH that promotes neuronal differentiation throughout the spinal cord. Thus, Olig2 specifies motor neurons not only through repressive interactions, which establish the pMN, but also by inducing the expression of transcription factors, like Lhx3. These transcription

factors then further promote motor neuron identity with the induction of pan-neuronal transcription factors, like Ngn2, to promote differentiation of motor neurons.

Although Olig2 is initially required for motor neuron specification, Olig2 is down-regulated as these cells become post-mitotic (Novitch et al. 2001; Mizuguchi et al. 2001; Lu et al. 2002; Lee et al. 2005; Copray et al. 2006). Several lines of evidence indicate that down-regulation of Olig2 is critical for motor neuron differentiation. Studies with ES cells have shown that overexpression of Olig2 preferentially produces oligodendrocytes but not motor neurons (Copray et al. 2006; Hu et al. 2012). Consistent with this finding, the relative expression level of Olig2 is correlated with neuronal or glial cell fate (Lee et al. 2005; Rabadán et al. 2012; Imayoshi et al. 2013). Although pMN cells co-express Olig2 and Ngn2, the expression levels are heterogeneous (Lee et al. 2005). Progenitors with high Ngn2 and low Olig2 become motor neurons, whereas the opposite relationship is seen for oligodendrocyte specification (Lee et al. 2005). This observation is further supported by in vitro studies that show that Olig2 protein levels oscillate over time and overall protein levels are associated with different cell fate outcomes (Imayoshi et al. 2013). Lee et al. found that Olig2 antagonizes the pro-neural activity of Ngn2; thus, down-regulating Olig2 expression is an essential step in differentiation of motor neurons (Lee et al. 2005). Despite the importance of down-regulating Olig2, little is known about how this is accomplished during development. Although some extrapolate from the early role of mir-17-3p in down-regulating Olig2 in the p2 domain that mir-17-3p also functions to down-regulate Olig2 expression in the pMN as motor neurons differentiate, this remains untested (Meijer et al. 2012). Therefore the question of how Olig2 expression is down-regulated in motor neurons remains unanswered.

After motor neuron generation from the pMN is complete, Olig2 once again switches roles. A subset of pMN progenitors are reserved and give rise to the vast majority of oligodendrocytes found in the spinal cord. At the onset of oligodendrogenesis, Ngn2 vanishes from the pMN but Olig2

expression is maintained. Because of this shift in the cell type produced, later in development, this domain is no longer referred to as the pMN but instead is often called the pre-oligodendrocyte, or pOL, domain. As with motor neuron generation, Olig2 is absolutely required for specification of oligodendrocyte precursor cells (OPCs). In Olig2 null mice, oligodendrocyte markers are absent at all stages examined by in situ hybridization, immunohistochemistry, and RT-PCR (Lu et al. 2002; Takebayashi et al. 2002a; Liu et al. 2007). Given the striking phenotype for both motor neurons and OPCs, it is not surprising that the Olig2 null is embryonic lethal by e18.5 (Lu et al. 2002; Takebayashi et al. 2002a). By this point in development, in wild-type embryos, OPCs have already been produced and both early oligodendrocyte markers, such as Sox10 and PDGFRa, and later markers, such as Plp and Mbp, are expressed. However, in order to address whether the loss of Olig2 results in profound delay in OPC production rather than a complete loss of these cells, spinal cord explants from these mice were cultured in vitro from e15.5 to the equivalent of P6 (Lu et al. 2002). No oligodendrocyte markers were seen in Olig2 null explant cultures at any stage, indicating that OPCs are not specified in the absence of Olig2 (Lu et al. 2002). These studies indicate that Olig2 is required for OPC specification, while misexpression studies in chick neural tube have demonstrated that Olig2 is also sufficient to produce ectopic OPCs (Zhou et al. 2001; Liu et al. 2007). Both early OPC markers, such as Sox10 and PDGFRa, and more mature oligodendrocyte markers, like MBP, are observed starting at 4 days post electroporation (dpe) (Zhou et al. 2001; Liu et al. 2007). However, at earlier experimental time-points (2dpe), no ectopic OPCs are seen unless Olig2 is co-electroporated with Nkx2.2 (Sun et al. 2001, 2003). The inability of Olig2 to generate premature OPCs at this earlier developmental time-point reflects an interesting difference in the transcriptional program that underlies spinal OPC generation in chick compared to mouse. In chick, the Nkx2.2 domain expands dorsally to overlap with Olig2 prior to the onset of oligodendrogenesis (Fu et al. 2002; Zhou et al. 2001; Sun et al. 2003). This coexpression of Olig2 and Nkx2.2 appears to

be required for OPC production in the chick spinal cord (Fu et al. 2002; Zhou et al. 2001; Sun et al. 2003). Thus, before the dorsal expansion of Nkx2.2 in chick, Olig2 is unable to induce precocious OPC generation. In contrast, there is very little overlap of Nkx2.2 and Olig2 in the mouse spinal cord when OPCs emerge from the Olig2+ domain (Fu et al. 2002). However, around birth most Olig2+ cells are also Nkx2.2+ indicating that there may be two distinct ventral-derived oligodendrocyte populations in mice (Fu et al. 2002). In both mouse and chick, however, Nkx2.2 misexpression alone is not sufficient to produce OPCs (Zhou et al. 2001). Additionally, Nkx2.2 knock-out mice actually have increased numbers of OPCs as a result of ventral expansion of Olig2 (Qi et al. 2001). Together, these studies show that Olig2 is both necessary and sufficient for OPC specification in the spinal cord.

Although the vast majority of spinal cord oligodendrocytes, estimates range from 80 to >90%, arise from the ventral Olig2+ domaina subset of oligodendrocytes is generated in the dorsal spinal cord (Cai et al. 2005; Vallstedt et al. 2005; Fogarty et al. 2005). The Nkx6 transcription factors, Nkx6.1 and Nkx6.2, are known to regulate Olig2 expression in the ventral spinal cord (Novitch et al. 2001; Cai et al. 2005). In Nkx6.1/6.2 null mice, Olig2 expression is lost in the pMN and no motor neurons or OPCs from the ventral spinal cord are produced (Cai et al. 2005; Vallstedt et al. 2005; Fogarty et al. 2005). However, around e14.5, Olig2+ cells appear in more dorsal regions of the neural tube, and by e18.5 these cells begin migrating out of the ventricular zone (VZ) (Cai et al. 2005; Vallstedt et al. 2005). Although knocking out Nkx6.1/6.2 results in embryonic lethality, explants from these null embryos eventually develop cells with mature oligodendrocyte markers that myelinate axons (Cai et al. 2005; Vallstedt et al. 2005). Additionally, differentiated oligodendrocytes are observed in explant cultures derived from the dorsal half of the neural tube, further evidencing that a population of oligodendrocytes are produced from the dorsal spinal cord and also require Olig2 for specification (Vallstedt et al. 2005).

Finally, Olig2 is implicated in astrocyte specification in the ventral spinal cord. Early work with Olig2 knockout mice suggested that Olig2 did not play a role in astrocyte development, because Olig2 was not co-expressed with any astrocytic markers during development and astrocyte generation appeared largely unperturbed in the absence of Olig2 (Lu et al. 2002; Takebayashi et al. 2002a). However, in vitro work demonstrated that isolated Olig2+ progenitors differentiated into astrocytes, as well as oligodendrocytes and neurons, in culture (Gabay et al. 2003). Additionally, Olig2 initially represses astrocyte fate by repressing astrocytic genes like GFAP, while subsequent derepression of these targets allows for their transcription (Gabay et al. 2003; Fukuda et al. 2004). This paradigm of initial repression followed by derepression of fate-specifying targets is reminiscent of the action of Olig2 in motor neuron specification. Although no gross defects in astrocyte specification were observed in Olig2 knockouts, these observations indicated that Olig2 still might play a role in astrocyte formation. Later work revealed that astrocytes are specified in a domain-dependent manner, much like other cell types in the spinal cord, indicating that the loss of Olig2 may only affect a specific population of astrocytes (Bachoo et al. 2004; Muroyama et al. 2005; Hochstim et al. 2008). Both domain-specific inspection of these knockout lines and further experiments with conditional Olig2 knockout mice show that Olig2 is involved in the specification of astrocytes from the ventral spinal cord (Setoguchi and Kondo 2004; Marshall et al. 2005; Masahira et al. 2006; Cai et al. 2007). Ablating Olig2 using an astrocytic Cre line decreased astrocytes in the white matter, as well as the astrocyte markers GFAP, GS, and S100b (Cai et al. 2007). Lineage tracing studies revealed that, in addition to producing motor neurons and OPCs, Olig2+ progenitors also give rise to astroctyes based on location, morophology, and expression of astrocyte markers such as GFAP and s100b (Masahira et al. 2006). Although earlier work indicated that Olig2 was not found in cells with astrocyte markers, Setoguchi and Kondo found that a small population of immature astrocytes in the mouse cortical VZ, where neural progenitors and newly born neurons

reside, have cytoplasmic Olig2 but no differentiated astrocytes were Olig2+ (Setoguchi and Kondo 2004). These results suggest that Olig2 may be expressed early in astrocyte development and subsequently down-regulated during astrocyte maturation (Gabay et al. 2003; Setoguchi and Kondo 2004). This hypothesis was born out by in vitro work with astroctye cultures, which showed that, much like the role of Olig2 in motor neuron specification, nuclear export and subsequent down-regulation of Olig2 is a key step in the differentiation of astrocytes (Setoguchi and Kondo 2004). In addition to providing a mechanistic basis for the down-regulation of Olig2, the observation that Olig2 is exported as a part of astrocyte development is noteworthy because the only other context where cytoplasmic translocation of Olig2 has been reported is following injury (Cassiani-Ingoni et al. 2006; Niu et al. 2012; Magnus et al. 2007).

Transcriptional Activity of Olig2

Given the importance of Olig2 for generating a variety of cell types, much of the work on Olig2 has explored the molecular mechanisms underlying these roles: the transcriptional activity of Olig2. Most bHLH transcription factors, like Olig2, are thought to function predominately as transcriptional repressors. Consistent with this, previous work has shown that Olig2 functions as a transcriptional repressor to establish the pMN and during motor neuron generation (Novitch et al. 2001; Lee et al. 2005). When the transcriptional repressor domain of Engrailed (EnR) was fused to the bHLH domain of ckOlig2 and misexpressed in chick neural tube, its activity recapitulated what is seen with full-length Olig2 (Novitch et al. 2001). Misexpression of this Olig2-EnR construct results in dorsal repression of Irx3 and ectopic Ngn2, Lhx3, MNR2, and Hb9 (Novitch et al. 2001). Conversely, when Olig2 is fused to the transcriptional activator domain VP16, no ectopic markers are seen (Novitch et al. 2001). In fact, within the pMN and ventral horn there is a reduction in Ngn2, Lhx3, Isl1/2, and MNR2 with this activator Olig2 construct (Novitch et al. 2001). Similar

results were obtained with N-terminal fusions of these domains with mouse Olig2 (Mizuguchi et al. 2001). With VP16-mOlig2 misexpression, a modest decrease in motor neuron markers is seen, but EnR-mOlig2 did not recapitulate the activity of Olig2 (Mizuguchi et al. 2001). These differences could be attributed to an N-terminal, rather than C-terminal, fusion altering the activity of Olig2. Also, these differences could stem from the use of different species of Olig2. This author, as well as others, has observed that misexpression of chick Olig2 results in a more dramatic expansion of ectopic motor neuron markers compared to mouse Olig2. Since the results seen in Mizuguchi et al. are not antithetical to those of Novitch et al. but rather less robust, the differences seem to reflect variability in phenotype severity between species. In addition to misexpression data indicating that Olig2 functions as a repressor, Olig2 has been shown to directly bind regulatory elements of genes involved in motor neuron specification and differentiation, such as Hb9 (Lee et al. 2005; Mazzoni et al. 2011). Binding of Olig2 to these loci represses their transcription, thus preventing premature differentiation of progenitors (Lee et al. 2005). Subsequent derepression at the appropriate developmental time, which is achieved through down-regulation of Olig2 expression, then allows for transcription of these genes (Lee et al. 2005). Together, these results suggest that Olig2 functions exclusively as a transcriptional repressor in motor neuron generation.

The fact that Olig2-EnR results in ectopic expression of differentiation markers and transcription factors associated with motor neuron identity, whereas Olig2-VP16 results in a decrease in motor neuron markers within in the pMN and the retention of progenitor markers, raises an interesting possibility that Olig2 may function as a transcriptional activator during OPC specification in contrast to its activity as a repressor during motor neuron specification. Using the same constructs for misexpression, Zhou et al. found that coelectroporation of Olig2-EnR and Nkx2.2 replicated the ability of wild-type Olig2 to generate ectopic Sox10+ and MBP+ cells in the chick neural tube, whereas Olig2-VP16 and Nkx2.2 did not (Zhou et al. 2001). Additionally, Olig2-

EnR, NICD, and Su(H) electroporation resulted in a more modest increase in the generation of premature OPCs (Zhou et al. 2001). Similar to what is seen with motor neurons, there is a decrease in OPC markers Sox10, PDGFRa, and MBP with Olig2-VP16 electroporation (Zhou et al. 2001). These results suggest that Olig2 functions as a transcriptional repressor in OPC generation, as it does in motor neuron production. However, Olig2 has also been shown to function as an activator of certain oligodendrocyte genes. For example, Olig2 has been shown to bind and activate the Sox10 enhancer both in cultured oligodendrocytes and in the developing spinal cord (Küspert et al. 2011). Furthermore, genome wide analysis by ChIP-seq revealed that Olig2 and Brg1, which functions primarily as a transcriptional activator, co-occupy targets known to be important for oligodendrocyte specification and differentiation (Yu et al. 2013). Genomic loci with Olig2-Brg1 co-occupancy were also associated with activating histone marks H3K27Ac and H3K4me3 (Yu et al. 2013). Although Olig2, as described above, is thought to function primarily as a transcriptional repressor, these results suggest that Olig2 may promote oligodendrocyte fate, at least in part, through gene activation.

The activity of Olig2 in astrocyte generation is similar to what is seen in motor neuron production. Luciferase assays demonstrate that the astrocytic specification gene GFAP is repressed in the presence of Olig2 (Fukuda et al. 2004). Further work showed that Olig2 blocks GFAP expression by sequestering p300 from complexing with STAT3, which is known to activate GFAP in astrocytes (Fukuda et al. 2004). Additionally, as in motor neurons, down-regulation of Olig2 at the appropriate time in development is essential for derepression of astrocyte genes in order to allow transcription and differentiation (Gabay et al. 2003; Fukuda et al. 2004).

Molecular Interactions of Olig2

Members of the bHLH family of transcriptions factors are known to bind DNA as obligate

dimers. Early work indicated that cell type-specific bHLH proteins, also called class A bHLHs, preferred dimerization with bHLH E-proteins and that these dimers are the only transcriptionally active dimers (Dilworth et al. 2004; Yang et al. 2009). Although Olig2 does interact with E-proteins, E12 and E47, Olig2 has also been shown to dimerize with other partners as well. In addition to heterodimerizing with E12 and E47, Olig2 heterodimerizes with Olig1 and Ngn2 and also strongly homodimerizes (Samanta and Kessler 2004; Lee et al. 2005; Li et al. 2008). Furthermore, Olig2 interacts with HLH proteins Id2 and Id4 (Samanta and Kessler 2004). Id proteins lack a basic domain for DNA binding and, for other bHLH transcription factors, interaction with Ids results in cytoplasmic translocation thereby rendering the bHLH transcriptionally inactive. Despite evidence that Olig2 dimerization may change due to temporal expression of dimer partners and phosphorylation of Olig2, the biological roles of these dimers had not been previously investigated (Lu et al. 2002; Zhou and Anderson 2002; Lee et al. 2005; Li et al. 2011). Since choice of dimerization partner has been shown to affect the activity for other bHLHs (Murre et al. 1989; Lassar et al. 1991; Murre et al. 1991; Neuhold and Wold 1993; Castanon et al. 2001), determining the function of each Olig2 dimer is critical to understanding how Olig2 mediates diverse developmental functions and was one of the aims of this dissertation.

Protein Structure and Post-translational Modifications of Olig2

As their name aptly describes, bHLH proteins consist of a basic DNA-binding domain followed by two amphipathic alpha-helices, which are linked via flexible loop. These alpha-helices interlock such that the basic domain and first helix are most closely associated with DNA and the second alpha-helix of two bHLHs interact (Ellenberger et al. 1994; Ma et al. 1994). When dimerized, the flexible loop allows these proteins to achieve this conformation. Many bHLH proteins contain an additional dimerization domain that is contiguous and carboxy-terminal to the second helix.

However, Olig2 belongs to a subclass of bHLH proteins that have no known secondary dimerization domains (Amoutzias et al. 2004). The basic region fits in the major groove and recognizes a hexanucleotide CANNTG motif, called an E-box. The residues in the basic domain that contact DNA are highly conserved across the bHLH family, yet a given bHLH tends to recognize specific E-box motifs (Bertrand et al. 2002; Amoutzias et al. 2004). A number of factors are thought to lend specificity to the interaction of a bHLH protein to a particular E-box, or set of E-boxes, including dimerization with different partners (Setoguchi and Kondo 2004).

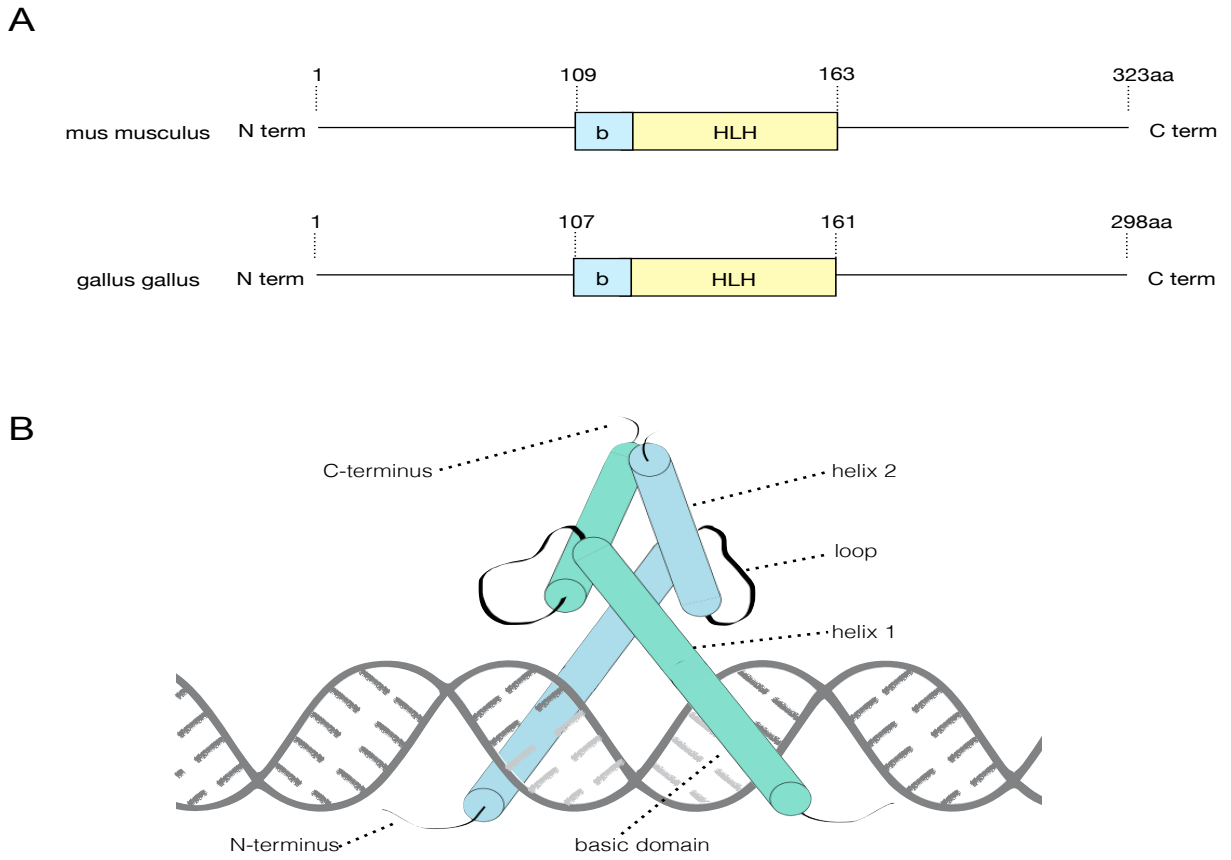
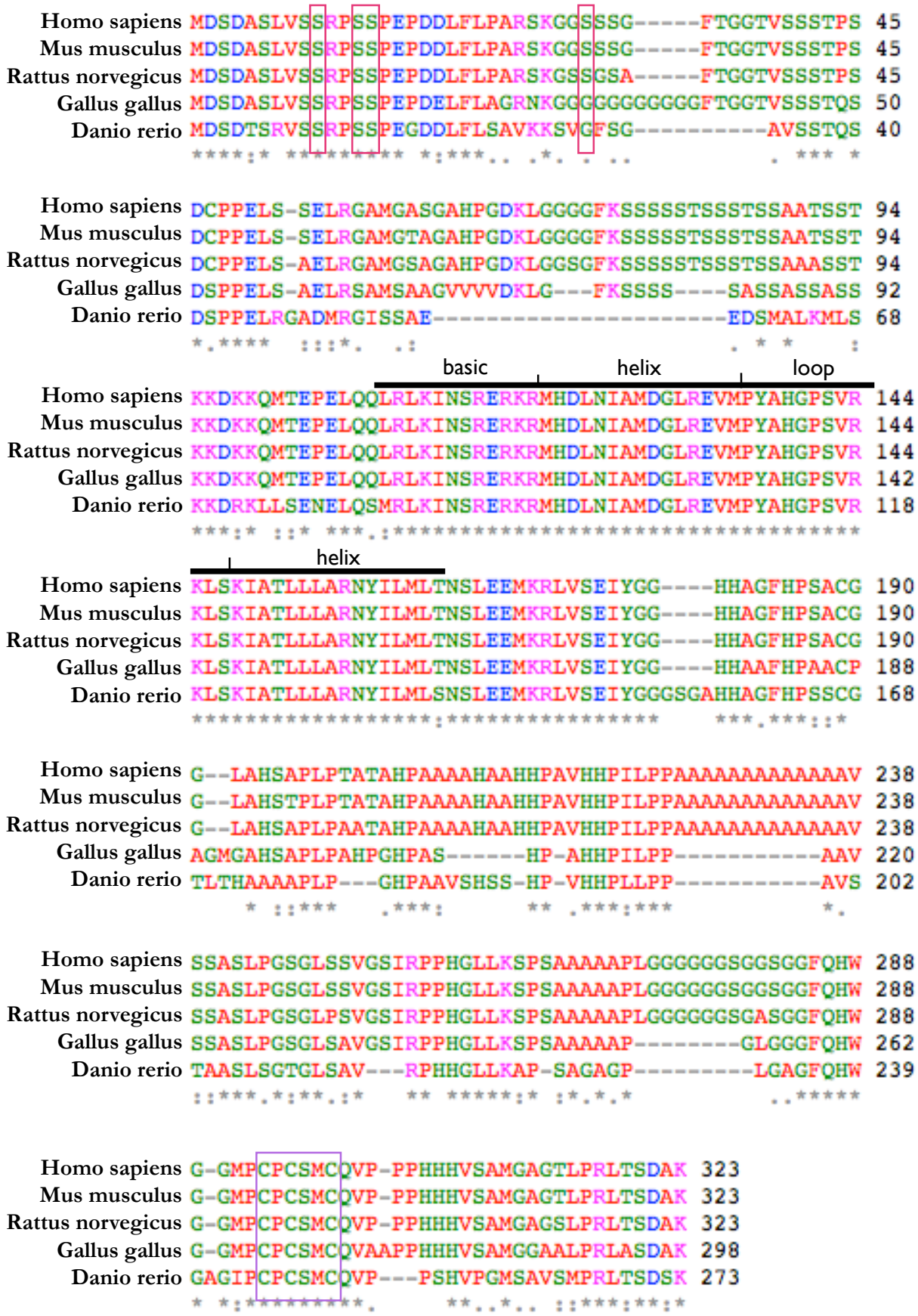


Figure 2. Olig2 structure and DNA binding.
A) Schematic of mouse (mus musculus) and chicken (gallus gallus) Olig2 indicating the basic domain (b), which binds DNA, and helix-loop-helix domain (HLH), which is involved in dimerization of bHLH proteins. B) Diagram of a bHLH dimer binding DNA. One bHLH protein is illustrated in teal and the other in light blue. bHLH protiens dimerize primarily through helix 2 of their HLH domains. The flexible loop then allows for helix 1 to achieve a conformation such that the basic domain, which is contiguous with helix 1, can contact DNA. The basic domains of a bHLH dimer each bind in the major groove and recognize a single E-box (CANNTG), represented by the light grey base pairs. There are no known structural domains of the N- and C-termini of Olig2.

Post-translational modifications are often a means for modulating the activity of a protein. This allows a given protein to behave differently at different times, in different sub-cellular compartments, or even in response to changes in signals. Because of the potent capacity of post-translational modifications to enable a single protein to accomplish disparate tasks, understanding the post-translational modifications of Olig2 is essential for understanding its diverse activities in different cell types. To date, phosphorylation is the only known post-translational modification of Olig2 and multiple sites have been found (Samanta and Kessler 2004; Li et al. 2011; Mehta et al. 2011; Sun et al. 2011). A triple phosphorylation motif has been shown to be important for the proliferative activity of Olig2 (Mehta et al. 2011; Sun et al. 2011). This N-terminal motif, which consists of S10, S13, S14, is phosphorylated in mitotic progenitors and declines as cells differentiate (Sun et al. 2011). Furthermore, phosphorylation of this motif is found in human gliomas (Sun et al. 2011). Phosphorylation of Olig2 at these residues plays a role in the antagonist relationship between Olig2 and p53, which is central to tumor formation (Mehta et al. 2011). Lastly, S147 has previously been identified as a phosphorylated site (Li et al. 2011). Li et al. propose that phosphorylation at S147 triggers the neuronal-glial switch during spinal cord development (Li et al. 2011). In our investigation of Olig2 phosphorylation, we also identified S147. A more thorough discussion of the similarities and differences between my findings and what has been previously reported can be found in Chapter 2.

Figure 3. Sequence alignment of Olig2.
Amino acid sequence of Olig2 from human to zebrafish created using the ClustalW2 multiple alignment tool. Known structural features (black), phosphorylated residues (red boxes), and a CXCXXC motif (purple box) have been overlaid on this alignment. Amino acid colors: red—small, hydrophobic, blue—acidic, magenta—basic, green—sulfhydryl, hydroxyl, amine, G. Star below a residue indicates that this amino acid is conserved across all sequences examined. Colon indicates that both size and hydropathy of the amino acid is conserved whereas a period indicates that either size or hydropathy is conserved.


basic
helix
loop
helix

Olig2 in Brain Development

As noted earlier, Olig2 is not just expressed in the developing spinal cord but in the brain, as well. Much like in spinal cord, Olig2 is initially expressed in the ventricular zone in the developing forebrain, mainly in the medial and lateral ganglionic eminencies (MGE, LGE) and anterior entopeduncular region (AEP)/anterior pre-optic area (POa), and is later found in white matter areas, such as the corpus callosum (Takebayashi et al. 2000; Lu et al. 2000, 2002). Mouse model studies reveal that Olig2 specifies oligodendrocytes in the forebrain through its activity as a repressor (Zhou and Anderson 2002; Lu et al. 2002; Takebayashi et al. 2002a; Marshall et al. 2005). In contrast to spinal cord development, where all oligodendrocytes arise from Olig2+ cells, in the brain, Olig1 is capable of specifying certain populations of oligodendrocytes in the ventral forebrain and hindbrain in the absence of Olig2 (Lu et al. 2002). Additionally, Olig2 is involved in a binary cell fate decision between interneuron and glial cells in the ventral forebrain (Miyoshi et al. 2007; Petryniak et al. 2007). These cells initially express the transcription factor Nkx2.1 and subsequently either begin to express Olig2 or Dlx1/2. These Olig2+ cells predominately give rise to OPCs whereas Dlx1/2+ cells become GABAergic interneurons, which tangentially migrate into the cortex. Although Dlx1/2 cells appear unaffected by the loss of Olig2, there is a significant increase in Olig2+ cells and OPCs in Dlx1/2 knockout mice (Miyoshi et al. 2007). Olig2 has also been found to play a role in astrocyte generation in the cortex (Marshall et al. 2005; Cai et al. 2007). Interestingly, Olig2 is involved in the production of basal forebrain cholinergic neurons, as well (Furusho et al. 2006). In Olig2 knockout animals, there is a significant reduction (about 40%) in cholinergic cells in the basal forebrain (Furusho et al. 2006). Lineage tracing experiments reveal that roughly 50% of these cells come from Olig2+ progenitors. Together, these results suggest that Olig2 plays similar roles throughout the developing CNS in generating both cholinergic neurons and oligodendrocytes.

The Other Oligs

In addition to Olig2, there are two other members of the Olig branch of bHLH transcription factors: Olig1 and Olig3. Olig3 is expressed in the p0 domain and in more dorsal progenitor domains and plays a role in dorsal interneuron specification, similar to the role that Olig2 plays in motor neuron cell fate (Takebayashi et al. 2002b; Müller et al. 2005; Filippi et al. 2005; Ding et al. 2005). Since Olig3 expression does not overlap with Olig2 and there appears to be no interplay between these proteins, Olig3 will not be discussed further. Olig1, however, is of consequence to the study of Olig2. The Olig1 and Olig2 genes lie adjacent to each other in all genomes assessed thus far and are thought to arise from a gene duplication event (Meijer et al. 2012). Consistent with the idea that Olig1/2 arose from gene duplication, there is high sequence homology between these two proteins (Meijer et al. 2012). Initially, Olig2 was believed to be the more ancestral gene because homologues had been found in species from zebrafish to human, whereas Olig1 had only been identified in mammals (Novitch et al. 2001; Rowitch et al. 2002). Olig1 homologues have since been found in non-mammalian vertebrates, including zebrafish, although no chicken Olig1 homologue has been found to date (Li et al. 2008). Furthermore, in silico analysis of Olig genes, as well as the entire bHLH family, indicates that Olig1 emerged earlier (Amoutzias et al. 2004, Bronchain et al. 2007). Regardless of which gene emerged first, duplicated genes often have redundant or overlapping functions, or more interestingly, acquire new characteristics (Wagner 1994, 2001; Brookfield 2003; Lynch and Katju 2004); thus, understanding the role of Olig1 in spinal cord development could elucidate interesting aspects of CNS cell fate specification.

In the neural tube, the closely related transcription factor Olig1 is initially co-expressed with Olig2 but disappears following the establishment of the pMN (Zhou et al. 2000; Mizuguchi et al. 2001; Wu et al. 2006). However, the role of this early Olig1 expression is unclear. Olig1 is not required for ventral patterning, as evidenced by normal patterning and pMN formation in both

Olig1 knockout and misexpression mice (Sun et al. 2001; Lu et al. 2002). Additionally, although Olig1 expression aberrantly persists in the presumptive pMN domain in Olig2 knockout mice, Irx3, which is expressed in the dorsally adjacent p2 domain, is no longer repressed and now abuts the Nkx2.2+ p3 domain (Lu et al. 2002). Taken together, these data indicate that, despite its early expression, Olig1 is not required for ventral patterning or capable of functioning redundantly with Olig2 in Irx3 repression.

One explanation for this early expression is that Olig1/2+ progenitors are reserved for oligodendrocyte generation later in development. However, this does not appear to be the case. Lineage tracing experiments with ROSA26/Olig1-cre mice reveal that cells that once expressed Olig1 become motor neurons as well as oligodendrocytes (Lu et al. 2002). Similarly, in Olig1-DTA mice, where Olig1 expressing cells are selectively ablated with diphtheria toxin, many motor neurons are lost suggesting that a portion of Olig1+ cells give rise to motor neurons (Wu et al. 2006). Despite early expression in motor neuron progenitors, however, Olig1 is dispensable for motor neuron generation. In Olig1 knockout mice, normal numbers of motor neurons are produced (Lu et al. 2002). Furthermore, Olig1 does not compensate for the loss of Olig2 in motor neuron production. Although Olig1 expression is maintained in pMN domain of Olig2-/- mice, motor neurons are not produced (Lu et al. 2002). Given that Olig1 does not seem to play a role in motor neuron production, it follows that Olig1 expression is extinguished prior to motor neuron generation in the pMN of wild-type embryos. After motor neuron generation is complete, Olig1 is once again expressed in Olig2+ progenitors in the ventral VZ (Mizuguchi et al. 2001; Lu et al. 2002). This expression coincides with the onset of oligodendrogenesis.

Similar to motor neuron specification, Olig1 is not required for OPC specification (Lu et al. 2002). In Olig2 knockout embryos, there are no oligodendrocyte markers at any stage of development, whereas, in the Olig1 null, Olig2 expression in OPCs appears to be unaffected, as are

early OPC markers, such as PDGFRa (Lu et al. 2002). Furthermore, just as Olig1 does not function redundantly to Olig2 in motor neuron specification, Olig1 also does not appear to function redundantly in OPC specification either. Electroporation of Olig2 in mesoderm tissue results in ectopic Sox10 expression (Zhou et al. 2000). With Olig1 misexpression, no ectopic Sox10 is observed (Zhou et al. 2000). However, Olig1 does play an important role in the oligodendrocyte lineage. In the absence of Olig1, there is a delay in the expression of more mature oligodendrocyte markers, such as Mbp and Plp, which suggests that Olig1 functions in oligodendrocyte maturation rather than specification (Lu et al. 2002). Like Olig2, Olig1 expression persists in mature oligodendrocytes (Lu et al. 2000; Zhou et al. 2000; Xin et al. 2005; Niu et al. 2012). However, Olig1 is localized in the cytoplasm following oligodendrocyte differentiation, and nuclear export of Olig1 has been shown to be a key step in the differentiation of oligodendrocytes (Niu et al. 2012). Although Olig2 appears to compensate for the loss of Olig1 in terms of oligodendrocyte differentiation, because there is only a delay in mature oligodendrocyte markers, Olig1 is indispensable for myelinogenesis (Xin et al. 2005). Post-natal analysis of Olig1 null mice reveals that, although oligodendrocytes contact axons, myelin is never produced (Xin et al. 2005). These axons eventually degenerate leading to global gliosis and death (Xin et al. 2005).

Despite the similarities between Olig1 and Olig2 in both sequence and temporal expression, it appears that these two proteins have highly divergent roles in development.

Olig2 in Injury and Disease States

Considering that Olig2 function is essential for motor neuron and oligodendrocyte specification, it is not surprising that Olig2 has been implicated in a variety of neurological disease states. The best studied of these will be discussed here.

There is considerable evidence that Olig2 is highly expressed in gliomas, which are tumors of

glial origin (Marie et al. 2001; Lu et al. 2001; Hoang-Xuan et al. 2002; Riemenschneider et al. 2004; Yokoo et al. 2004; Ligon et al. 2004, 2007; Tabu et al. 2007; Preusser et al. 2007). For this reason, Olig2 has often been used as a diagnostic marker (Marie et al. 2001; Yokoo et al. 2004; Preusser et al. 2007). However, Olig2 is not just a marker of gliomas but plays an active role in tumorgenesis (Ligon et al. 2007; Mehta et al. 2011).

Olig2 is also involved in the formation of reactive astrocytes in brain and spinal cord injury. Following CNS injury, glia invade the injured tissue and become reactive, forming a scar. Olig2 is not only expressed in these cells (Buffo et al. 2005; Cassiani-Ingoni et al. 2006; Magnus et al. 2007) but highly unregulated in response to injury (Chen et al. 2008). As reactive astrocytes originate from mature astrocytes, which down-regulate Olig2 prior to differentiation, re-expression of Olig2 is thought to play a role in reactive gliosis (Chen et al. 2008). Consistent with this hypothesis, selectively ablating Olig2 in astrocytes decreases proliferation of reactive astrocytes and formation of a glial scar (Chen et al. 2008). Notably, Olig2 is localized in the cytoplasm in reactive astrocytes (Cassiani-Ingoni et al. 2006; Magnus et al. 2007).

Both Olig2 and the related gene Olig1 are triplicated in Down Syndrome. Although roughly 300 genes are consistently triplicated in Down Syndrome, Olig1/2 are thought to be causative in the cognitive deficits seen in Down Syndrome (Chakrabarti et al. 2010). Because Olig2 is essential for generating a variety of cell types in both the brain and spinal cord, triplication of Olig2 in Down Syndrome could result in multiple phenotypes. In particular, I was interested whether Olig2 triplication is involved in the motor deficits seen in Down Syndrome since Olig2 is required for motor neuron production. A more in depth introduction to this question, as well as preliminary findings of this study, are presented in Appendix A.

Rationale

Although the roles of Olig2 are well known, how these disparate functions are accomplished by a single transcription factor is not well understood. Understanding how Olig2 is able to mediate these temporally restricted roles in spinal cord development is the focus of this work. I explore both dimerization (in Chapter 1) and phosphorylation (in Chapter 2) as potential mechanisms by which the activity of Olig2 is altered during development.

Chapter 1

Olig2 Dimerization

Partner Choice Determines Target Gene Binding and Cell Fate

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Running title: Olig2 Dimer Roles in Spinal Cord Development

ABSTRACT

In the developing spinal cord, Olig2 is responsible for patterning the motor neuron progenitor domain and is essential for both motor neuron and oligodendrocyte specification, yet how Olig2 is able to switch between these roles is largely unknown. Here we investigate dimerization as a possible mechanism to alter the behavior of Olig2 across development. Our data show that the Olig2 homodimer is required to generate motor neurons whereas both Olig2-Olig2 and Olig2-Olig1 can generate oligodendrocyte precursors. Furthermore, our data show that different Olig2 dimers are recruited to distinct genomic loci involved in cell fate specification and differentiation, providing a mechanistic basis underlying divergent roles of Olig2 during development.

INTRODUCTION

Throughout development, a variety of molecules, including transcription factors, are reused at different times and places to accomplish different tasks. A major question in developmental biology, with important implications for our ability to address disease states, is how these molecules are able switch between roles in development. The transcription factor Olig2 plays many key, and often paradoxical, roles in proliferation of progenitors and cell fate specification of both neurons and glia. In the developing spinal cord, Olig2 is responsible for patterning the motor neuron progenitor (pMN) domain and is essential for both motor neuron (MN) and oligodendrocyte precursor (OPC) specification (Novitch et al. 2001; Sun et al. 2001; Mizuguchi et al. 2001; Zhou et al. 2001; Fu et al. 2002; Zhou and Anderson 2002; Lu et al. 2002; Takebayashi et al. 2002a; Sun et al. 2003). Although the diverse roles that Olig2 plays in the developing CNS have been known for some time, how Olig2 switches between these roles during development is still poorly understood.

Olig2 belongs to the basic helix loop helix (bHLH) family of transcription factors, which require dimerization to bind DNA. Thus, choice of dimerization partner is one mechanism by which

this single transcription factor could accomplish different tasks. As is the case with other bHLHs, each dimer could potentially possess distinct properties, such as target gene binding, that enable it to perform different roles across development. For example, Twist homodimers are required for mesoderm development, whereas Twist heterodimers with Daughterless, a drosophila E-protein homologue, repress myogenesis by sequestering Daughterless from forming an activating dimer with MyoD (Castanon et al. 2001). For MyoD, another bHLH, the biological function of homo- and heterodimer forms is opposite that of Twist: MyoD homodimers are transcriptionally inactive while heterodimers with E47 are potent activators of muscle specifying genes (Lassar et al. 1991; Neuhold and Wold 1993; Murre et al. 1989).

Previous work has demonstrated that Olig2, like Twist and MyoD, heterodimerizes with E-proteins E12 and E47, as well as cell-type specific bHLHs Olig1 and Ngn2 (Samanta and Kessler 2004; Lee et al. 2005). Additionally, Olig2 displays a strong homodimerization preference (Lee et al. 2005). Recently, Olig2 phosphorylation has been shown to affect choice of dimerization partner (Li et al. 2011). However, the functional ramifications of this alteration are unclear. Whether these Olig2 dimers have distinct biological roles in spinal cord development, remains an unanswered question.

Here we show that different Olig2 dimers drive the generation Olig2-directed cell types. Our data reveals that the Olig2 homodimer alone directs MN generation in chick spinal cord. In contrast, both Olig2-Olig2 and Olig2-Olig1 are capable generating OPCs. Interestingly, while both the Olig2 homodimer and Olig2-Olig1 heterodimer bind early oligodendrocyte (OL) target genes, only Olig2-Olig1 is specifically recruited to genes that are known to be highly upregulated during OL maturation. These data suggest that Olig2-Olig1 may play a unique role in OL maturation. Additionally, we have identified a novel secondary dimerization domain within the C-terminus of Olig2 that mediates the homodimerization preference of Olig2.

RESULTS AND DISCUSSION

Olig2 dimers have distinct functions *in vivo*

To assess the biological activity of Olig2 dimers in the developing spinal cord, we created constitutively dimerized constructs for Olig2-Olig2, Olig2-Olig1, Olig2-Ngn2, and Olig2-E47. These forced dimer constructs were made by fusing an epitope tagged coding sequence for Olig2 to a dimerization partner via a sequence that encodes for a flexible polypeptide linker (Fig 1a). The length and sequence of the polypeptide linker has been shown to allow for normal dimerization between linked proteins in an exclusive and constitutive manner (Neuhold and Wold 1993). Using *in ovo* electroporation, we expressed these Olig2 dimers in chick neural tube at HH10-12, then confirmed expression of each Olig2 dimer and examined their ability to generate ectopic MNs and OPCs at 3 days post-electroporation (dpe) and 5dpe, respectively, by immunohistochemistry.

We first tested whether Olig2-Olig2 replicates the activity of Olig2 alone in chick neural tube. Olig2 misexpression alone results in dorsal, ectopic expansion of both MNs and V2a interneurons (INs) by 3dpe and premature OPC generation by 5dpe (Fig S1a,d; (Novitch et al. 2001; Zhou et al. 2001). Similar to expression of Olig2 alone, Olig2-Olig2 generated both ectopic MNs, marked by Hb9 (Fig 1b,f), and premature OPCs, marked by Sox10 (Fig 2a,e), as well as ectopic Chx10+ V2a INs (Fig 1b,g). These results indicate that the Olig2 homodimer is capable of driving MN and OPC fates in the developing spinal cord. Considering the homodimerization preference of Olig2 (Lee et al. 2005), it follows that the Olig2 homodimer most closely resembles what is seen with Olig2 misexpression alone.

In contrast to Olig2 misexpression, either alone or as a homodimer, misexpression of Olig1 alone did not produce either ectopic MNs or V2a INs (Fig S1b). Olig1 alone also failed to trigger formation of Sox10+ OPCs (Fig S1e; Zhou et al. 2000). These results prompted us to test whether the Olig2-Olig1 heterodimer contributes to the fate specification of any Olig2-directed cell types.

Interestingly, Olig2-Olig1 generated ectopic OPCs (Fig 2b,e) but not MNs (Fig 1c,f). Additionally, Olig2-Olig1 misexpression results in ectopic V2a IN production (Fig1c,g). Our results indicate that Olig1 is able to promote OPC fate by forming a heterodimer with Olig2 and highlight the differential biological activity between Olig2-Olig2 and Olig2-Olig1. Our findings also provide mechanistic basis underlying the observation in loss-of-function studies that Olig1 contributes to OL development but it is insufficient for OL specification in the absence of Olig2 function in developing spinal cord (Lu et al. 2002).

Neither Olig2-Ngn2 nor Olig2-E47 generated ectopic MNs (Fig 1d-f) or OPCs (Fig 2c-e). Electroporation with either Ngn2 or E47 alone also did not result in ectopic MNs (Fig S1c; Mizuguchi et al. 2001). Although we cannot exclude the possibility that Olig2-Ngn2 or Olig2-E47 are not biologically relevant Olig2 dimers, these results suggest a role for these dimers that is independent from transcriptional activity. The role of these dimers may be twofold. First, when Olig2 levels are high, the interaction of Olig2 with Ngn2 and E47 may serve to decrease Ngn2-E47 dimer formation thereby preventing Ngn2-E47 from binding and activating post-mitotic MN genes, as previous work has suggested (Lee et al. 2005). Second, as cells transition from a progenitor to post-mitotic state, Olig2-Ngn2 and Olig2-E47 may act to sequester Olig2 away from homodimerization. Olig2 must be down regulated in order to allow for differentiation of MNs. Since both Ngn2 and E47 are highly expressed in the intermediate zone of the neural tube (Lee and Pfaff 2003; Holmberg et al. 2008), increased concentrations of these bHLHs during the transition from pMN progenitors to MNs may increase heterodimer formation and, concomitantly, decrease Olig2 homodimers, thereby facilitating in the down regulation of Olig2.

Together, our data uncover distinct biological activities of the Olig2 dimers in driving MN and OPC fates. Among these dimers, the Olig2 homodimer is uniquely capable of generating MNs during spinal cord development. Our misexpression data also indicate that Olig2-Olig1 is capable of

functioning redundantly to Olig2-Olig2 in OPC specification.

The carboxyl-terminus of Olig2 contains a novel secondary dimerization domain that mediates homodimerization

Our results, which indicate an essential biological role of the Olig2 homodimer in MN and OPC formation, coupled with the strong homodimerization affinity of Olig2 (Lee et al. 2005), raised the question of whether Olig2 contains any unique features which impart this preference. To investigate what mediates the homodimerization preference of Olig2 over heterodimerization interactions, we performed yeast two hybrid assays with several Olig2 fragments and known dimerization partners. Full-length Olig2 interacted strongly with Olig proteins and to a lesser degree with Ngn2 and E47 (Fig 3b,c; S2a,b).

Since bHLH proteins have been shown to interact primarily through the second helix of their HLH domains (Ellenberger et al. 1994; Ma et al. 1994),we predicted that Olig2 fragments containing only the canonical bHLH domain (109-163aa) would be sufficient to bind dimerization partners with high affinity. Interestingly, however, an additional 24 amino acids just carboxy-terminal to the bHLH domain (164-187aa) are needed for strong interaction between Olig2 and its dimerization partners (Fig S2c). For this reason, we chose to include this sequence, which we have termed the 'X domain,' in Olig2 bHLH fragments and exclude this sequence from C-terminus constructs in subsequent experiments (Fig 3a). Notably, a sequence just C-terminal to the bHLH domain has been reported to enhance dimerization of E-proteins, including E47 (Goldfarb et al. 1998).

Unexpectedly, in yeast two hybrid assays, the carboxyl-terminus of Olig2 (187-323aa) alone was capable of interacting with Olig2 but not with any other dimerization partners (Fig 3b,c; S2a,b). These results suggest that the interaction between Olig2 via the C-terminal domain may contribute to the homodimerization preference of Olig2. To test whether this C-terminal interaction occurs in

mammalian cells, we performed GST pull-down assays from HEK293T cells co-transfected with Flag-tagged Olig2 dimerization partners and GST-Olig2 full-length or C-terminus constructs (Fig 3a,d; S3). As in the yeast two hybrid assays, in mammalian cells, the C-terminus of Olig2 interacted with Olig2 but not with other bHLH proteins (Fig 3d). Furthermore, we found that the Olig2 C-terminus is capable of self-interaction (Fig 3d). These results were also confirmed by co-immunoprecipitation assays using both *in vitro* translated proteins and whole cell lysates (data not shown). Our results uncover that the C-terminus of Olig2 contains a novel secondary dimerization domain that specifically mediates homodimerization, but not heterodimer formation, likely contributing to the strong homodimerization property of Olig2.

In order to further assess the role of the C-terminus secondary dimerization domain in Olig2 function, we created an Olig2Nt-Olig1Ct chimera, in which the C-terminus of Olig2 was replaced with that of Olig1 (Fig 3a). We reasoned that, if the C-terminus of Olig2 is responsible for the strong homodimerization characteristic of Olig2, then swapping this domain for one of a similar protein, Olig1, would decrease the homodimerization preference of Olig2. This may, in turn, compromise the ability of Olig2 to generate MNs given that this is a unique property of the Olig2 homodimer. To test this possibility, we misexpressed Olig2Nt-Olig1Ct in chick neural tube using *in ovo* electroporation and examined MN generation at 3dpe. Unlike Olig2, Olig2Nt-Olig1Ct does not generate ectopic Hb9+ MNs (Fig 3e,f). This data suggests that the intact C-terminus of Olig2 is necessary for inducing MN fate, likely by enhancing the homodimerization preference of Olig2.

Together, our data clearly illustrates that the Olig2 C-terminus contains a secondary dimerization domain that confers homodimerization preference. Although Olig2 belongs to a group of bHLH proteins that are not known to have any secondary interaction domains, other classes of bHLH transcription factors have additional dimerization domain C-terminal to the HLH domain, such as PAS, Orange, and leucine zipper domains, that regulate their activity (Vervoort and Ledent

2001). It is interesting to note that these additional dimerization domains of other bHLH transcription factors render specificity to their interactions (Amoutzias et al. 2004), much like the C-terminal domain of Olig2 that we have identified. Further studies are needed to identify the key residues involved in this interaction.

Olig2 homo- versus heterodimers preferentially bind distinct genomic loci

Our finding that only the Olig2 homodimer is capable of directing MN fate but both Olig2-Olig2 and Olig2-Olig1 promote OPC fate (Fig 1,2) prompted us to investigate the molecular mechanisms that underlie the differential activities of two dimers in generating Olig2-directed cell types. We considered the possibility that these dimers are recruited to distinct genomic targets that are important for differing roles in cell fate specification and differentiation, as well as common targets that are involved in their shared function in promoting OPC fate. Based on *in vivo* activity of Olig2-Olig2 versus Olig2-Olig1 in chick spinal cord and the known roles of Olig2 and Olig1, we hypothesized that these dimers are differentially bound to Olig2 targets such that Olig2-Olig2 binds both pMN and OPC targets whereas Olig2-Olig1 binds OPC and OL maturation targets. To investigate this possibility, we performed chromatin immunoprecipitation (ChIP) with each dimer and examined binding to Olig2 targets that may be differentially bound between MNs, OPCs, and maturing OLs using quantitative PCR.

In order to select targets to test, we looked for genes that are known to be highly regulated or specifically expressed in these cellular contexts (Lee et al. 2005; Dugas et al. 2006). Then, we compared the binding pattern of Olig2 to these targets in pMN cells, OPCs, immature OLs (iOLs), and mature OLs (mOLs) using published Olig2 ChIP-seq datasets (Mazzoni et al. 2011; Yu et al. 2013). By integrating ChIP-seq and gene expression data sets, we generated a list of high-confidence, direct target sites of Olig2 that display cell context-specific Olig2 binding (Fig S4). These targets can

be divided into three groups: pMN, early OL, and late OL. The pMN group contains genomic loci where Olig2 is recruited in pMN cells but not in OL lineage cells (Fig S4a). These loci are also associated with genes expressed in MNs but not in OPCs for OLs. For instance, Olig2 binds the Hb9 enhancer, a MN-specific gene, in pMN cells but is not recruited to the same gene locus in OPCs or OLs (Lee et al. 2005; Mazzoni et al. 2011). Both the early and late OL groups contain loci that are occupied by Olig2 in OL lineage but not pMN cells (Fig S4b,c). However, the early OL group displays Olig2-bound ChIP-seq peaks in OPCs whereas the late OL group does not. These targets only have Olig2 ChIP-seq binding peaks in iOL and mOL datasets. For example, Cnp is an Olig2 ChIP-seq target in both OPC and iOL data sets. In contrast, Olig2 binds RhoG, a gene specifically expressed in OL differentiation (Dugas et al. 2006), only in later stages of OL maturation but not in pMN or OPC cellular contexts.

To test whether Olig2-Olig2 and Olig2-Olig1 dimers show any differences in binding to the targets identified as described above, we performed ChIP followed by qPCR with each of these dimers. We expressed Flag-epitope tagged Olig2-Olig2 or Olig2-Olig1 forced dimers in P19 mouse embryonic cells followed by ChIP assays using anti-Flag antibodies. The relative binding occupancy of each Olig2 dimer to our selected Olig2 target loci was then determined using qPCR (Fig S5). Next, we compared the relative binding occupancy of Olig2-Olig2 versus Olig2-Olig1 to evaluate binding preference for these target loci (Fig 4a). Both Olig2-Olig2 and Olig2-Olig1 dimers bind to each Olig2 target compared to an established negative control genomic region that does not recruit Olig2 (Fig S5).

Interestingly, Olig2-Olig2 and Olig2-Olig1 exhibited differential binding affinity for several targets that corresponds with their differing roles in spinal cord development (Fig 4, S5). The Olig2 homodimer shows a strong preference for MN gene regions, such as Hb9, Isl1, and Lhx3 (Fig 4). Consistently, these genes have Olig2 binding peaks in pMN, but not OL, lineage cells in the ChIP-

seq data sets (Mazzoni et al. 2011; Yu et al. 2013). Additionally, Olig2-Olig2 preferentially binds early OL group targets Cnp, Plp, and Rap2a (Fig 4). Conversely, Olig2-Olig1 preferentially binds late OL group targets that are known to be highly upregulated during oligodendrocyte maturation (Dugas et al. 2006) and that have Olig2 ChIP-seq peaks only in iOL and mOL datasets. The genes in this category include Rab6, Rac1, RhoG, ApoD, Myrf1 and YY1 (Fig 4). In addition to these differentially bound targets, Olig2-Olig2 and Olig2-Olig1 bind to Chx10, Hes5, and a subset of early OL group targets with little difference in binding preference (Fig 4). These common early OL group targets, Sox10, Cdc42 and Axin, are genes that have Olig2 peaks throughout all stages of oligodendrocyte differentiation from OPC to mOL (Fig S4b). Since both dimers generated ectopic Sox10+ OPCs in chick neural tube (Fig 2a,b,e), it is not surprising that Olig2-Olig2 and Olig2-Olig1 showed similar binding at this locus in ChIP assays. This consistency supports the reliability of our ChIP results to assess functionally relevant differences in gene binding. Another gene in this common target of both dimers is Chx10, a gene expressed in V2a INs. Although the biological importance of Olig2 binding to Chx10 has yet to be determined, it is well correlated with our observation that both these Olig2 dimers generate ectopic Chx10+ cells when misexpressed in chick spinal cord (Fig 1b,c,g). Together, these data indicate that Olig2 homo- and Olig2-Olig1 heterodimers have different binding preferences for the selected target genes, which likely contributes the distinct activities of these dimers.

This study elucidates the roles of Olig2 dimers in spinal cord development and provides mechanistic evidence for how these dimers contribute to Olig2-directed cell fate specification (Fig 5). Our results from both *in vivo* and *in vitro* experiments, indicate a unique and essential role for the Olig2 homodimer in MN specification. We found that the Olig2 homodimer preferentially binds MN fate specifying genes and promotes MN formation. Although Olig2 levels are high early in

neural tube development, Olig1 levels are also initially high (Wu et al. 2006). The C-terminal secondary dimerization domain of Olig2 that mediates homodimer preference may function to promote Olig2 homodimer formation at this time during development. As levels of proneural factors, Ngn2 and E47, begin to rise in pMN cells, Olig2-Ngn2 and Olig2-E47 heterodimer formation may sequester these proteins to prevent premature MN differentiation through the action of the Ngn2-E47 heterodimer (Lee and Pfaff 2003; Lee et al. 2004, 2005). Then, as Olig2 levels decline and Ngn2 levels rise (Lee et al. 2005), Ngn2-E47 dimers may become more prevalent while Olig2 homodimers decrease. Following MN specification, our data indicate that the Olig2 homodimer and heterodimer with Olig1 are capable of functioning redundantly in OPC specification. However, given the strong homodimerization preference of Olig2 and the low level of Olig1 expression at the onset of OPC generation, it is unclear what the relative contribution of this heterodimer is to specification of this cell type. Loss of function studies demonstrate that Olig1 is dispensable for OPC formation but is required for OL maturation and myelinogenesis (Lu et al. 2002; Xin et al. 2005). Our ChIP results, which indicate that Olig2-Olig1 preferentially binds genomic loci that are iOL and mOL-specific Olig2 ChIP-Seq peaks, suggest a role for Olig2-Olig1 in OL maturation. Although Olig2 displays a homodimerization preference *in vitro*, as development proceeds Olig2 expression declines while Olig1 levels concurrently rise (Zhou et al. 2000). Thus, temporal changes in levels of these two proteins may lead to a shift in the predominate dimer present in a maturing OL from Olig2 homodimer to Olig2-Olig1 heterodimer.

FIGURE LEGENDS

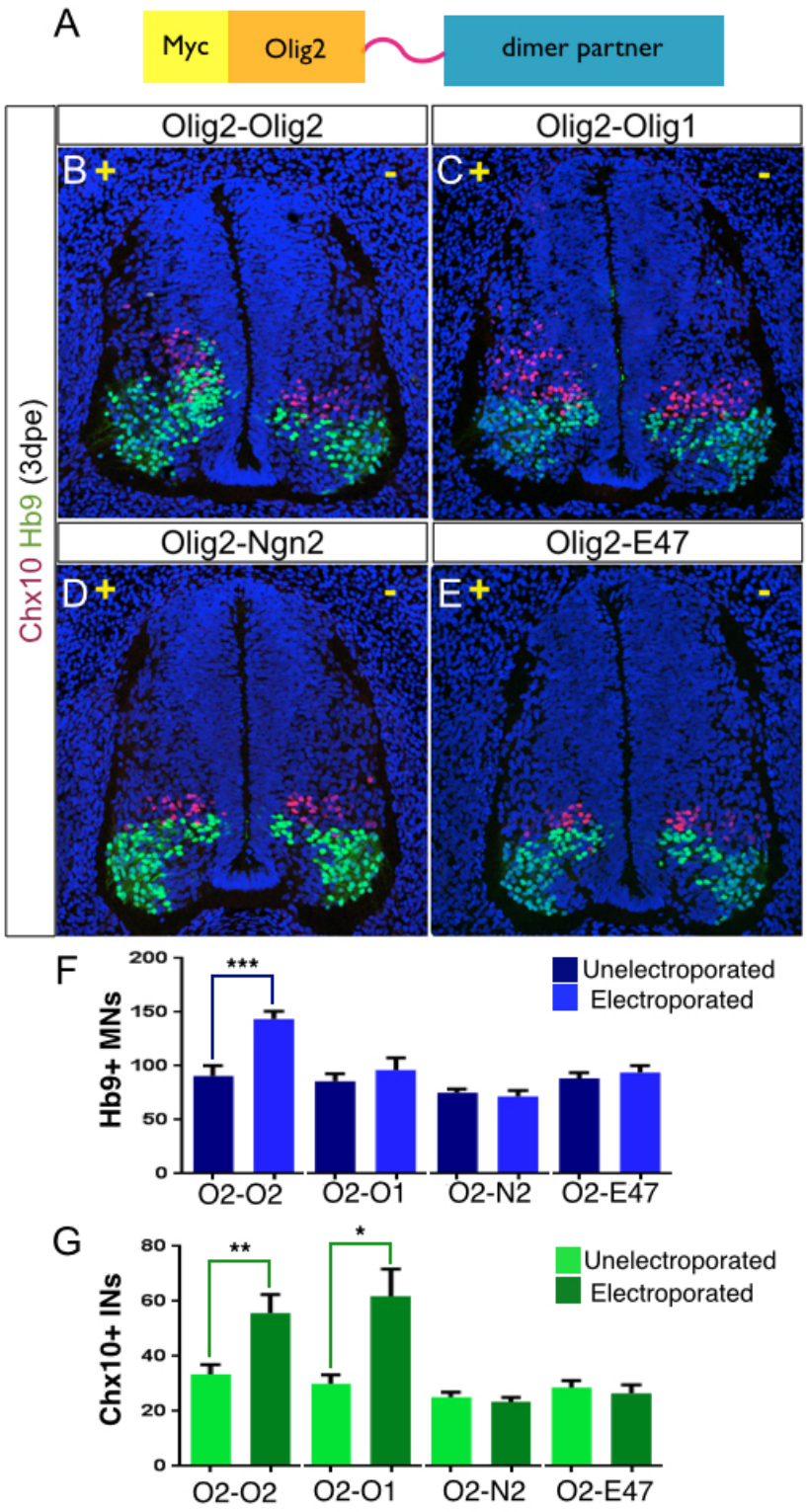


Figure 1. Only the Olig2 Homodimer Produces Ectopic MNs.

(A) Schematic of forced dimer constructs used. (B-E) Effects of Olig2 dimer misexpression in chick embryo neural tube on MNs, marked by Hb9, and V2a INs, marked by Chx10. Images representative of 5-8 embryos per dimer. Plus sign indicates electroporated side of the spinal cord; minus sign is the control side. (F-G) Quantification of MN and V2a IN cells with Olig2 dimer misexpression. Mean ± SEM were calculated by pooling 2-3 sections from at least 5 embryos per dimer. O2=Olig2 O1=Olig1 N2=Ngn2 (F) A significant increase in MNs is seen with Olig2-Olig2 misexpression, *** $p<0.001$ paired Student's t-test. Misexpression with Olig2-Olig1, Olig2-Ngn2, and Olig2-E47 do not result in ectopic MNs. (G) Both Olig2-Olig2 and Olig2-Olig1 result in ectopic Chx10+ cells, * $p<0.05$ and ** $p<0.01$ paired Student's t-test, but not Olig2-Ngn2 or Olig2-E47.

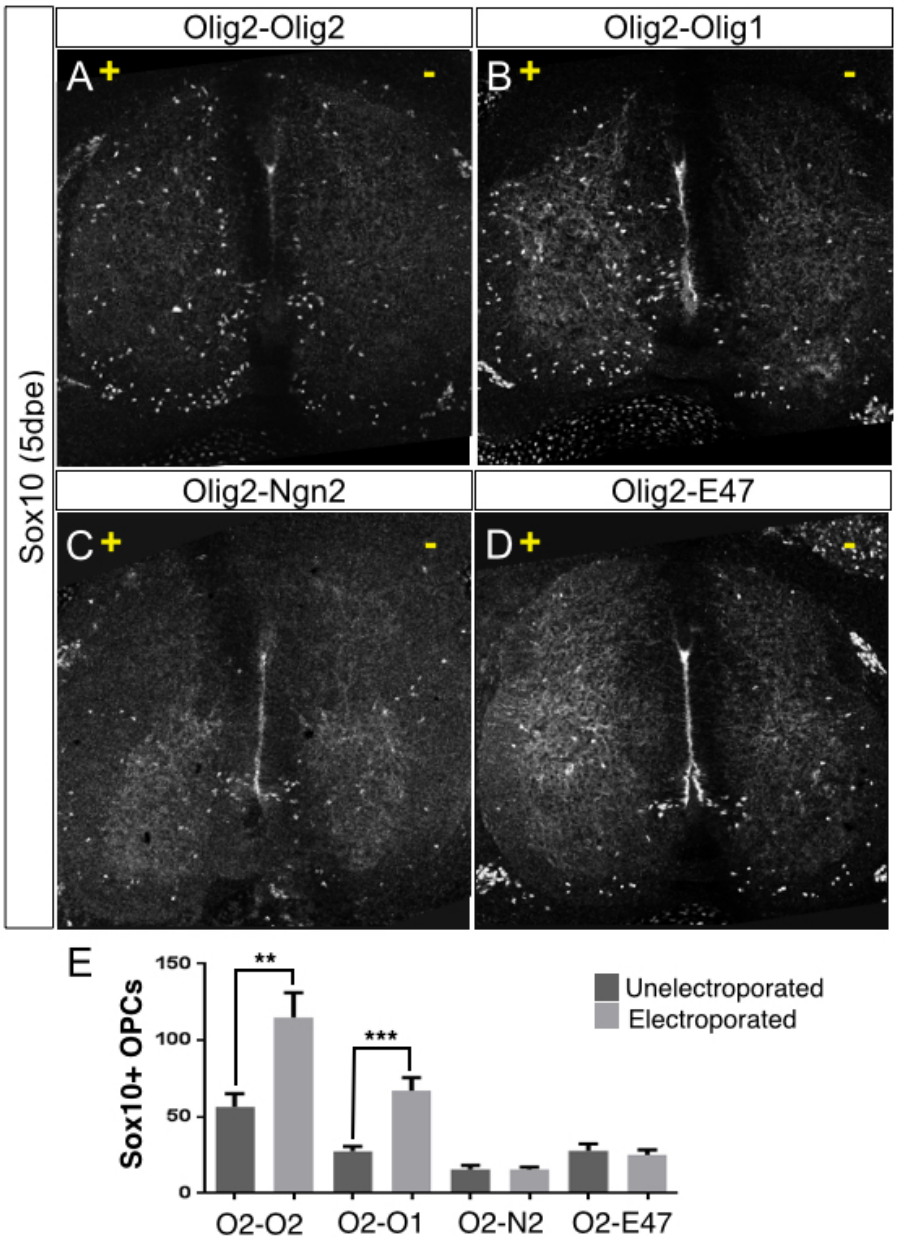

Olig2-Olig2
Olig2-Olig1
Olig2-Ngn2
Olig2-E47
Sox10 (5dpe)
A
B
C
D
+
-
E
Sox10+ OPCs
150
100
50
0
**
***
Unelectroporated
Electroporated
O2-O2
O2-O1
O2-N2
O2-E47

Figure 2. Both Olig2-Olig2 and Olig2-Olig1 Result in Premature OPC Generation.

(A-D) The effect of Olig2 dimer misexpression on Sox10+ OPC generation in 5dpe chick neural tube. (E) Analysis of Sox10+ OPCs. Values calculated from pooling 3 sections from 5 to 9 embryos per dimer, plotted as mean ± SEM. Both Olig2-Olig2 and Olig2-Olig1 misexpression result in increased number of Sox10+ cells, ** $p<0.001$ and *** $p<0.001$ respectively paired Student's t-test. Neither Olig2-Ngn2 or Olig2-E47 produce additional Sox10+ OPCs.

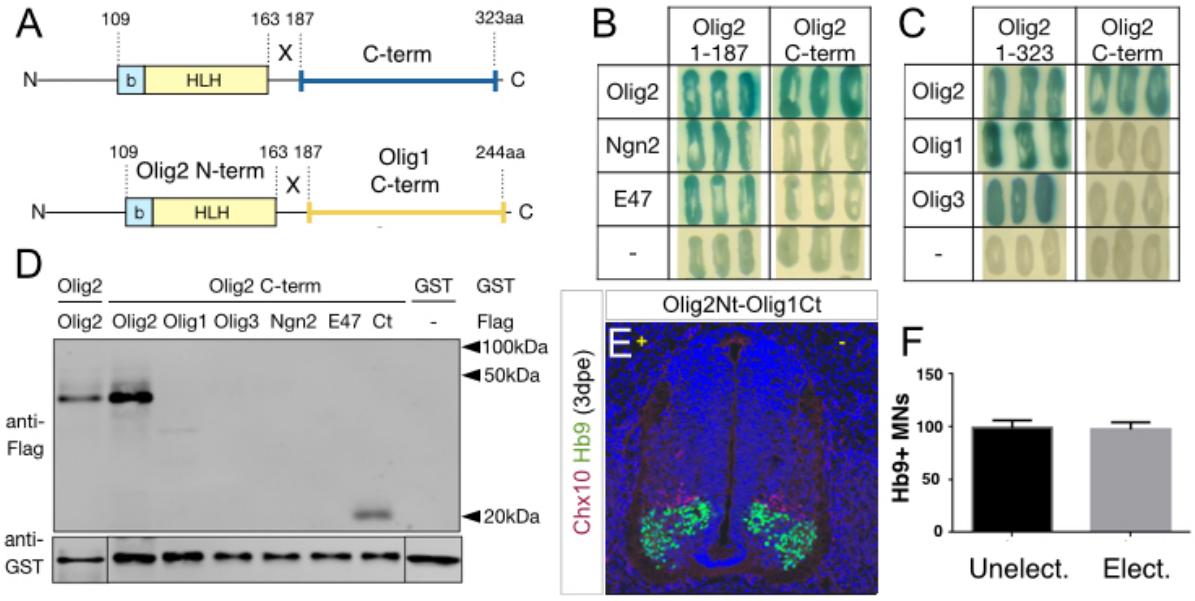

A
109
163 187
323aa
X
C-term
N
b
HLH
C
109
Olig2 N-term
163 187
X
Olig1
C-term
244aa
N
b
HLH
C
B
Olig2
1-187
Olig2
C-term
Olig2
Ngn2
E47
-
C
Olig2
1-323
Olig2
C-term
Olig2
Olig1
Olig3
-
D
Olig2
Olig2 C-term
GST
GST
Olig2 Olig2 Olig1 Olig3 Ngn2 E47 Ct
-
Flag
100kDa
50kDa
20kDa
anti-
Flag
anti-
GST
Olig2Nt-Olig1Ct
E
+
-
Chx10 Hb9 (3dpe)
F
Hb9+ MNs
150
100
50
0
Unelect.
Elect.

Figure 3. Olig2 Contains a Novel Secondary Dimerization Domain

(A) Diagram of Olig2 highlighting the region (187-323aa) used for C-term only constructs and schematic of the Olig2Nt-Olig1Ct chimera in which the C-terminus of Olig2 was replaced with that of Olig1. (B-D) Protein interactions between bHLH factors and Olig2 or Olig2 C-term assessed using both yeast two hybrid and GST pull-down. Olig2 interacts with known dimerization partners Olig2, Ngn2, and E47 (B) as well as Olig1 and Olig3 (C) in yeast two-hybrid assays, whereas the C-term only interacts with Olig2. (D) In GST pull down assays with GST-Olig2 C-terminus and Flag-tagged dimerization partners, Olig2 C-terminus only interacts with Olig2 and other C-term constructs. (E) HB9+ MNs and Chx10+ V2a INs following Olig2Nt-Olig1Ct misexpression. (F) Mean ± SEM was calculated by pooling 2-3 sections from 8 embryos. No statistically significant difference in HB9+ MNs is seen at 3dpe with Olig2Nt-Olig1Ct electroporation (paired Student's t-test).

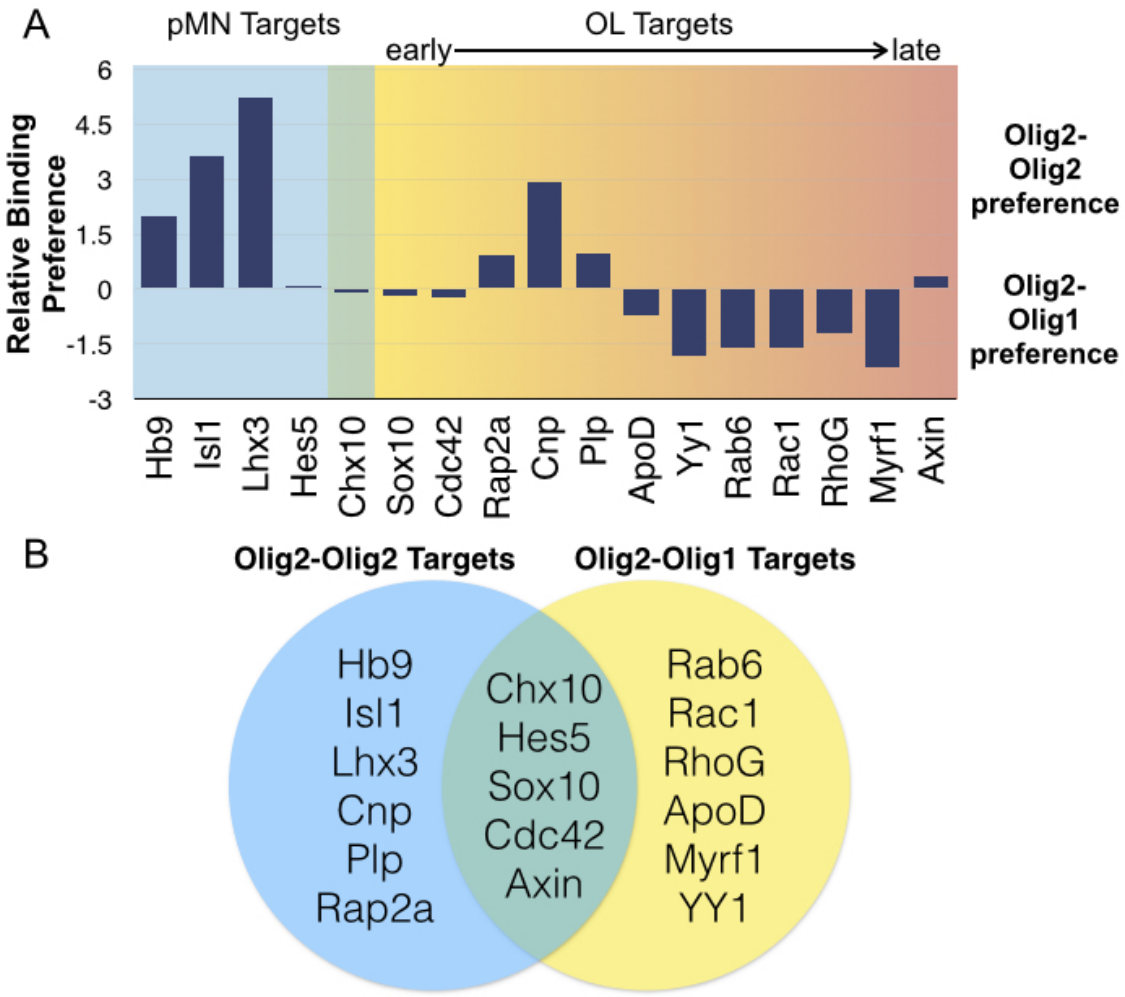

A
pMN Targets
OL Targets
early
late
Relative Binding Preference
6
4.5
3
1.5
0
-1.5
-3
Olig2-Olig2 preference
Olig2-Olig1 preference
Hb9
Isl1
Lhx3
Hes5
Chx10
Sox10
Cdc42
Rap2a
Cnp
Plp
ApoD
Yy1
Rab6
Rac1
RhoG
Myrf1
Axin
B
Olig2-Olig2 Targets
Olig2-Olig1 Targets
Hb9
Isl1
Lhx3
Cnp
Plp
Rap2a
Chx10
Hes5
Sox10
Cdc42
Axin
Rab6
Rac1
RhoG
ApoD
Myrf1
YY1

Figure 4. Olig2-Olig2 and Olig2-Olig1 Are Recruited to Distinct Genomic Loci

ChIP was performed in P19 cells expressing either Olig2-Olig2 or Olig2-Olig1 and binding to target loci was quantified by real-time PCR. (A) Data are expressed as log2 of the ratio of relative binding occupancy (ΔΔCt) between dimers. Positive values indicate Olig2-Olig2 binding preference and negative values indicate a preference for Olig2-Olig1. Values near zero are common targets. (B) Diagram summarizing binding preference.

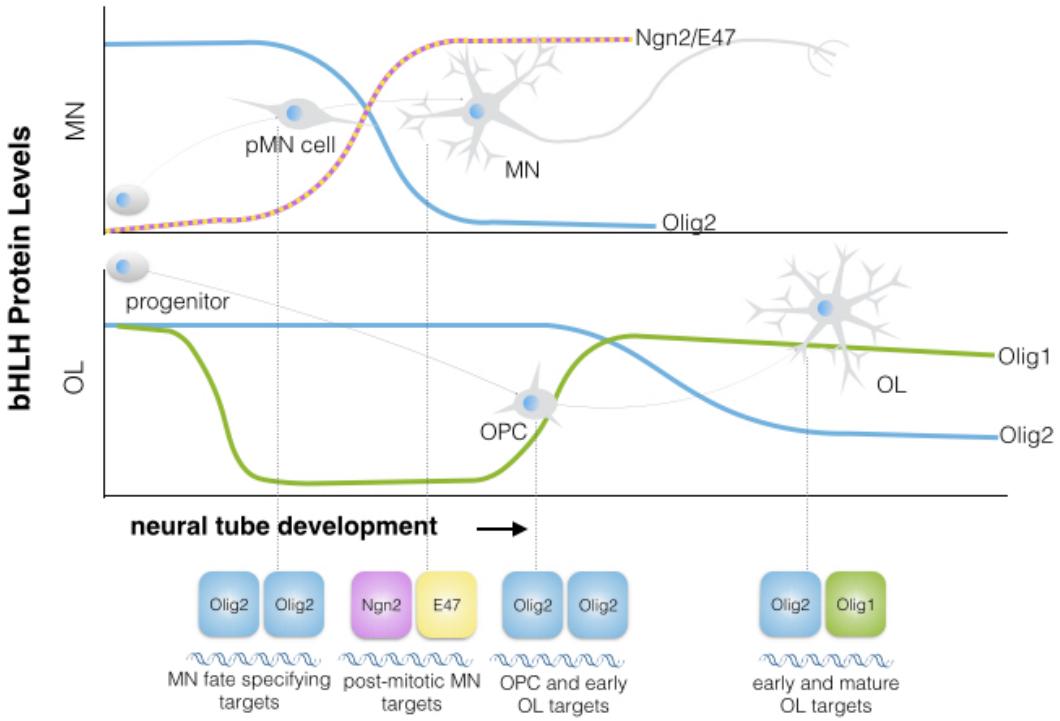

bHLH Protein Levels
MN
pMN cell
Ngn2/E47
MN
Olig2
OL
progenitor
Olig1
OL
OPC
Olig2
neural tube development
Olig2
Olig2
Ngn2
E47
Olig2
Olig2
Olig2
Olig1
MN fate specifying targets
post-mitotic MN targets
OPC and early OL targets
early and mature OL targets

Figure 5. Proposed roles of Olig2 dimers in spinal cord development.

Early in development when Olig2 levels are high, Olig2-Olig2 binds MN fate specifying targets and gives rise to MNs, presumably through derepression of these targets at the appropriate developmental time. Formation of the Olig2-Ngn2 and Olig2-E47 dimers may serve to prevent Ngn2-E47 formation, which could trigger activation of post-mitotic MN genes and premature differentiation. As these progenitors are committed to MN cell fate, Ngn2 levels rise and Olig2 is down-regulated. At this time, Olig2-Ngn2 and Olig2-E47 sequesters Olig2 from homodimerization, allowing Ngn2-E47 to bind DNA. Cells with continued expression of Olig2 are reserved for oligodendrocyte generation. Olig2-Olig2 preferentially binds early OL genes. Then as Olig1 levels increase, a temporal shift in the predominate Olig2 dimer from Olig2-Olig2 to Olig2-Olig1 may occur. Olig2-Olig1 preferentially binds late OL genes and likely drives OL maturation and differentiation.

SUPPLEMENTAL FIGURES

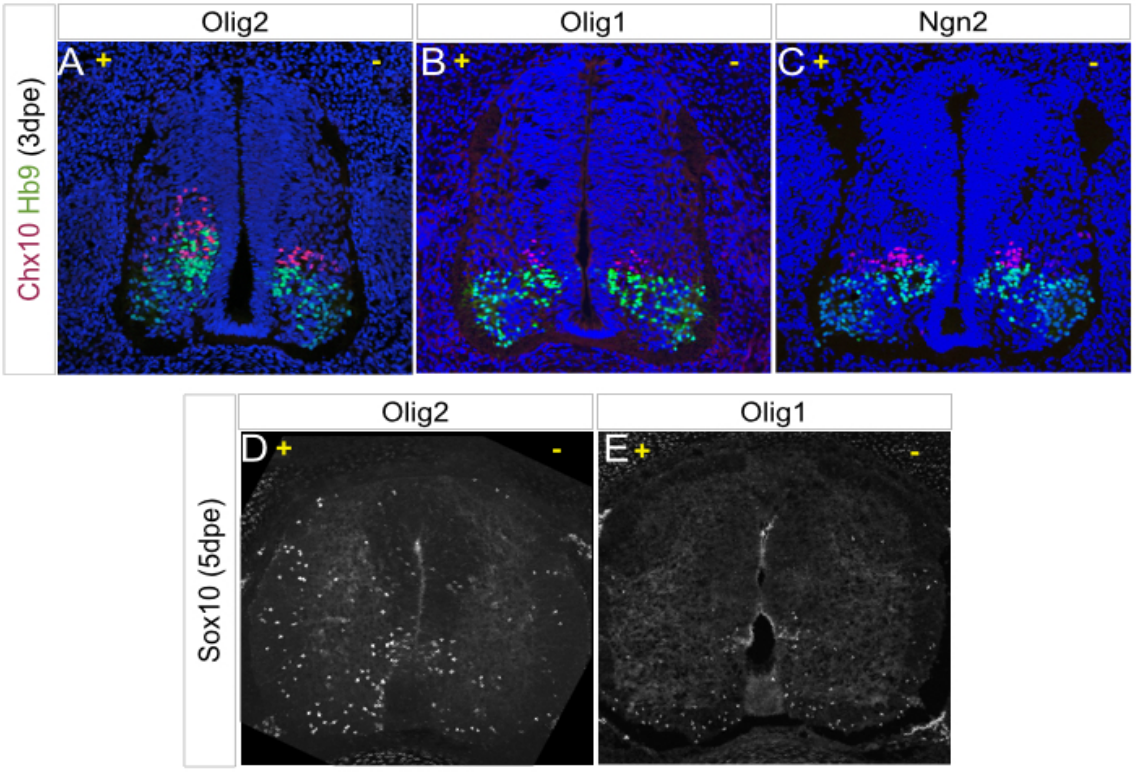
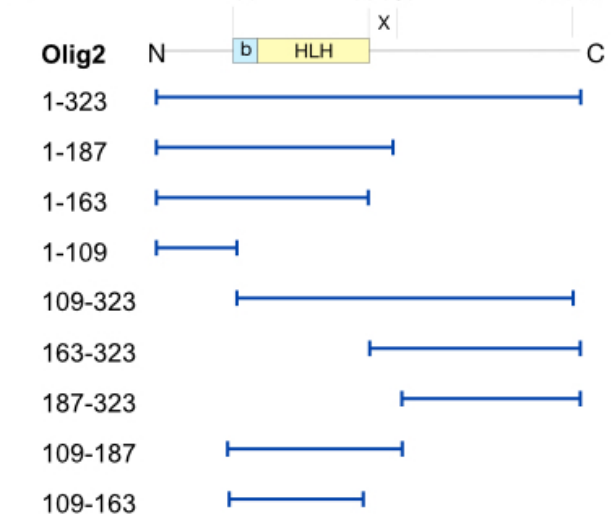


Figure S1. Misexpression of single bHLHs in chick spinal cord

(A-C) Effects of misexpression of individual bHLH constructs at 3dpe. Only Olig2 (A), but not other Olig2 dimerization partners Olig1 (B) and Ngn2 (C), produces ectopic HB9+ MNs and Chx10+ V2a INs. (D-E) Ability to generate ectopic OPCs assessed at 5dpe. Olig2 (D) but not Olig1 (E) produces ectopic Sox10+ OPCs by 5dpe.

A



B

Olig2	Ngn2	E47	Olig1	Olig3
+++	+	+	+++	+++
+++	+	++	NT	NT
+	-	-	NT	NT
-	-	-	NT	NT
+++	+	+	NT	NT
+++	-	-	NT	NT
+++	-	-	-	-
+++	+++	+++	NT	+++
++	-	+	NT	

C

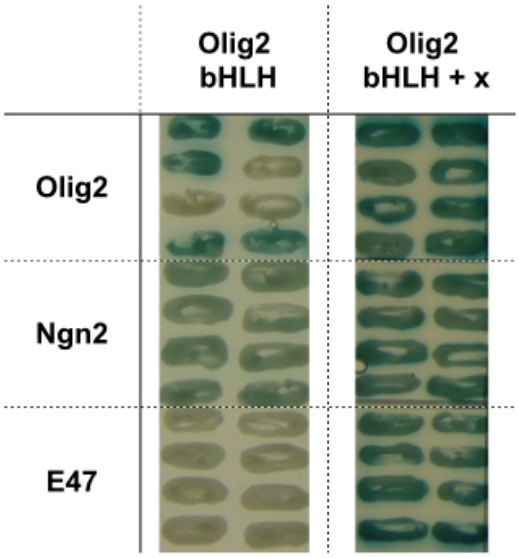


Figure S2. Residues adjacent to the bHLH domain enhance dimerization of Olig2

(A) Constructs used for yeast two hybrid and their relative affinity for each Olig2 binding partner. NT= not tested (B) Yeast two hybrid results illustrate that Olig2 bHLH constructs that include the X region (163-187aa) interact more strongly with dimer partners Olig2, Ngn2 and E47 than the Olig2 bHLH alone.

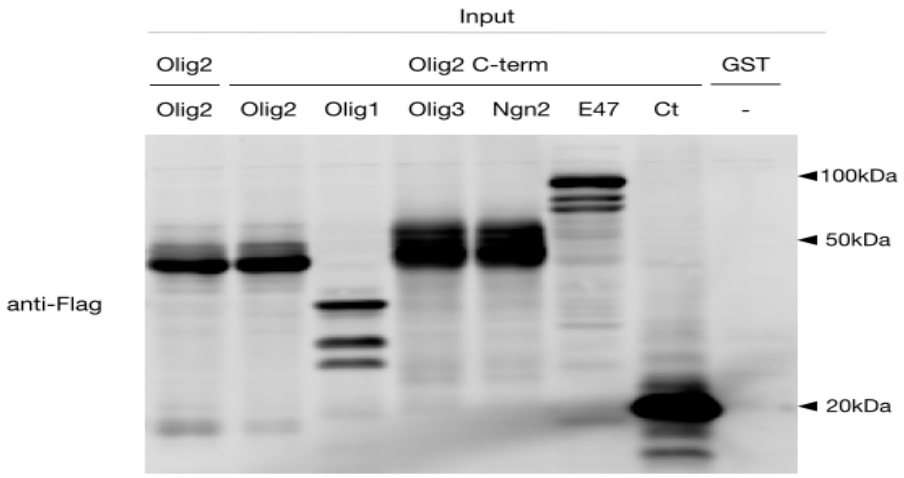

Input
Olig2
Olig2 C-term
GST
Olig2
Olig2
Olig1
Olig3
Ngn2
E47
Ct
-
anti-Flag
100kDa
50kDa
20kDa

Figure S3. Inputs for GST Pull down

Western blot of 5% input with anti-flag antibody showing expression of Olig2 interaction partners.

A

B

C

	Gene	pMN	OPC	iOL	mOL
pMN Group	**Hb9**	O2-O2			
	Isl1	O2-O2			
	Lhx3	O2-O2			
	Hes5	Common			
Early OL Group	**Chx10**		Common	Common	
	Sox10		Common	Common	Common
	Cdc42		Common	Common	Common
	Rap2a		O2-O2	O2-O2	
	Cnp		O2-O2	O2-O2	
	Plp1		O2-O2	O2-O2	
Late OL Group	**ApoD**			O2-O1	
	Yy1			O2-O1	
	Rab6			O2-O1	
	Rac1			O2-O1	O2-O1
	RhoG			O2-O1	
	Myrf			O2-O1	
	Axin				Common

Figure S4. Olig2 Target Loci Groups for Dimer ChIP Assays

(A-C) ChIP target loci group subdivisions are based on the cell context (see column headings) where Olig2-binding in ChIP-seq was observed. These group classifications have been combined with Olig2 dimer binding preference from ChIP (O2-O2=Olig2-Olig2; O2-O1=Olig2-Olig1). (A) pMN group targets display Olig2-binding peaks in pMN but not OL ChIP-seq data sets. (B-C) Early OL group targets (B) are delineated by having Olig2-binding peaks in OPCs whereas late OL group targets (C) do not.

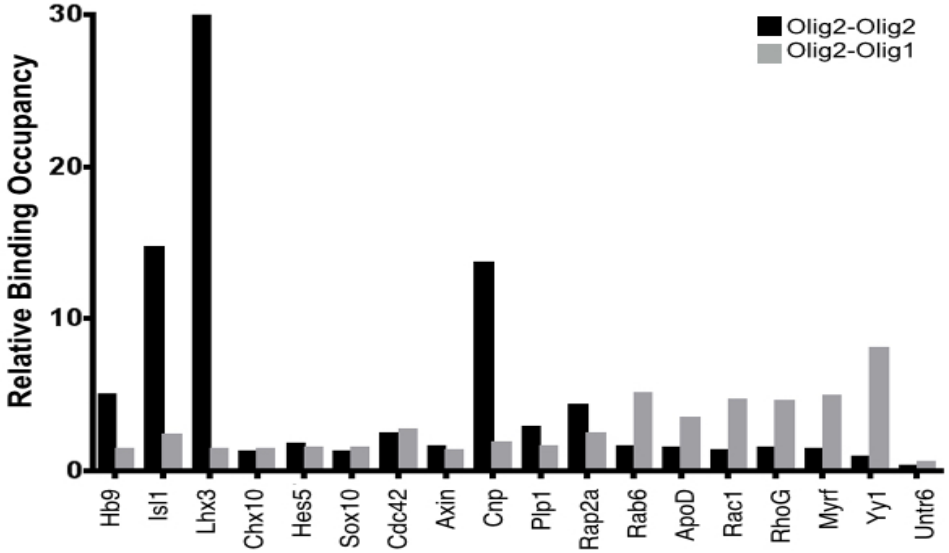

Olig2-Olig2
Olig2-Olig1
Relative Binding Occupancy
30
20
10
0
Hb9
Isl1
Lhx3
Chx10
Hes5
Sox10
Cdc42
Axin
Cnp
Plp1
Rap2a
Rab6
ApoD
Rac1
RhoG
Myrf
Yy1
Untr6

Figure S5. Relative binding occupancy of Olig2 homo- and hetero- dimers to pMN and OL targets. Relative binding occupancy of Olig2-Olig2 and Olig2-Olig1 to genomic loci bound by Olig2 during MN and OPC cell fate specification, as well as OL maturation (see methods for calculation). Untr6 serves as a negative control region for Olig2 binding.

MATERIALS AND METHODS

DNA Constructs

Forced dimer constructs for *in ovo* electroporation consist of myc-tagged full-length chicken Olig2 fused to a full-length dimerization partner (chicken Olig2, mouse Olig1, chicken Ngn2, or human E47) via the sequence 5'-GGAGGATCAGGTATGCATGGAGGTTCAGGTGGA-3' that encodes for a flexible linker. These fusions were cloned into RCASBP(B) using Clontech InFusion per manufacturer instructions. Mouse versions of forced dimers for cell transfection were cloned into a modified version of pcDNA3 vector (Invitrogen) that includes a 3X-Flag-NLS in the same manner. RCASBP(B) myc Olig2Nt-Olig1Ct was created using Clontech InFusion and consists of 6X-myc tag ckOlig2 (1-185 aa) fused to the carboxyl-terminus of Olig1 (163-244 aa). Full-length mouse Olig2 or Olig2 C-terminus (188-323aa) were sub-cloned into pEBG to create GST-fusion proteins. Full-length dimerization partners (mouse Olig1, Olig2, Olig3, Ngn2, or human E47) and Olig2 C-terminus were sub-cloned into pcDNA3 3X-Flag-NLS. For yeast two hybrid, these same inserts were sub-cloned into LexA- and B42 vectors (Gyuris et al. 1993).

In ovo electroporation and Immunohistochemistry

Fertilized chicken eggs were electroporated as previously described (Thaler et al. 2002) at HH10-12 and collected at 3 or 5dpe. Embryos were fixed in 4% PFA and cryosectioned at 12um. Then, immunohistochemisty was performed in a 0.1% fish skin gelatin blocking buffer with

overnight incubation at 4ºC using the following primary antibodies: guinea pig anti-Chx10 (Thaler et al. 1999), mouse anti-MNR2/Hb9 (DSHB 5C10), mouse anti-myc (Millipore and DSHB 9E10), rabbit anti-Olig2, rabbit anti-Sox10 (Abcam), rabbit anti-Olig1 (Millipore), rabbit anti-Ngn2 (Ma et al. 2008). Immunofluorescent images were acquired with a Nikon A1 Confocal. Cell counts were done using the ImageJ plugin and GraphPad Prism was used to perform paired Student's t-tests as described in figure legends. Data is presented as mean ± SEM.

Yeast Two Hybrid

Yeast two hybrid assays were performed as previously described (Lee et al. 1998). For each experiment, at least three independently derived colonies were used.

GST Pull-down and Western blot

HEK293T cells were transfected using calcium phosphate method and lysed 48 hours later. GST pull-downs were performed overnight at 4ºC followed by SDS-PAGE and Western blotting. Blots were then probed sequentially with mouse anti-Flag M2 (Sigma) and mouse anti-GST (Clontech) primary antibodies in Odyssey blocking buffer overnight at 4ºC, probed with Licor IRdye 680LT or 800CW secondary antibodies for 1hr at room temperature, and imaged using Licor Odyssey. Odyssey software was then used to quantify relative band intensity.

ChIP and RT-PCR

P19 cells were transfected using Invitrogen Lipofectamine 2000 per manufacturer instructions and lysed 48 hours post transfection. The Fast ChIP method (Nelson et al. 2006) was used for chromatin immunoprecipitation using mouse anti-Flag M2 (Sigma) and Dynabeads (Invitrogen) for immunoprecipitation. Quantitative RT-PCR was then used to evaluate binding of Olig2 forced

dimers to genomic regions of interest using SYBR Green and QuantStudio 7 (Invitrogen). See supplement for a list of primers used (Table S1). Technical replicates were averaged to generate a single Ct value. Relative binding occupancy was calculated as follows: $2^{\Delta\Delta Ct}=2^{(C_{t,X} - C_{t,R})_{IgG} - (C_{t,X} - C_{t,R})_{IP}}$ where $C_{t,X}$ is IgG/IP Ct value and $C_{t,R}$ is IN Ct value. Relative binding occupancies were plotted as mean ± SEM from 4-5 biological replicates. Binding preference was calculated as a ratio of Olig2-Olig2:Olig2-Olig1 relative binding occupancies then graphically represented as log2 of this ratio.

ChIP Primers

Gene	Peak Location	Forward Primer	Reverse Primer
Sox 10	chr15:78995771-78997012	TTCATCCCTCCACTCTTTGG	ACTCGTGGCCAGCTTTCTAA
Rap2a	chr14:120906419-120907466	CACATAACGCACCAGACACC	GGACCATAAGGCCAATGCTA
Rab6	chr7:107772992-107773584	TGAACAGGAGCACCAATGAG	CACAGGTCACAGGCAGAGAA
Axin	chr17:26325212-26326214	CCACAGACAGCTGACCTTCA	ACTGGGTACCTCAGCATTGG
Cdc42	chr4:136884938-136886400	GCGGCATTAAAATCACAGGT	CCTTCCTGCCTCTGTCTCAC
Isl1	chr13:117,084,140	TCAGGTGCTGTGTTTGCTGT	GGCCCTTCTCTTTCAGGAGT
Lhx3	chr2:26,030,950	GATGATCCACCCTCAGCAAT	GTCCATCAGAGACCCAGAGC
Hb9 (Mnx1)	chr5:29,811,955	TGGGAAATTCCACTGAAAGG	GGTGTCCAGAAATCCACAGG
Hes5	chr4:154,334,796	GGGAAAAGGCAGCATATTGA	CACGCTAAATTGCCTGTGAA
Chx10 (Vsx2)	chr12:85918267-85919358	CTCTGGGAATCGATTTGGAA	GGAGATGACTGGCCTAACCA
Plp1	chrX:133373428-133374863	AAGCAGGGAGCATGAAAAGA	CACATGTAAGGCGAGAAGCA
Cnp	chr11:100442418-100443646	GTCCTGTCTCCCTTCTGCTG	CCTTGCTGAGAGGTCTTTGG
YY1	chr12:110039843-110040163	AGATGCTGTTGGCTGGACTT	AGTGGCAAGAGTGCCTTGTT
Rac1	chr5:144268632-144269446	ACCTTTGCACGGACATTTTC	TTTTGAGTCTCTGGCCGACT
ApoD	chr16:31334637-31335187	CAAACAAGTGGAGCGATGTG	CTCGGACTGCTAGCTTCCAC
RhoG	chr7:109393036-109393926	GCCCAAGAGACACTGGAGAG	CTTTTCCTGGCCTGTCTTTG
Myrf	chr19:10294433-10295397	AGGACAGGTGTTCTGCAACC	CCACACTCCCTGCTACCATT

Chapter 2

Novel Olig2 Phosphorylation Governs Dimerization, Subcellular Localization, and Cell Fate Specification

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Running title: Novel Olig2 Phosphorylation

ABSTRACT

Olig2 is essential for ventral patterning, motor neuron generation and oligodendrocyte specification in the developing spinal cord. Recent work is now elucidating how the activity of Olig2 is regulated across development. Here, we have identified a phosphorylated motif, S147/T151, which is necessary for both motor neuron and oligodendrocyte cell fate specification. Phosphorylation at these sites favors homodimerization and is associated with mitotic cells. Additionally, we have found a novel phosphorylated site at S265 that is a critical regulatory site for Olig2 function. Our data suggest that S265 phosphorylation triggers nuclear export and degradation of Olig2. Furthermore, we propose that CaMKIV is responsible for phosphorylating Olig2 at S265. Together, this work elucidates the unique roles of these phosphorylation sites for Olig2 activity and provides mechanistic insight regarding how Olig2 accomplishes disparate temporal roles in the developing spinal cord.

INTRODUCTION

Transcription factors play a key role in generating diverse cell types through gene activation and repression. Many of these transcription factors are recycled throughout development to achieve disparate ends. Olig2, a bHLH transcription factor, is required for the fate specification of several distinct cells types during CNS development. Early in the developing spinal cord, Olig2 both establishes the motor neuron progenitor (pMN) domain through repressive interactions with transcription factors expressed in adjacent progenitor domains, Irx3 and Nkx2.2, and maintains the progenitor status of these cells by repressing post-mitotic motor neuron genes (Novitch et al. 2001; Takebayashi et al. 2002a; Lee et al. 2005). Olig2 then promotes motor neuron (MN) differentiation through derepression of motor neuron gene transcription (Novitch et al. 2001; Park et al. 2002; Lee et al. 2005). However, Olig2 must be down-regulated to allow for terminal differentiation of motor

neurons (Lee et al. 2005; Novitch et al. 2001). Later in spinal cord development, Olig2 is responsible for specifying oligodendrocyte precursor cells (OPCs) (Zhou et al. 2001; Lu et al. 2002; Takebayashi et al. 2002a; Park et al. 2002; Liu et al. 2007). Unlike Olig2's proneural role, Olig2 is persistently expressed in OPCs and oligodendrocytes (OLs) (Lu et al. 2000; Zhou et al. 2000; Takebayashi et al. 2000).

In light of the complex temporal activity of Olig2, we sought to better understand how Olig2 mediates these diverse roles. Our previous work has implicated dimerization as one factor that alters Olig2 gene binding and cell fate specification in the developing spinal cord (Chapter 1). We discuss how temporal changes in dimer partner concentrations could contribute to Olig2 dimer formation (Chapter 1). In addition to this, other strategies may be employed to control which Olig2 dimer forms. Post-translational modification, in particular phosphorylation, is a well-established means of altering dimerization. For example, phosphorylation of the bHLH transcription factor E47 prevents homodimerization and promotes heterodimer formation with MyoD (Lluís et al. 2005). Phosphorylation at S147 has been shown to affect the interaction of Olig2 with dimerization partners Olig2, Olig1, and Ngn2 (Li et al. 2011). Furthermore, multiple phospho-sites have been found to regulate Olig2 activity in specific cellular contexts (Setoguchi and Kondo 2004; Li et al. 2011; Mehta et al. 2011; Sun et al. 2011; Meijer et al. 2014). However, the dynamics of how phosphorylation, specifically at multiple phosphorylation sites, regulates the pleiotropic roles of Olig2 are still not fully understood. Many proteins are elegantly regulated by phosphorylation at multiple sites. For this reason, we wanted to investigate whether Olig2 activity is regulated by phosphorylation at any other sites during spinal cord development.

In this study, we identify three phosphorylated residues in Olig2, S147, T151, and S265, which are phosphorylated in a hierarchical manner to govern the temporal activity of Olig2 during spinal cord development. We show that Olig2 is initially phosphorylated at S147 and T151. These sites

function as a single motif that is required for both MN and OPC specification. Subsequent phosphorylation at S265 halts the cell fate specification activity of Olig2 and is important for the switch from proliferation to differentiation. Our data indicate that Olig2 is translocated to the cytoplasm when phosphorylated at S265, which provides a mechanistic basis for how phosphorylation this critical regulatory site could result in down-regulation of Olig2 expression. Additionally, we find that CaMKIV is expressed in differentiating MNs and that co-expression of Olig2 and CaMKIV results in nuclear to cytoplasmic translocation of Olig2. We find that not only is CaMKIV capable of phosphorylating Olig2 at S265, but our data suggest that CaMKIV is responsible for phosphorylating Olig2 at this site in vivo. Together, we show that these three hierarchical sites are essential for the concise regulation of Olig2 as it plays distinct roles in the developing spinal cord.

RESULTS

Olig2 is phosphorylated at S147, T151, and S265

SDS-PAGE followed by Western blot analysis of Olig2 revealed that Olig2 protein migrates as two bands, which suggests that Olig2 is post-translationally modified (Fig 1B,C). Since phosphatase treatment of this lysate ablated the upper, slower migrating band, this indicated that Olig2 is phosphorylated (Fig 1B). We then identified ten putative phosphorylated residues within Olig2 by phosphorylation consensus sequence. These residues were located across the Olig2 sequence. Thus, to test whether any of these residues are phosphorylated, we first examined Olig2 phosphorylation in Olig2-dN, Olig2-dC, Olig2-N, and Olig2-C constructs by in vitro kinase assays followed by Western blot. Only the Olig2-N (N-terminus alone construct) lacked phosphorylation, indicating that there are phosphorylated residues in both the bHLH domain and C-terminus of Olig2. In order to identify which residues are phosphorylated, we created serine (S)/threonine (T) to alanine (A) mutants for each of the potential phospho-sites in the bHLH domain and C-terminus. If Olig2 is phosphorylated at a given residue in our system, then mutating this S/T to an unphosphorylatable A will reduce or even abolish Olig2 phosphorylation on a Western blot. These experiments revealed that Olig2 is phosphorylated at S147, T151, and S265 (Fig 1A). Sequence analysis illustrates that these residues are highly conserved across species.

In order to monitor the phosphorylation status of Olig2 at each residue as well as to establish whether there is a relationship between these sites, we generated phospho-specific antibodies for each of the three residues. We confirmed the specificity of these affinity-purified antibodies by Western blot analysis comparing wild-type Olig2 to A mutants for each of the three sites (Fig 1D). These Western blots demonstrate that the phospho-specific antibodies recognize wild-type but not unphosphorylated Olig2 (Olig2 3A). There is also no phospho- band for the corresponding A mutant of each antibody, confirming the specificity of these antibodies to their phosphorylated

residue (Fig 1D). These experiments also indicated that phosphorylation of Olig2 is hierarchical in nature (Fig 1D). If either S147 or T151 is mutated to alanine, there is no phosphorylation at either site. Additionally, S147A and T151A mutants are not phosphorylated at S265. Conversely, with the S265A mutant, both S147 and T151 are still phosphorylated. Together, these results indicate that phosphorylation at S147 and T151 precedes and is necessary for phosphorylation at S265. Given the close proximity of S147 and T151, the results of these Western blots suggest interconnectivity between these two sites.

Next, in order to address whether the sites we identified in vitro are biologically relevant, we assessed whether these sites are phosphorylated in vivo. To determine whether Olig2 is phosphorylated at these residues in vivo, we immunoprecipitated Olig2 from mouse embryonic spinal cord over a range of developmental stages (Fig 1E). Spinal cords were collected from e9.5 to e18.5 in order to encompass both MN and OPC fate specification, in which Olig2 plays distinct biological roles. Both total Olig2 and phospho-Olig2 levels were quantified from Western blots. This analysis revealed that not only is Olig2 phosphorylated at all three residues in spinal cord but that the phosphorylated fraction of Olig2 increases as development proceeds for all three residues (Fig 1F). Additionally, since Olig2 is known to be involved in neuronal and glial specification in telencephalon development, we examined Olig2 phosphorylation in mouse cortical lysates. Like in the spinal cord, we found that Olig2 is also phosphorylated across cortical development.

Olig2 is phosphorylated at S147, T151, and S265 in pMN cells and OPCs

These Western blots demonstrated that Olig2 is phosphorylated at S147, T151, and S265 in vivo and gave us temporal information about Olig2 phosphorylation. However, these Western blots did not provide insight into the phosphorylation status of Olig2 or subcellular localization of this phosphorylation in pMNs and OLs. To investigate this, we turned to cultured cells. Due to the

difficulty of isolating a large, pure population of pMN cells from mouse spinal cord, we generated pMN cells through directed differentiation of ES cells (Fig S1A-D'; Wichterle et al. 2002). This allowed us to examine Olig2 phosphorylation from the onset of Olig2 expression until its down-regulation during MN differentiation. Similar to our findings from Western blot analysis, Olig2 is phosphorylated at all three residues in MN progenitors (Fig 2A-C'). However, the sub-cellular localization of these phosphorylated forms of Olig2 differ. Both S147 and T151 phosphorylation are nuclear, whereas phospho-S265 signal is predominately perinuclear (Fig 2C). Additionally, phospho-Olig2+ cells tended to express low levels of Olig2 and cells that were Olig2+ high were phospho-Olig2- (Fig 2A-C'). Furthermore, phosphorylation at S147 and T151 is associated with mitotic Olig2+ cells, based on both phospho-histone H3 and DAPI stain (Fig 2D-E', data not shown).

In addition to looking at Olig2 phosphorylation in MN progenitors, we also examined phosphorylation in oligodendrocytes using primary cultures. OPCs were isolated from rat brains by immunopanning for PDGFRa and were either examined at the OPC stage or OLs differentiated in culture (Fig S2A-E). This allowed us to assess Olig2 phosphorylation across OL maturation. Similar to pMN cells, in OPCs Olig2 expression is both nuclear and cytosolic (Fig 2F,J). We found that phospho-S147 and -T151 are predominately nuclear throughout OL maturation (Fig 2G,H,K,L). Phospho-S265 has nuclear localization, as well as strong signal in OL processes (Fig 2I,M). This cytoplasmic localization becomes more pronounced as the processes of these developing OLs becomes more elaborate.

Phosphorylation at S147 and T151 is required for MN and OPC production

After establishing that Olig2 is phosphorylated at the three residues that we have identified both in vivo and in relevant cell types, we next wanted to investigate what the functional ramifications of Olig2 phosphorylation during spinal cord development. To elucidate the biological

role that phosphorylation plays in Olig2-mediated cell fate specification, we employed in ovo electroporation. We misexpressed unphosphorylatable alanine (A) and phospho-mimetic glutamic acid (E) mutant Olig2 constructs in chick neural tube at HH stage 11-12, before the onset of MN generation, then collected embryos at 3 days post-electroporation (dpe), after MN generation is complete, and 5dpe, during OPC generation and migration (Fig 3, S3). Since we know that Olig2 wild-type misexpression results in both ectopic Hb9+ MNs and Chx10+ cells, as well as premature OPC generation (Novitch et al. 2001; Zhou et al. 2000), we compared the effects of misexpressing phospho-mutants to Olig2 wild-type on patterning, proliferation, cell fate specification, and differentiation by immunostaining (Fig 3A,E,I; S3A).

Unlike Olig2 wild-type, neither Olig2 S147A or T151A produce ectopic MNs or ectopic Chx10+ cells at 3dpe (Fig 3F,G,M). Ectopic expression of either mutant also failed to trigger premature OPC generation by 5dpe (Fig 3J,K,N; S3B,C). Additionally, neither of these A mutants repressed Irx3 transcript dorsally by in situ hybridization (Fig 3B,C). These results suggest that phosphorylation at S147 and T151 are indispensable for cell fate commitment of both MNs and OPCs. Our finding that mutating either site abolishes phosphorylation at the other site and both A mutants have same phenotype suggests that the two sites function in concert. In order to confirm this finding, we attempted to make phospho-mimetic E mutants for S147 and T151. Given the interdependency of these two sites, E mutant for a single site may not be sufficient to recapitulate the activity of pS147/pT151 so, in addition to creating S147E and T151E mutants we also generated a S147E/T151E double mutant construct. Unfortunately, none of these constructs behaved differently from phospho-deficient mutants in our experiments. This result could indicate that mutating S147 and T151 to glutamic acid did not successfully generate a constitutively phosphorylated version of Olig2.

Phosphorylation at S265 inactivates Olig2

In contrast to S147A and T151A, electroporation of S265A resulted in a dramatic expansion of MNs and OPCs, as well as Chx10+ V2a INs (Fig 3H,L,M; S3D). Interestingly, there were significantly more ectopic MNs and OPCs with S265A than Olig2 wild-type misexpression (Fig 3O,P S3D). Additionally, there is substantial dorsal repression of Irx3 transcript with S265A misexpression (Fig 3D). These data suggest that phosphorylation at S265 mediates the switch from specification of progenitors to differentiation. Based on Western blot analysis, S265A is still phosphorylated at S147 and T151, which are necessary for cell fate specification of MNs and OPCs (Fig 1D). Without phosphorylation at S265, Olig2 seems capable of committing more progenitors to MN and OPC fates, likely through phosphorylation at S147 and T151, but continues to do so unheeded.

To examine the activity of Olig2 when it is phosphorylated at S265, we created a phospho-mimetic mutant for this residue, S265E (Fig S4A). Whereas S265A behaves like a hyperactive version of Olig2, the phospho-mimetic mutant for S265 exhibits compromised activity compared to wild-type Olig2. There is a significant reduction in ectopic MNs and OPCs when this S265E mutant is misexpressed in chick neural tube (Fig S4B,C).

Both Western blot analysis and electroporation data indicate that a hierarchical relationship exists between the phosphorylation sites such that phosphorylation at S147 and T151 precedes S265 (Fig 1D). Although S265 phosphorylation appears to follow pS147/pT151 endogenously, the S265E construct may still have dynamic phosphorylation at S147 and T151. Thus, electroporating with this construct may not fully recapitulate the activity of Olig2 when all three residues are phosphorylated. To monitor the activity of Olig2 when all three residues are phosphorylated, we electroporated an Olig2 3E (S147E, T151E, S265E) construct, in which all three S/T have been mutated to a constitutive phosphomimetic E. Electroporation with this construct did not result in dorsal ectopic

expansion of MNs, like wild-type Olig2 (Fig S4D,E). However, there were ectopic Hb9+ MNs found along the midline of the ventricular zone, where progenitors usually reside (Fig S4E). Although we did not observe premature OPC production with this construct, expression of Olig2 3E is confined to the progenitor domain by 4dpe so the expression of this construct may not have been sustained to see an effect on OPC production. Additionally, the electroporated side of the spinal cord was dramatically smaller with 3E misexpression. This led us to investigate whether proliferation or differentiation was affected by immunostaining for progenitor and post-mitotic neuronal markers. We found that, while the Sox2+ progenitor domain was unperturbed, the NeuN+ lateral zone was dramatically smaller on the side of the spinal cord electroporated with the Olig2 3E construct (Fig S4F,G). This indicates that Olig2 3E does not cause premature differentiation or depletion of progenitors.

Phosphorylation alters Olig2 dimerization

There are a number of ways that phosphorylation could affect the activity and molecular interactions of Olig2. Since we know that bHLH proteins bind DNA as obligate dimers, we first investigated whether phosphorylation affects dimer partner choice by GST pull-down assays. Olig2 strongly homodimerizes and also heterodimerizes with Olig1, Ngn2, and E47 (Samanta and Kessler 2004; Lee et al. 2005). We co-transfected HEK293T cells with Flag epitope-tagged Olig2 dimerization partners (Olig2, Olig1, Ngn2, or E47) and wild-type or A mutant GST-fusions of Olig2. We found that S147A and T151A have decreased interaction with Olig2 and increased interaction with heterodimer partners Ngn2 and E47 (Fig 4A,C-E). Although similar results were seen for S265A, the difference in dimer partner preference between Olig2 wild-type and S265A was less pronounced than what was seen with S147A and T151A (Fig 4A,C-E). For Olig1 interactions, no consistent trend was observed (Fig 4B,E). This could indicate that interaction with Olig1 is not

directly related to phosphorylation at S147, T151, or S265.

Although we also used E mutant constructs in these experiments as well, we did not notice a significant difference between wild-type and E mutants in terms for dimerization preference, presumably because previous experiments have demonstrated that Olig2 wild-type is already phosphorylated at all three residues in vitro.

Phosphorylation at S147/T151 and S265 have opposite effects on Olig2 stability

Immunostaining from both Olig2+ pMN-like cells and OPCs indicates cytoplasmic localization of phospho-S265. This observation, in conjunction with the dramatic production of MNs and OPCs with S265A electroporation, led us to question whether phosphorylation at S265 triggers nuclear export of Olig2. We then asked whether this phosphorylation and subsequent nuclear export is linked to degradation of Olig2. To test this possibility, we performed cycloheximide chase experiments to determine the effect of phosphorylation on Olig2 protein half-life in vitro. HEK293T cells transfected with Olig2 wild-type and mutant plasmids were treated with the protein synthesis inhibitor, cycloheximide, and protein half-life was assayed by Western blot. Both S147A and T151A were less stable than Olig2 wild-type, whereas S265A was more stable (Fig 4F-G). Conversely, S265E was less stable than wild-type and Olig2 3E had an even shorter half-life (Fig S5A,B).

Kinases

Beyond understanding the biological role of Olig2 phosphorylation at S147, T151, and S265, we also sought to identify the kinases are responsible for phosphorylating Olig2 at these residues. We used several online services (NetPhos, KinasePhos, Phospho Motif, ELM, PPSP) to identify potential kinases for Olig2 at S147, T151, and S265 based on known consensus motifs. Then we

combined these results to generate a list of top predictions for each residue based on both consensus motif and CNS expression.

Next, we tested whether inhibiting individual putative kinases affected Olig2 phosphorylation and whether these kinases were capable of phosphorylating Olig2 in vitro. To address these questions, we first expressed Olig2 wild-type in HEK293T cells then treated these cells with available inhibitors for putative kinases. We then compared phosphorylation of Olig2 between cells treated with kinase inhibitors or vehicle by Western blot analysis. These results indicated that inhibitors of Gsk3-beta, PKA, PKC, ROCK, CK2, and CaMKs decrease Olig2 phosphorylation (Fig S6A-C). To test whether these kinases are capable of phosphorylating Olig2 in vitro, we co-expressed Olig2 with constitutively active (ca) versions of candidate kinases. We then immunoprecipitated for total Olig2 protein and examined phosphorylation at each residue by Western blot (Fig 5F). This analysis revealed that Olig2 phosphorylation is increased for all three residues by expression of ca-CaMKIV but not ca-PKA (Fig 5F). We also used these constitutively active constructs for in ovo electroporation and observed that the number of phospho-S265+ cells increased with expression of ca-CaMKIV and ca-Gsk3-beta, but not ca-PKA (Fig 5A-D').

Of these kinases, CaMKIV is of particular interest. Both in situ hybridization and immunostaining show that CaMKIV is specifically expressed in the ventral horn of the developing spinal cord, where MNs reside (Fig 5H; S6A-C). When we co-immunostained for CaMKIV and Olig2, we found that differentiating MNs located in the intermediate zone co-express CaMKIV and Olig2 (Fig 5G). These cells are committed to a MN cell fate and are migrating out of the ventricular, progenitor zone and into the their post-mitotic positions in the lateral neural tube. This suggests that CaMKIV expression is coincident with the transition from committed to post-mitotic MN, which also coincides with the down-regulation of Olig2 expression. Furthermore, when ca-CaMKIV is co-expressed with Olig2 in HEK293T cells, Olig2 is translocated to the cytoplasm similar to when

Olig2 is phosphorylated at S265 (Fig 5E).

FIGURE LEGENDS

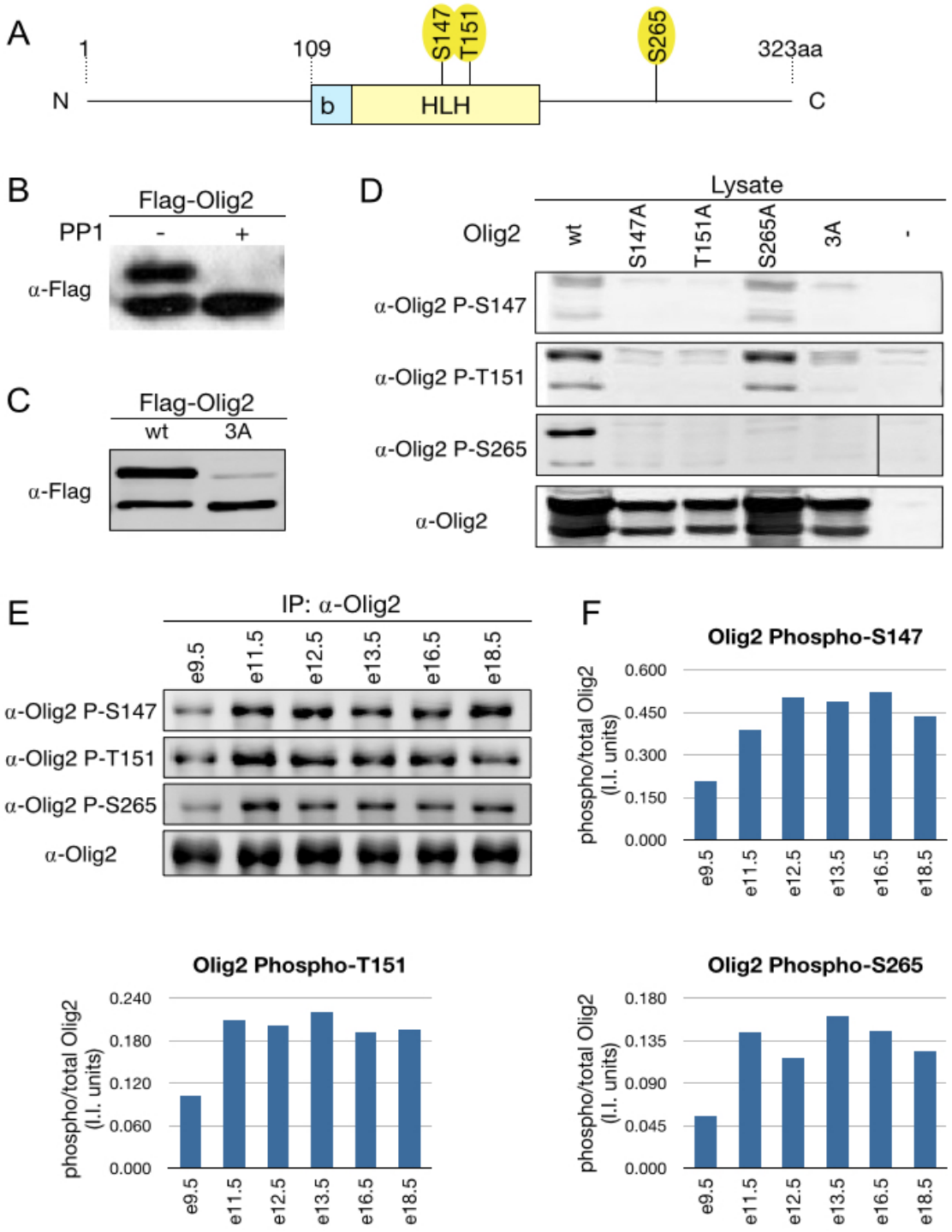


Figure 1. Olig2 is phosphorylated at S147, T151, and S265. A) Schematic of Olig2. Both S147 and T151 are located within the bHLH domain. S265 is in the C-terminus of Olig2. B) HEK293T cells were transfected with Flag-Olig2. Untreated or PP1 phosphatase treated lysates were separated by SDS-PAGE followed by Western blot analysis and proved with anti-Flag antibodies. Olig2 resolves as two bands. The upper, slower migrating band is ablated by PP1. C) Cells transfected with Flag-Olig2 wild-type (wt) or a triple unphosphorylatable mutant (3A) were analyzed by immunoblotting with anti-Flag antibodies. Mutating all three residues to A largely ablates the upper band. D) Lysates from HEK293T cells transfected with wt, single A, 3A, or vector alone were separated by SDS-PAGE and analyzed by immunoblotting with phospho-specific antibodies raised against each phospho-residue, as well as with a total Olig2 antibody. Olig2 wt is phosphorylated at S147, T151, and S265. Neither 3A or vector alone have phospho- bands for any of the three residues. Phospho-antibodies do not recognize the single A-mutant for the residue that they were raised against. When either S147 or T151 is mutated to A, Olig2 is unphosphorylated at all three residues. S265A is still phosphorylated at S147 and T151. E-F) Mouse spinal cord lysates were collected from e9.5 to e18.5. Endogenous Olig2 was then immunoprecipitated with an anti-Olig2 antibody, separated by SDS-PAGE, and immunoblotted with anti-Olig2 and anti-phospho-Olig2 antibodies. Olig2 is phosphorylated at all three residues in the developing mouse spinal cord (E) and the fraction of phosphorylated Olig2 increases over time (F).

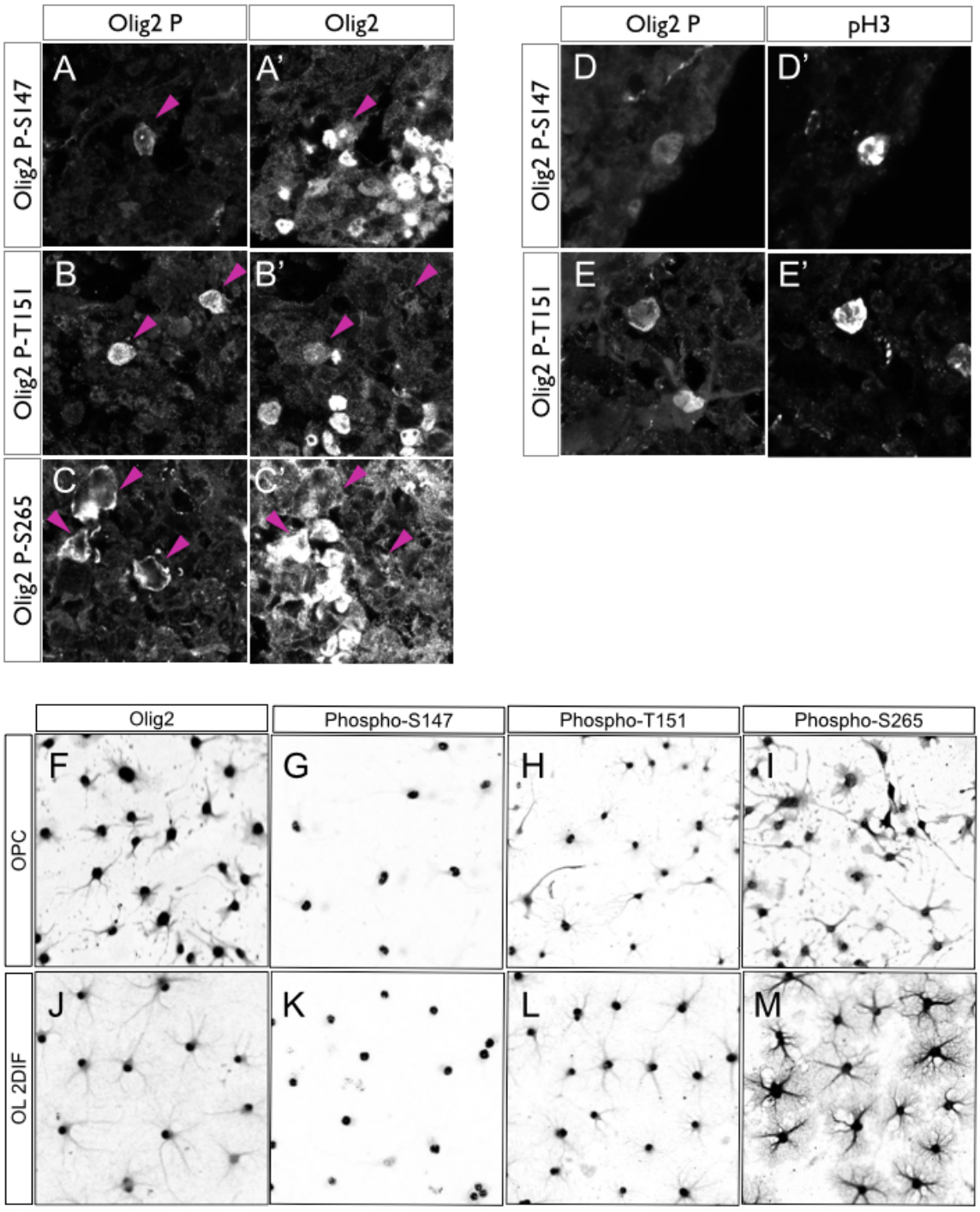

Olig2 P
Olig2
pH3
Olig2 P-S147
Olig2 P-T151
Olig2 P-S265
A
A'
B
B'
C
C'
D
D'
E
E'
Olig2
Phospho-S147
Phospho-T151
Phospho-S265
OPC
OL 2DIF
F
G
H
I
J
K
L
M

Figure 2. Olig2 is phosphorylated in pMN cells, OPCs, and OLs. A-C) A subset of Olig2+ cells (A'-C') are phosphorylated at S147 (A), T151 (B), and S265 (C) in ESC derived pMN cells. EBs shown were collected after 4 days in culture with RA + HH-Ag, sectioned and immunostained. Phospho-S147 (A) and phospho-T151 (B) localization is nuclear whereas phospho-S265 (C) is perinuclear. (D-E') Phospho-S147 (D) and -T151 (E) are often mitotic pH3+ cells (D'-E') as well. F-M) Olig2 is also phosphorylated across OL maturation in primary cultured cells. Both total (F) and phospho-Olig2 (G-I) are predominantly nuclear at the OPC stage. By 7 days in differentiation conditions (7DIF) both Olig2 (I) and phospho-S265 (M) staining are extensively localized in the processes of OLs whereas phospho-S147 (K) and phospho-T151 (L) are still predominately nuclear.

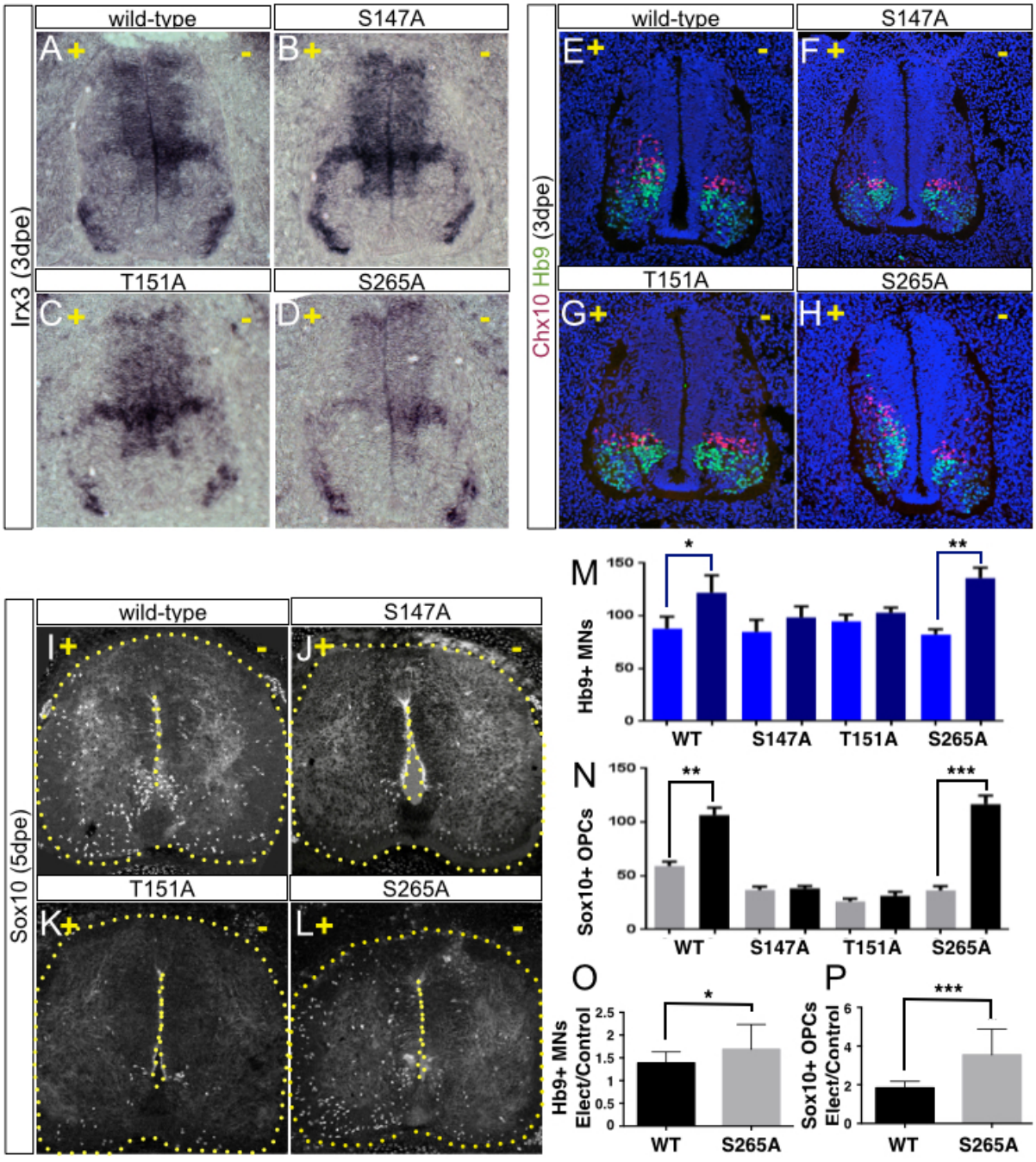
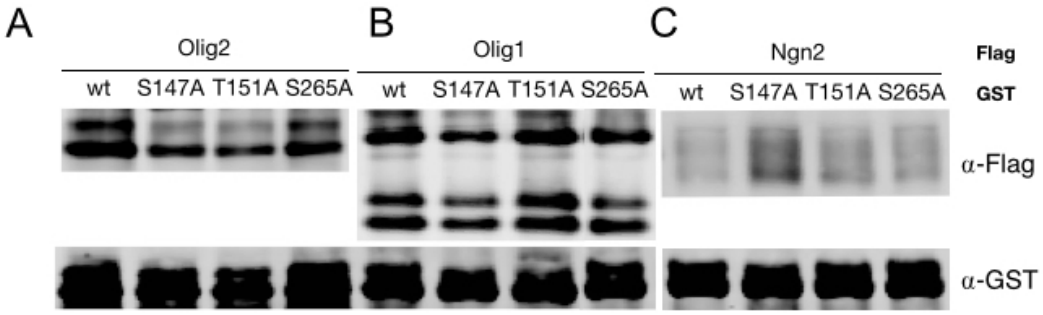
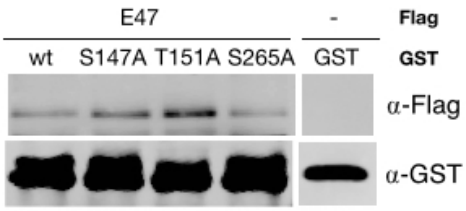

wild-type
S147A
T151A
S265A
Irx3 (3dpe)
A
B
C
D
+
-
Chx10 Hb9 (3dpe)
E
F
G
H
Sox10 (5dpe)
I
J
K
L
M
Hb9+ MNs
150
100
50
0
*
**
WT
S147A
T151A
S265A
N
Sox10+ OPCs
**
***
O
Hb9+ MNs
Elect/Control
2.5
2
1.5
1
0.5
0
P
Sox10+ OPCs
Elect/Control
6
4
2
0

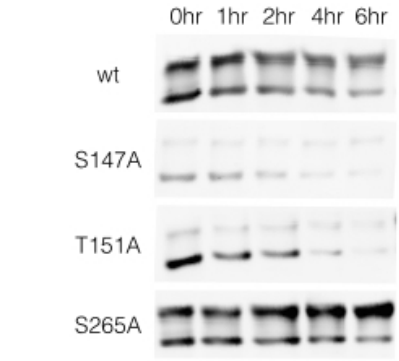
Figure 3. Biological roles of Olig2 phosphorylation. A-L) Olig2 wild-type or A mutant constructs were electroporated into chick neural tube and collected at 3dpe to examine the effect on ventral patterning and MN specification and at 5dpe to examine OPC production. Wild-type misexpression dorsally represses Irx3 transcript (A) and results in ectopic Hb9+ MNs and Chx10+ V2a INs by immunostaining(E). Irx3 expression is unchanged with S147A (B) or T151A (C) and no ectopic MNs or V2a INs are seen (F,G). S265A misexpression resulted in dorsal repression of Irx3 (D) and ectopic Hb9+ and Chx10+ cells (H). By 5dpe, ectopic Sox10+ OPCs are seen with wild-type misexpression (I) and S265A (L) but not with S147A (J) or T151A (K). (M-N) Quantification of MNs and OPCs after Olig2 wt or A mutant electroporation. Mean ± SEM from electroporated and unelectroporated sides of the neural tube were calculated by pooling 3 sections from 8 embryos per construct. Olig2 wt and S265A misexpression result in a significant increase in MNs (M), $^{**}p < 0.01$ and $^{***}p < 0.001$ respectively, and OPCs (N), $^{**}p < 0.01$ and $^{***}p < 0.001$, by Student's t test. No statistically significant difference was detected with S147A or T151A misexpression for either MNs or OPCs. O-P) There are significantly more ectopic Hb9+ MNs (O) and Sox10+ OPCs (P) with S265A misexpression than wild-type Olig2, $^{*}p < 0.05$ and $^{***}p < 0.001$ respectively.





E

Lane	Flag/GST (I.I.)
O2-O2	2.41
O2-S147A	2.31
O2-T151A	2.13
O2-S265A	1.91
O1-O2	1.73
O1-S147A	1.28
O1-T151A	2
O1-S265A	1.11
Ngn2-O2	0.12
Ngn2-S147A	0.36
Ngn2-T151A	0.21
Ngn2-S265A	0.15
E47-O2	0
E47-S147A	0.005
E47-T151A	0.0014
E47-S265A	0.033
GST only	0.004



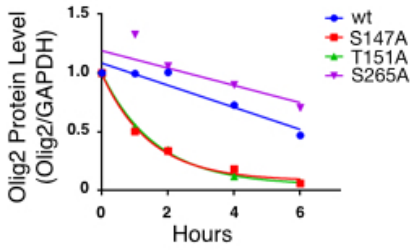
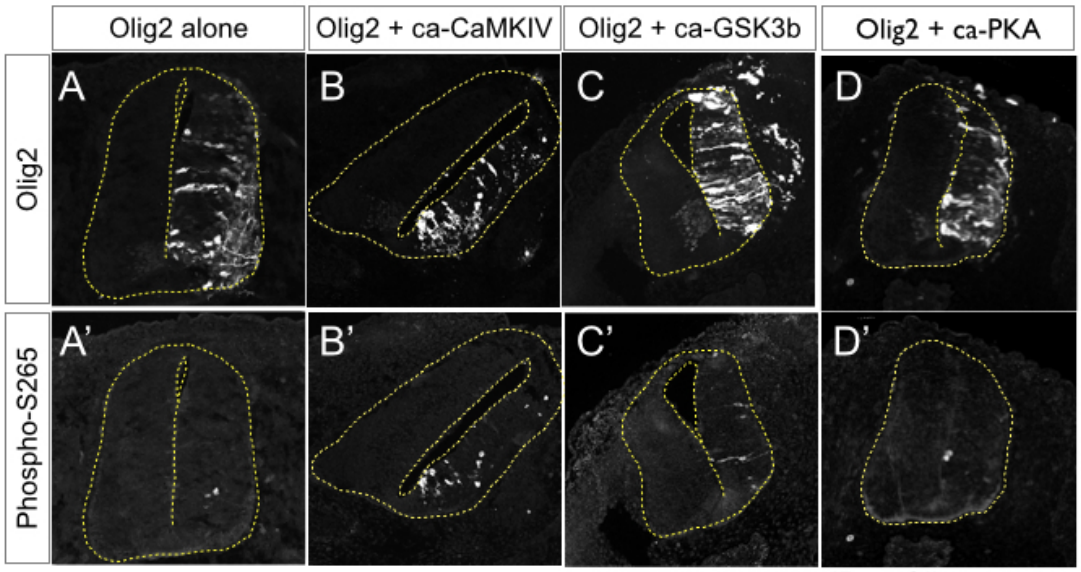
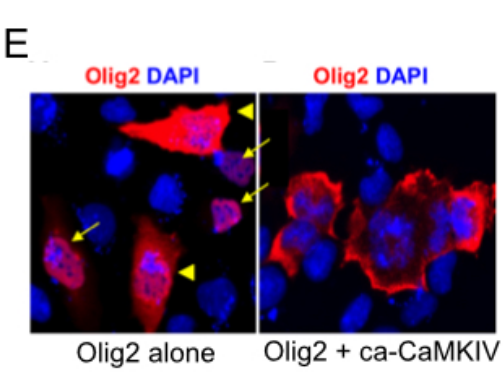
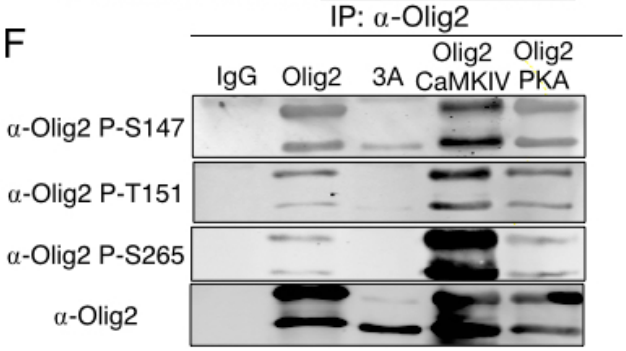
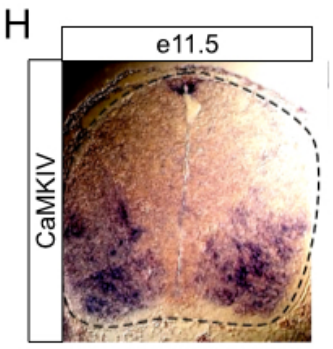


Figure 4. Phosphorylation alters interaction with dimerization partners and stability of Olig2. A-D) Interaction between Olig2 phospho-mutants and dimerization partners was assessed by GST pull-down assays with GST-Olig2 mutants and Flag-tagged dimerization partners. Compared to wild-type S147A and T151A have decreased interaction with Olig2 (A) and Olig1 (B) and increased interaction with Ngn2 (C) and E47 (D). S265A is more comparable to wild-type. E) Band intensities from Olig2 dimer partner immunoblots were obtained from Licor Odyssey software. A ratio of Flag/GST was calculated to normalize to pull-down efficiency. F) Protein half-life of Olig2 A mutants was compared to wild-type. HEK293T cells were transfected with Flag-Olig2 wt, S147A, T151A, or S265A then treated with cycloheximide for 0, 2, 4, or 6 hours. Immunoblots were then probed with anti-Flag. G) Band intensity was quantified using Licor Odyssey then a ratio of Olig2 to GAPDH, as loading control,was plotted against time to examine differences in stability. S147A and T151A are substantially less stable than Olig2 wild-type. S265A is slightly more stable.


Olig2 alone
Olig2 + ca-CaMKIV
Olig2 + ca-GSK3b
Olig2 + ca-PKA
Olig2
Phospho-S265
A
B
C
D
A'
B'
C'
D'


E
Olig2 DAPI
Olig2 DAPI
Olig2 alone
Olig2 + ca-CaMKIV


F
IP: α-Olig2
IgG
Olig2
3A
Olig2 CaMKIV
Olig2 PKA
α-Olig2 P-S147
α-Olig2 P-T151
α-Olig2 P-S265
α-Olig2


H
e11.5
CaMKIV

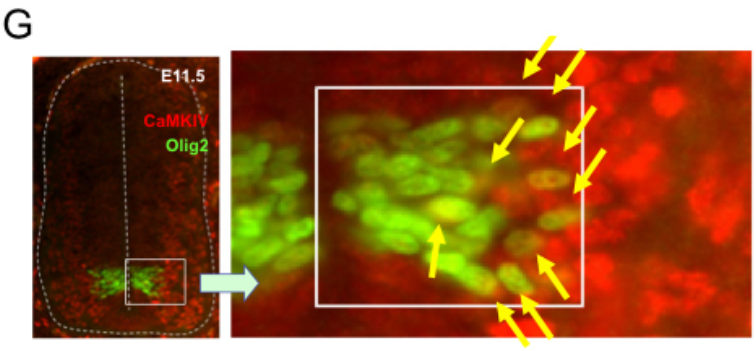

G
E11.5
CaMKIV
Olig2

Figure 5. Putative Olig2 Kinases. A-D) The number of phospho-S265+ cells is increased with constitutively active (ca) CaMKIV (B, B') or ca-Gsk3b (C, C') co-electroporation with Olig2 compared to expressing Olig2 alone (A, A'). Co-electroporating Olig2 with ca-PKA (D,D') does not increase the number of phospho-S265+ cells. E) In HEK293 cells, ~50% cells have nuclear HA-Olig2 (arrows), whereas in ~50% Olig2 is both nuclear and cytoplasmic (arrow heads). F) With the addition of ca-CaMKIV, Olig2 becomes almost exclusively cytoplasmic. G) CaMKIV is highly and specifically expressed in the ventral horn, where post-mitotic MNs reside, in e12.5 mouse spinal cord. H) In e11.5 mouse spinal cord, Olig2 is expressed in progenitors in the ventricular zone and CaMKIV is expressed in MNs in the ventral horn by immunostaining. Cells in the intermediate zone co-express Olig2 and CaMKIV (arrows).

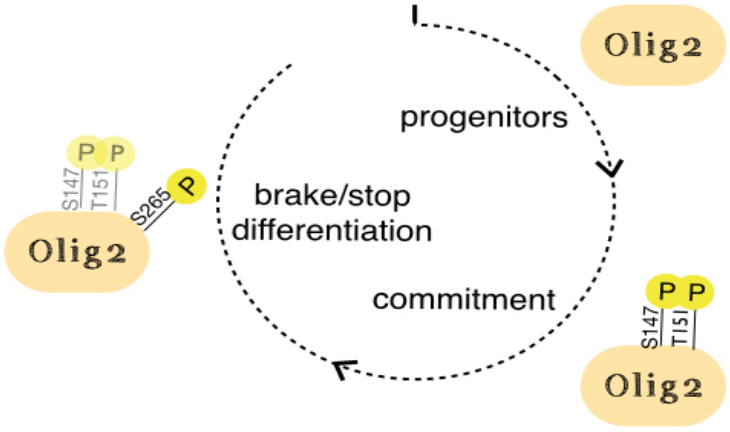

Olig2
progenitors
P P
S147
T151
S265
P
brake/stop
differentiation
Olig2
commitment
P P
S147
T151
Olig2

Figure 6. Proposed model of temporal Olig2 phosphorylation. Olig2 is expressed in progenitors. Phosphorylation at S147 and T151 is required for the ability to generate MNs and OPCs. Additionally, phosphorylation at these two sites permits subsequent phosphorylation at S265. Once phosphorylated at S265, Olig2 is no longer exported

SUPPLEMENTAL FIGURES

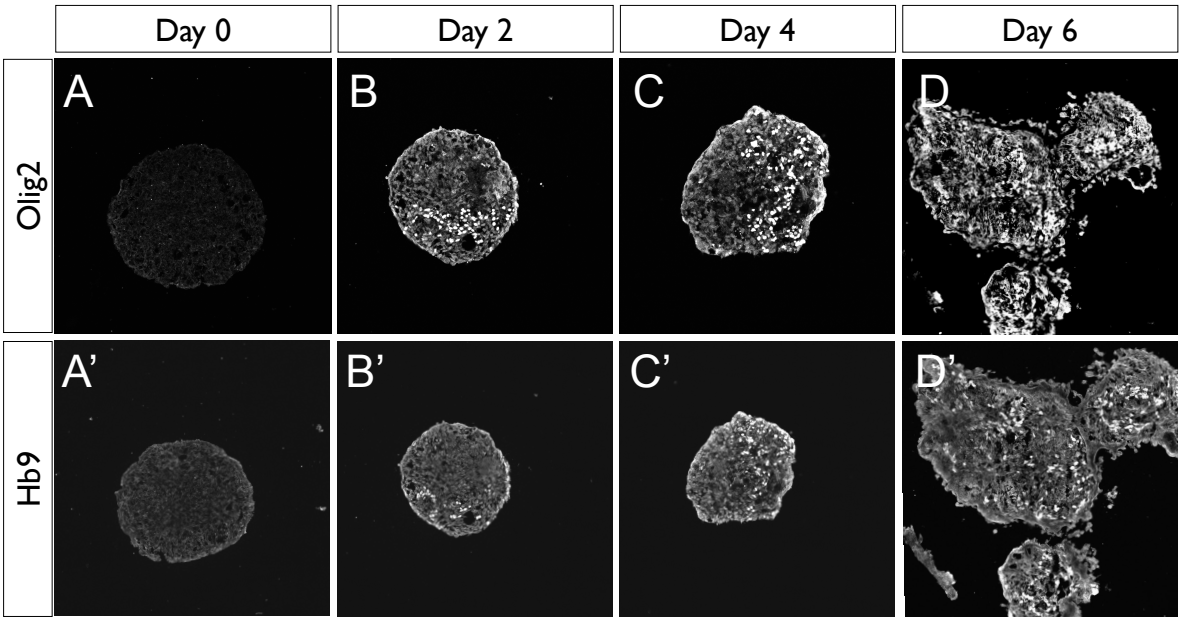


Figure S1. Induction of Oig2 and Hb9 in ESC-derived pMN EBs. EBs collected at the onset of RA and HH-Ag treatment have no Olig2+ (A) or Hb9+ (A') cells. By 2 days, some cells are Olig2+ (B) and Hb9+ (B'). After 4 (C) and 6 days (D) with RA + HH-Ag many cells are Olig2+ (C,D) and Hb9+ (C',D') demonstrating successful induction of MN markers in this system.

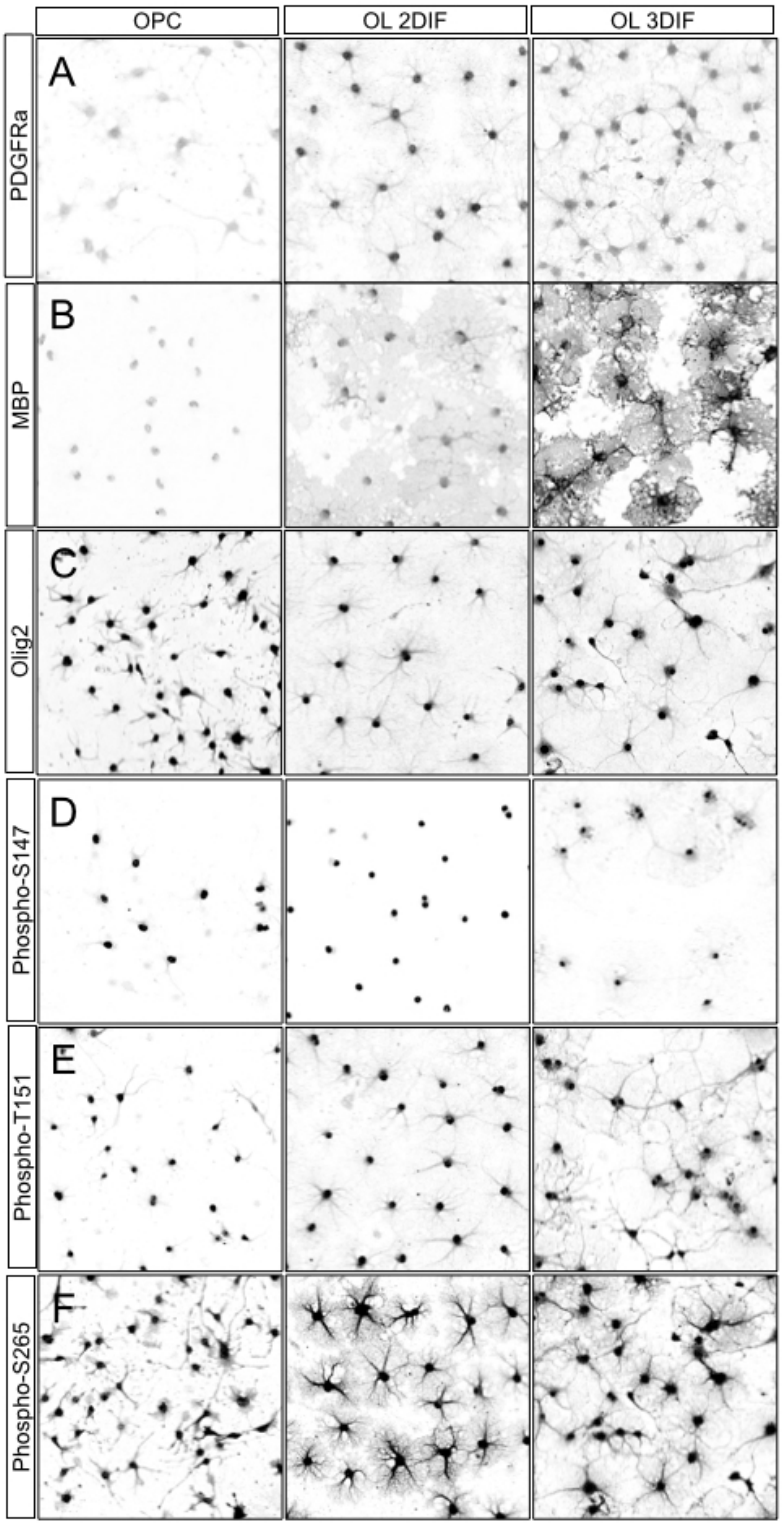

OPC
OL 2DIF
OL 3DIF
PDGFRa
MBP
Olig2
Phospho-S147
Phospho-T151
Phospho-S265
A
B
C
D
E
F

Figure S2. Olig2 is phosphorylated across OL maturation. Cultured OLs were collected at the OPC stage and after 2 and 3DIF. Cells were stained with PDGFRa (A), an early OL marker, and MBP (B), a more mature OL marker. C) OLs are Olig2+ at all stages of maturation. Olig2 is located in the nucleus, as well as the cell body and processes of OLs. D-F) OLs are also phospho-Olig2+ at all stages examined. Both phospho-S147 (D) and -T151 (E) are predominately nuclear throughout OL differentiation. In contrast, phospho-S265 (F) is extensively localized in the cell body and processes of OLs as well as the nucleus.

A

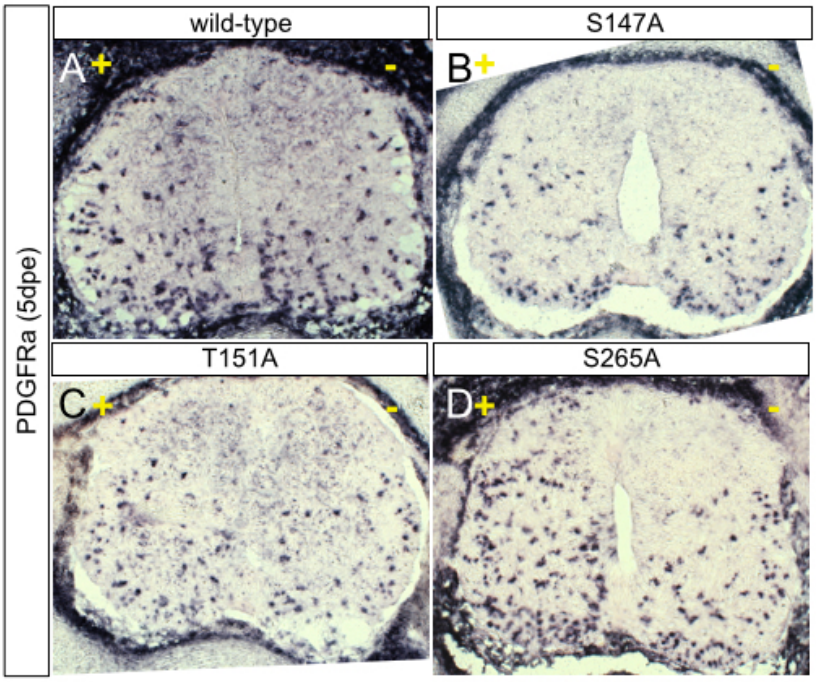
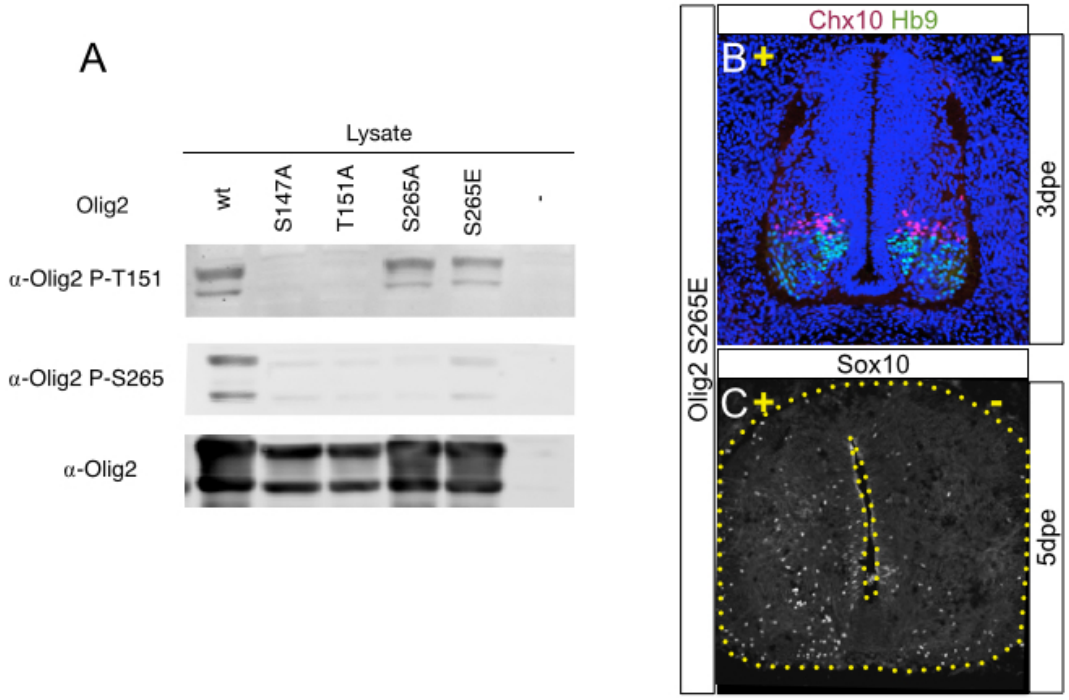


Figure S3. PDGFa expression in chick embryos electroporated with Olig2 phospho-mutants. Olig2 A mutants have consistent effects on another early OL marker, PDGFRa, as with Sox10. A) There are more PDGFRa expressing cells by in situ hybridization with Olig2 wild-type misexpression. Ectopic PDGFRa expression is not seen with S147A (B) or T151A (C). Many more PDGFRa expressing cells are seen with S265A.


A
Lysate
Olig2
wt
S147A
T151A
S265A
S265E
-
α-Olig2 P-T151
α-Olig2 P-S265
α-Olig2
Chx10 Hb9
B
+
-
3dpe
Olig2 S265E
Sox10
C
+
-
5dpe

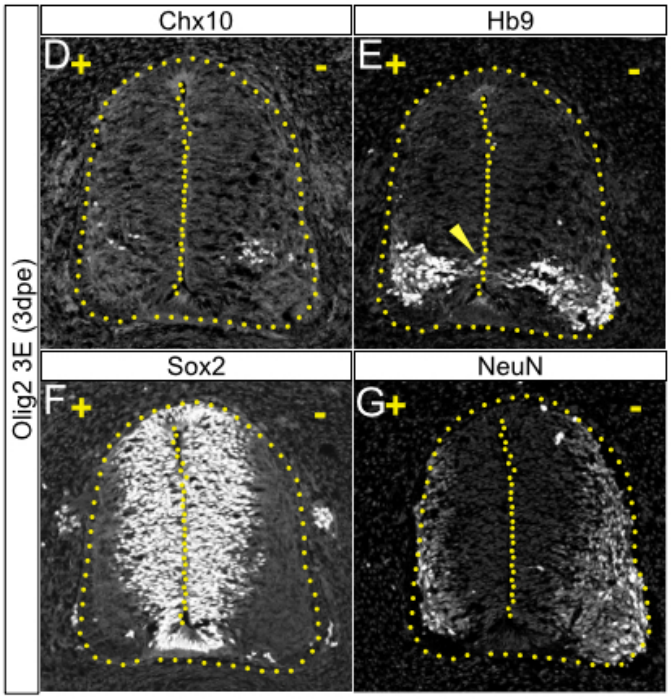
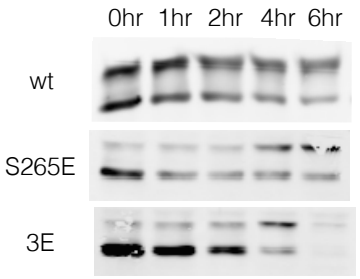

Chx10
Hb9
D
+
-
E
+
-
Olig2 3E (3dpe)
Sox2
NeuN
F
+
-
G
+
-

Figure S4. Misexpression of phosphomimetic constructs. A) Phosphomimetic construct S265E is still phosphorylated at T151 like S265A. Additionally, anti-phospho-S265 weakly recognizes S265E. B-C) Misexpression of Olig2 S265E does not lead to ectopic Chx10+ V2a INs or Hb9+ MNs (B) and is less efficient than Olig2 wild-type at generating ectopic Sox10+ OPCs (C). D-G) Olig2 3E misexpression results in reduced Chx10+ (D) and Hb9+ cells (E), as well as Hb9+ cells ectopically located next to the ventricle where progenitors reside (arrow). 3E misexpression also reduces the size of the electroporated side of the spinal cord. This reduction does not appear to result from depletion of progenitors, as the Sox2 progenitor domain is largely unperturbed (F). However, NeuN+ post-mitotic neurons are reduced (G) on the electroporated side of the spinal cord compared to the control side.

A


0hr 1hr 2hr 4hr 6hr
wt
S265E
3E

B

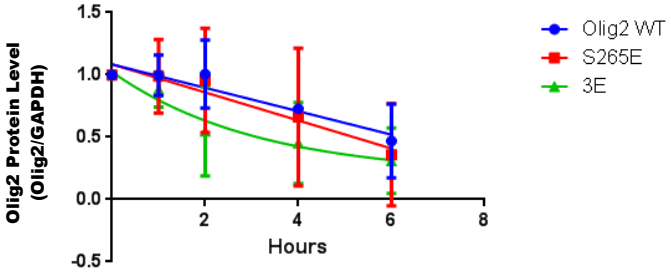

Olig2 Protein Level
(Olig2/GAPDH)
1.5
1.0
0.5
0.0
-0.5
2
4
6
8
Hours
Olig2 WT
S265E
3E

Figure S5. Protein half-life of Olig2 E mutants compared to wild-type. A) HEK293T cells were transfected with Olig2 wt, S265E, or 3E, treated with cycloheximide for the indicated times, and analyzed by immunoblot with anti-Olig2 and anti-GAPDH antibodies. B) Both S265E and 3E are less stable than Olig2 wild-type.

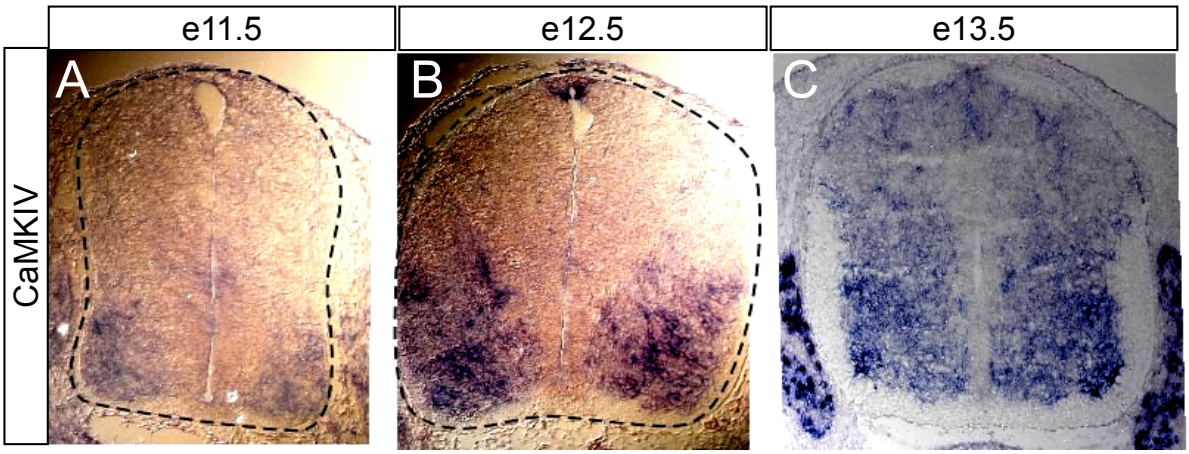
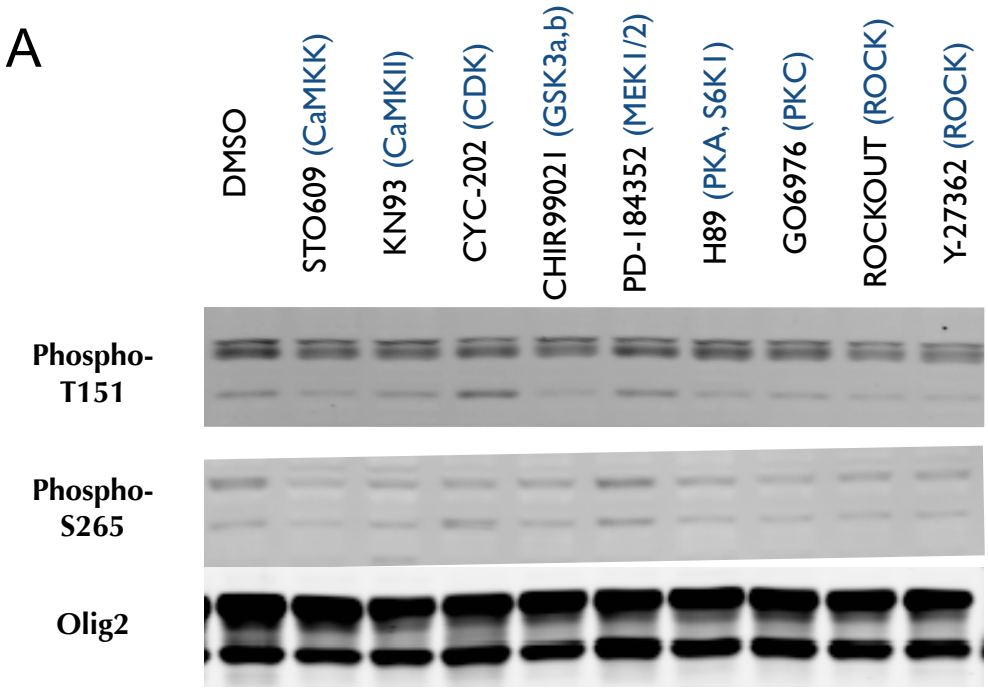
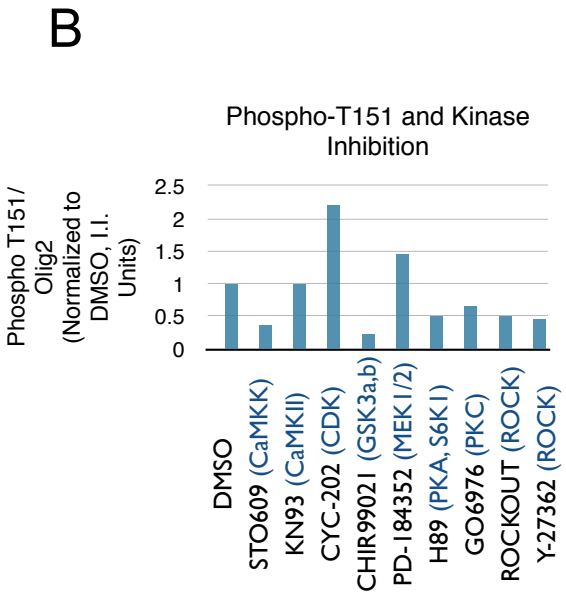

e11.5
e12.5
e13.5
CaMKIV
A
B
C

Figure S6. CaMKIV expression in neural tube development. A-C) Cross sections of mouse embryonic spinal cord were examined for CaMKIV expression by in situ hybridization. At e9.5 (A) and e12.5 (B), CaMKIV is specifically and highly expressed in the ventral horn where MNs reside. By e13.5, CaMKIV is more broadly expressed in the post-mitotic neurons but still shows high expression in MNs.


A
DMSO
STO609 (CaMKK)
KN93 (CaMKII)
CYC-202 (CDK)
CHIR99021 (GSK3a,b)
PD-184352 (MEK1/2)
H89 (PKA, S6K1)
GO6976 (PKC)
ROCKOUT (ROCK)
Y-27362 (ROCK)
Phospho-T151
Phospho-S265
Olig2


B
Phospho-T151 and Kinase Inhibition
Phospho T151/Olig2 (Normalized to DMSO, I.I. Units)
2.5
2
1.5
1
0.5
0
DMSO
STO609 (CaMKK)
KN93 (CaMKII)
CYC-202 (CDK)
CHIR99021 (GSK3a,b)
PD-184352 (MEK1/2)
H89 (PKA, S6K1)
GO6976 (PKC)
ROCKOUT (ROCK)
Y-27362 (ROCK)

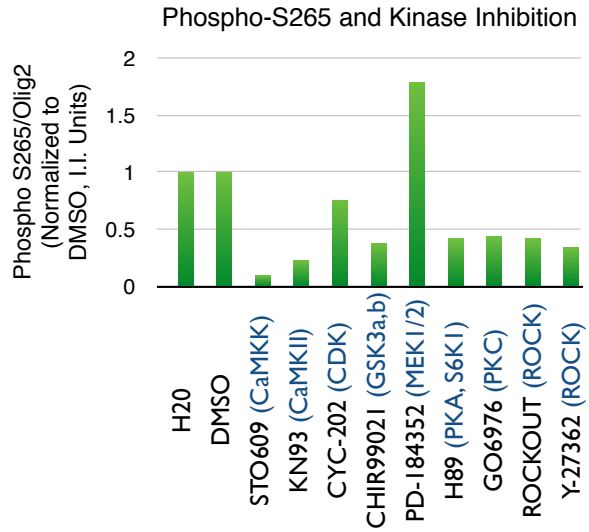

C
Phospho-S265 and Kinase Inhibition
Phospho S265/Olig2 (Normalized to DMSO, I.I. Units)
2
1.5
1
0.5
0
H20
DMSO
STO609 (CaMKK)
KN93 (CaMKII)
CYC-202 (CDK)
CHIR99021 (GSK3a,b)
PD-184352 (MEK1/2)
H89 (PKA, S6K1)
GO6976 (PKC)
ROCKOUT (ROCK)
Y-27362 (ROCK)

Figure S7. Screen of putative kinases with pharmacological inhibitors. A) HEK293T cells were transfected with Olig2 wild-type and treated with pharmacological inhibitors for putative kinases 24hrs post transfection. Changes in Olig2 phosphorylation was assayed by Western blot with anti-phospho and anti-total Olig2 antibodies. B-C) Phospho/total Olig2 band intensity was normalized to DMSO, vehicle, treatment and plotted. B) Inhibition of CaMKs, GsK3b, PKA, PKC, and ROCK decrease phospho-T151. C) Inhibition of CaMKs, GsK3b, CDKs, PKA, PKC, and ROCK decrease phospho-S265.

DISCUSSION

In this study, we show how processive phosphorylation of Olig2 at S147/T151 and then at S265 coordinate to govern the developmental activity of Olig2 in the spinal cord (Fig 6). Our data show that phosphorylation at S147 and T151 function in concert and both are required for both MN and OPC specification. Phosphorylation at these sites permits subsequent phosphorylation at S265, which serves as a critical regulatory site in the switch between the proliferative and pro-neural functions of Olig2. We also uncover mechanisms that account for these differing abilities. We have previously reported that the Olig2 homodimer is essential for MN specification and also produces OPCs and that the homodimer preferentially binds MN and OL fate specifying targets (Chapter 1). Here we show that, phosphorylation at S147/T151 increases homodimerization. Thus, the cell fate specification activity of S147/T151 is mediated, at least in part, through increased homodimerization preference. Subsequent S265 phosphorylation results in cytoplasmic translocation of Olig2 thereby down-regulating Olig2 expression and derepressing post-mitotic gene transcription, which allows for differentiation.

S147/T151 phosphorylation motif is required for proliferative and cell fate specifying functions of Olig2

Olig2 is required for the production of motor neurons and oligodendrocytes in the spinal cord. We show that Olig2 is phosphorylated at S147 and T151 in both MN progenitors and OPCs and that phosphorylation of these residues is necessary for the function of Olig2 in specifying these cell types. Mutating either of these sites to an unphosphorylatable A residue abolishes the ability of Olig2 to generate either MNs or OPCs in chick neural tube. Consistent with these results, S147 was previously shown to be critical for MN generation (Li et al. 2011). In contrast to our findings though, these authors show that in S147 phospho-null mice a small number of Sox10+ and

PDGFRa+ OPCs emerge by e18.5, indicating that the OPC specification is not lost but diminished and delayed (Li et al. 2011). However, our data, as well as that of Li et al, demonstrate that S147A has increased interaction with heterodimer partners Ngn2 and E47 and decreased interaction with Olig2 and Olig1 (Fig 4A-E; (Li et al. 2011). We have previously shown that only Olig2 homodimers are capable of generating MNs (Chapter 1). Additionally, only Olig2-Olig2 and Olig2-Olig1 can specify OPCs in chick neural tube (Chapter 1). Not only do the results of this Olig2 dimer study parallel those regarding Olig2 phosphorylation, together, they argue strongly that phosphorylation at S147 and T151 are required for both MN and OPC specification by increasing homodimer preference.

Additionally, both our in vitro data and results from chick neural tube electroporation indicate that S147 and T151 function as a single motif to regulate the cell fate specification function of Olig2. There is evidence that proximal phospho-sites function in concert, such that a particular change in a protein's behavior can be ascribed to a cluster rather than an individual phosphorylation site (Schweiger and Linial 2010). Additionally, these sites tend to be phosphorylated by the same kinase (Schweiger and Linial 2010). Because phosphorylation is often employed to radically affect the activity of a protein, as is the case with phospho-S147 and T151 for Olig2, it is thought that combining the phosphorylation of multiple sites increases the robustness of this effect. Furthermore, our data show that mutating either S147 or T151 to A results in a marked drop in Olig2 protein stability. These results further indicate the crucial importance of this motif to the function of Olig2 and suggest that mutations to either site would substantially disrupt MN and OPC generation.

Finally, consistent with the idea that S147/T151 are essential for the proliferative and cell fate specification function of Olig2, we found that Olig2 is phosphorylated at S147 and T151 predominately in mitotic cells (Fig 2D-E). Additionally, we noted that phospho-positive cells are

often Olig2-low (Fig 2A-C). This observation is well correlated with recent work, which shows that Olig2 levels oscillate over time in relation to cell cycle (Imayoshi et al. 2013).

Phosphorylation at S265 is an inactivating switch for Olig2

Our data demonstrate that phosphorylation of Olig2 is hierarchical and that phosphorylation of S147/T151 permits phosphorylation at S265 (Fig 1D). In contrast to S147/T151 phosphorylation, which is required for commitment to MN and OPC fate, phosphorylation at S265 halts the proliferative activity of Olig2. When Olig2 is phosphorylated at S147 and T151 but not at S265, as is the case with the S265A, we observed a significant increase in the production of ectopic cells compared to Olig2 wild-type misexpression (Fig 3H, L, O-P). Conversely, with S265E misexpression, which is constitutively phosphorylated at S265, resulted in a marked decrease the in the ability to produce MNs and OPCs compared to both Olig2 wild-type and S265A (Fig S4B-C). These results suggest that phosphorylation at S265 serves as a brake mechanism for the cell fate specification activity elicited by phosphorylation at S147/T151.

Interestingly, misexpression results with the triple phosphomimetic mutant (3E) also suggest that terminal phosphorylation at S265 halts the cell fate commitment role of Olig2 because, unlike with wild-type Olig2, dorsal ectopic MNs are not produced (Fig 4D-E). Consistent with the idea that S265 phosphorylation switches Olig2 from roles of progenitor maintenance and cell fate specification to differentiation, some ectopic MNs are observed in the ventricular zone where progenitors reside, suggesting that early phosphorylation of S265 results in premature differentiation of progenitors. However, widespread premature differentiation is not observed with this construct. When Olig2 is constitutively phosphorylated at all three residues, instead of allowing for temporally sequential phosphorylation of Olig2, the cell fate specification appears to be bypassed. These results indicate that timing of phosphorylation is important for Olig2 function. Furthermore, our

observation that Olig2 3E is only expressed in progenitors in the ventricular zone indicates that terminal phosphorylation at S265 may be related to the down-regulation of Olig2 in differentiating cells.

Immunostaining of both MN progenitors and OPCs demonstrate that phospho-S265 is localized in the cytoplasm (Fig 2C,I,M). These data suggest that phosphorylation at S265 results in nucleocytoplasmic translocation of Olig2. Furthermore, protein half-life experiments reveal that Olgi2 is significantly more stable when not phosphorylated at S265, indicating that phosphorylation at this site may play a role in the down-regulation of Olig2 expression. Since down-regulation of Olig2 expression is necessary for MN differentiation, understanding how this transition from proliferative to pro-neural activity is achieved in vivo answers an important developmental question. Interestingly, the region around this phospho-site (261-LKSPS-265) is a predicted phosphorylation-dependent, proline-directed sumoylation target based on consensus motif, which may provide a basis for how phosphorylation at S265 results in translocation of Olig2. Although nuclear export of Olig1 has been shown to be a critical step in the maturation of OLs (Niu et al. 2012), nucleocytoplasmic translocation of Olig2 has previously only been observed in disease states (Cassiani-Ingoni et al. 2006). To the best of our knowledge, this is the first report of nuclear export of endogenous Olig2 under non-injured conditions.

Lastly, we provide evidence that CaMKIV may be responsible for Olig2 phosphorylation at S265. We show that CaMKIV expression increases phopho-S265 in HEK293T cells and increases the number of phospho-S265+ cells in neural tube electroporation. Additionally, we show that expression of CaMKIV in vitro results in translocation of Olig2 to the cytoplasm. This finding parallels our observation from immunostaining that phospho-S265 signal is largely cytoplasmic. Although CaMKIV is predominately expressed in post-mitotic MNs, which no longer express Olig2, CaMKIV and Olig2 are co-expressed in newborn MNs migrating out of the ventricular zone as they

transition from progenitors to post-mitotic cells.

MATERIALS AND METHODS

DNA Constructs

For in ovo electroporation, RCASBP(B) myc-tagged chicken Olig2 (Novitch et al. 2001) was used as a wild-type expression construct. Additionally, this construct was used as a template to create S/T to A or E mutants using QuickChange II XL site directed mutagenesis kit per manufacturer instructions (Stratagene). The residues referenced are for mouse Olig2; the corresponding and conserved residues in chick Olig2 are as follows: mouse S147 = chick S145, mouse T151 = chick T149, mouse S265 = chick S247.

Mouse Olig2 mutants for cell transfection were generated by overlap extension PCR to introduce A or E mutations. These constructs were then subcloned into pEBG for GST pull-downs or into a modified version of pcDNA3 vector (Invitrogen) that includes a 3X-Flag-NLS for immunoprecipitation or whole cell lysate experiments.

In situ hybridization probes for Irx3 (gift from Bennett Novtich), PDGFRa (gift from Ben Deneen), and CaMKIV (ARK genomics) were generated from previously described plasmids.

In ovo electroporation and immunohistochemistry

Fertilized chicken eggs were electroporated at HH10-12 as previously described (Thaler et al. 2002). Embryos were collected at 3 or 5dpe, fixed in 4% PFA, and cryosectioned at 12um. Then, immunohistochemisty was performed in a 0.1% fish skin gelatin, 0.1% TX-100, PBS blocking buffer overnight at 4ºC with primary antibodies followed by Alexafluor secondaries. Sections were also counterstained with DAPI. Immunofluorescent images were acquired with a Nikon A1 Confocal. The ImageJ plugin was used for cell counts and GraphPad Prism was used to perform paired

Student's t-tests as described in figure legends. Data is presented as mean ± SEM.

Primary Antibodies for immunohistochemistry

The following antibodies were used: rabbit anti-Olig2, guinea pig anti-Chx10 (Thaler et al. 1999), mouse anti-MNR2/Hb9 (chick tissue, DSHB 5C10), guinea pig anti-Hb9, mouse anti-myc (Millipore and DSHB 9E10), rabbit anti-Sox10 (Abcam), mouse anti-MBP (Covance), rat anti-PDGFRa (BD Biosciences CD140), mouse anti-NeuN (Millipore, MAB377B), goat anti-Sox2 (Santa Cruz Biotechnology (sc-17320), goat anti-Gata3 (Santa Cruz Biotechnology (sc-1236), rabbit anti-phospho-histone H3 (Abcam), rabbit anti-CaMKIV (Cell Signaling). Phospho-specific Olig2 antibodies were generated and affinity purified by Bethyl Laboratories. Immunizing phospho-peptides were (CGPSVRKLpSKIATL for P-S147, CRKLSKIATLLLAR P-T151, CHGLLKSPpSPAAAAA P-S265) conjugated to KLH as a carrier and injected into sheep.

In situ hybridization

Digoxigenin-labeled riboprobes for in situ hybridization were generated by in vitro transcription labeling from the plasmids previously described then purified using a G50 column (GE Healthcare). Embryos were collected and sectioned in the same manner as for immunohistochemistry except 18um sections were collected. In situ hybridization was then performed as previously described () except the hybridization oven was kept at 72 degrees overnight, goat serum was used as a blocking agent in antibody staining steps in lieu of BSA, and slides were coverslipped with Mowiol mounting media.

ESC Culture and Differentiation

Mouse ES cell derived MNs were generated as previously described (Wichterle et al. 2002).

Briefly, mouse ES cells were cultured on a MEF monolayer in DMEM-KO media with 10% FBS, 0.1 mM non-essential amino acids (Gibco), 2 mM glutamine (Invitrogen), 0.1 mM â-mercaptoethanol (Sigma), and LIF. Then embryoid bodies were formed via the hanging drop method and transferred to low attachment plates for differentiation after two days. In order to direct MN differentiation, embryoid bodies were cultured in media containing 0.5uM RA + 1uM hedgehog agonist for 0-6 days as indicated in figures.

Primary cultured OPCs

Immunopanning, culture, and differentiation of rat OPCs was performed as previously described (Dugas and Emery 2013).

Immunoprecipitation and GST Pull-down Assays

HEK 293t cells were transfected using calcium phosphate method and lysed 48 hours later. GST pull-downs were performed overnight at 4ºC followed by SDS-PAGE and Western blotting. Blots were then probed sequentially with mouse anti-Flag M2 (Sigma) and mouse anti-GST (Sigma) primary antibodies in Odyssey blocking buffer overnight at 4ºC, probed with Licor IRdye 680LT or 800CW secondary antibodies for 1hr at room temperature, and imaged using Licor Odyssey. Odyssey software was then used to quantify relative band intensity.

Cycloheximide

Protein half-life of Olig2 wt and phospho-mutants were determined by cycloheximide time-course. HEK293T cells were transfected with Olig2 constructs listed above using Lipofectamine 2000 (Invitrogen) and treated with cycloheximide (40uM) 24 hours later. Cells were then lysed at 0, 1, 2, 4, and 6 hours and stored at -20ºC. Protein level was then assessed by running 50ug of lysate, as

determined by Bradford assay, on SDS-PAGE followed by Western blotting. Blots were probed overnight at 4ºC in Odyssey blocking buffer using mouse anti-Flag M2 for Olig2 and rabbit anti-GAPDH as a loading control. Liquor IRdye secondaries were used and blots were imaged on a Licor Odyssey. Odyssey software was used to quantify relative band intensity of both Olig2 mutants and GAPDH then Olig2 was normalized to GAPDH for analysis.

Pharmacological Kinase Inhibition

HEK293T cells were transfected with Olig2 wt using Lipofectamine 2000 (Invitrogen) and treated with protein kinase inhibitors, or DMSO for vehicle only treatment, 24 hours later. Dosage for individual inhibitors was chosen based on reported IC50 values for the candidate kinase in question. Following treatment and lysis, changes in Olig2 phosphorylation were analyzed by immunoblotting. Blots were probed with anti-phospho T151 or S265 and anti-pan Olig2 antibodies, as well as anti-GAPDH to assess equal loading across lanes. Western blots were then imaged and quantified using Licor Odyssey. A ratio of phospho- to total Olig2 was calculated for each inhibitor and vehicle. These ratios were normalized to vehicle only treatment, such that vehicle only is a ratio of 1, and graphed.

Conclusions

Summary

The bHLH transcription factor Olig2 performs essential, and often paradoxical, roles during development to specify diverse cell types, including motor neurons and oligodendrocytes. In order to promote the specification of these distinct cell types, Olig2 must differentially regulate gene expression in different cellular and developmental contexts. The work presented in this dissertation provides new insight into how Olig2 is regulated across spinal cord development in order to accomplish different tasks. The study presented in Chapter 1 reveals that choice of Olig2 dimerization partner directs cell specification and differentiation through recruitment of different Olig2 dimers to distinct genomic loci. Then, in Chapter 2, I demonstrate that phosphorylation of Olig2 at S147/T151 is required for motor neuron and oligodendrocyte specification. Phosphorylation at S147/T151 influences dimerization preference of Olig2, which provides a mechanistic basis for these distinct functions. Additionally, I propose that phosphorylation at S265, a putative CaMKIV site, serves as a switch to inactivate Olig2. These data show that, when phosphorylated at S147/T151 but not S265, Olig2 engages in proliferative and cell fate specification functions. When subsequently phosphorylated at S265, Olig2 becomes localized to the cytoplasm and is likely no longer transcriptionally active. As Olig2 inactivation is essential for motor neuron differentiation, S265 phosphorylation could be a mechanism to quickly and efficiently facilitate this process in differentiating pMN cells. Together, these data support to a model where external cues, relayed via protein kinases, regulate the temporal phosphorylation of Olig2. Differential phosphorylation of Olig2, in turn, governs Olig2 dimerization and subsequent binding of gene regulatory elements and sub-cellular localization to mediate the proliferative, cell fate specifying, and pro-neural functions of Olig2.

Conclusions and Future Directions

The overarching goal of this dissertation was to investigate the mechanisms by which Olig2 is capable of mediating disparate functions during CNS development. I approached this question by primarily focusing on phosphorylation and dimerization of Olig2, which I believe coordinate to govern the temporally dependent developmental roles of Olig2.

In Chapter 2, I present that Olig2 is initially phosphorylated at S147 and T151. Both of these sites are required for the production of both motor neurons and oligodendrocytes. Additionally, mutating either residue to an unphosphorylatable residue abolishes phosphorylation at the other residue. I propose that these two residues function as a single motif because of their interdependence and identical functions. Furthermore, cooperation between proximal phosphorylation sites is a strategy employed by a variety of proteins. Although the role of S147 was previously reported (Li et al. 2011), the fact that S147 is actually part of motif is an important observation, especially in light of the growing interest in using Olig2 phospho-sites as targets for therapeutics. Although I did not identify the kinase responsible for phosphorylating these residues, I did narrow down the list of kinases predicted by consensus motif using a combination of expression data, pharmacological inhibition of candidate kinases, and co-electroporation of dominant negative or constitutive active kinases with Olig2 in chick neural tube. Together, these data suggest that Gsk3b, PKC, or ROCK may be involved in S147/T151 phosphorylation, but further studies will be needed to verify the endogenously acting kinase. Additionally, my data show that phosphorylation at S147 and T151 influence Olig2 dimerization preference, providing a mechanistic basis for their effect on Olig2 activity.

Unlike other bHLH dimers, the role of individual dimers in producing Olig2-derived cell types was previously unexplored for Olig2 (Castanon et al. 2001; Yang et al. 2009; Dilworth et al. 2004; Neuhold and Wold 1993). Because many class A bHLH proteins form transcriptionally active

dimers with E-proteins and Olig2 has been shown to interact with E12 and E47, it was assumed that Olig2 dimers with E12 and E47 were responsible for the cell fate specification roles of Olig2 (Samanta and Kessler 2004). However, Olig2 was also shown to have high-affinity interactions with itself (Lee et al. 2005; Li et al. 2008), which raised the question of whether the Olig2 homodimer plays specific developmental roles and whether different Olig2 dimers have distinct roles. To investigate this question, I created constuitively dimerized constructs for each known Olig2 dimer using a flexible tether, which has previously been reported (Chapter 1, Neuhold and Wold 1993). I found that Olig2 dimers do have distinct functions (Chapter 1). In contrast to what was previously hypothesized, the Olig2-E47 heterodimer is not necessary for, or even capable of, generating motor neurons or oligodendrocytes. The same results were seen for the heterodimer with Ngn2, a pro-neural bHLH transcription factor that has been shown to be essential for motor neuron generation (Lee et al. 2005). Furthermore, misexpression data with these dimers suggest that Olig2-E47 and Olig2-Ngn2 may function to sequester Olig2 away from transcriptional activity and may be involved in the down-regulating Olig2 expression.

These results are well correlated with my study of Olig2 phosphorylation (Chapter 2). Neither S147A nor T151A produce ectopic motor neurons or oligodendrocyte precursors. Additionally, both phospho-null mutants show decreased homodimerization and increased interaction with heterodimer partners E47 and Ngn2. Following the cell fate specification activity of Olig2, which is controlled by initial phosphorylation at S147 and T151, subsequent phosphorylation at S265 may be coupled with dephosphorylation at S147/T151 to facilitate the down-regulation of Olig2 through increased dimerization with Ngn2 and E47.

My results also demonstrate that Olig2 homodimers are necessary for motor neuron specification and to produce oligodendroctye precursor cells. Although not unheard of, cell fate specification by bHLH homodimers is rare. Beyond being a novel finding, this observation could

have important therapeutic implications. Because the Olig2 homodimer is responsible for generating both motor neurons and oligodendrocytes in the spinal cord, the expression of another bHLH is less relevant and, thus, simplifies the design of therapeutics to target Olig2. In addition to uncovering the biological roles of the Olig2 homodimer, this work also provides evidence for the strong homodimerization preference of Olig2. I show that the C-terminus of Olig2 is capable of self-interaction but does not interact with other dimerization partners. This finding is especially intriguing since there is no predicted domains or motifs in the C-terminus of Olig2, aside from the S265 phosphorylation site. Although I did investigate whether changes in phosphorylation at S265 account for the secondary dimerization domain, this does not seem to be the case. S265A exhibits reduced interaction with the Olig2 C-terminus but mutating this residue does not abolish its interaction through the C-terminus, which indicates that other residues are likely involved in this interaction. Initial attempts to crystalize Olig2 have been unsuccessful, as have attempts to narrow down the critical residues involved in this interaction using liquid phase NMR. Future studies into this residue will be critical for understanding the strong preference of Olig2 to form homodimers, and thereby could provide insight into the dynamic and unique regulation of this essential transcription factor.

Finally, I show that Olig1-Olig2 heterodimers can also produce oligodendrocyte precursors. However, given that expression levels of Olig1 are relatively low at the onset of oligodendrogenesis, the Olig2 homodimer is likely primarily responsible for the specification of oligodendrocytes during development. Furthermore, my analysis of target gene binding between Olig2-Olig2 and Olig2-Olig1 reveals that the Olig2 homodimer preferentially binds fate specifying and early expressed oligodendrocyte target genes, as well as motor neuron targets. In contrast, Olig2-Olig1 preferentially binds oligodendrocyte gene loci that are involved in maturation and myelinogenesis of oligodendrocytes. These differences in genomic binding preferences provide a mechanistic basis for

the divergent roles of these dimers during development.

During development, specialized cell types not only need to be produced, but they also need to be generated at a precise time and in the proper number. In addition to uncovering phosphorylation sites that ensure that Olig2 specifies motor neurons and oligodendrocytes, we found a novel phosphorylation site, S265, that governs the second aspect of this important developmental balance. I show that S265 is only phosphorylated following S147/T151 phosphorylation and disabling this temporally sequential phosphorylation disrupts the activity of Olig2. As previously described, initial phosphorylation at S147/T151 is essential for the progenitor maintenance and cell fate commitment roles of Olig2. When S265 cannot be subsequently phosphorylated, as is the case with the S265A mutant, Olig2 continues to produce motor neurons and oligodendrocytes unchecked, resulting in a rampant overproduction of both cell types. However, when Olig2 can be phosphorylated at S265, phospho-S265 Olig2 is localized in the cytoplasm in both motor neuron progenitors and oligodendrocyte precursor cells. Consistent with this finding, a fraction of total Olig2 is observed in the cytoplasm of these same cells. Prior to this work, cytoplasmic translocation of Olig2 had only been reported in injury models (Magnus et al. 2007) and during astrocyte specification (Setoguchi and Kondo 2004). In contrast, nucleocytoplasmic translocation of Olig1, a closely related bHLH, has been shown to play a role in oligodendrocyte maturation (Niu et al. 2012). Thus, although Olig2 was not thought to be localized in the cytoplasm, this observation is congruous with other findings. Furthermore, nucleocytoplasmic translocation of a transcription factor is a commonly employed means of inactivating transcriptional activity. Because phosphorylation at S265 appears to switch the activity of Olig2 from proliferation to differentiation, I propose that translocation of Olig2 as the mechanism for this change in activity.

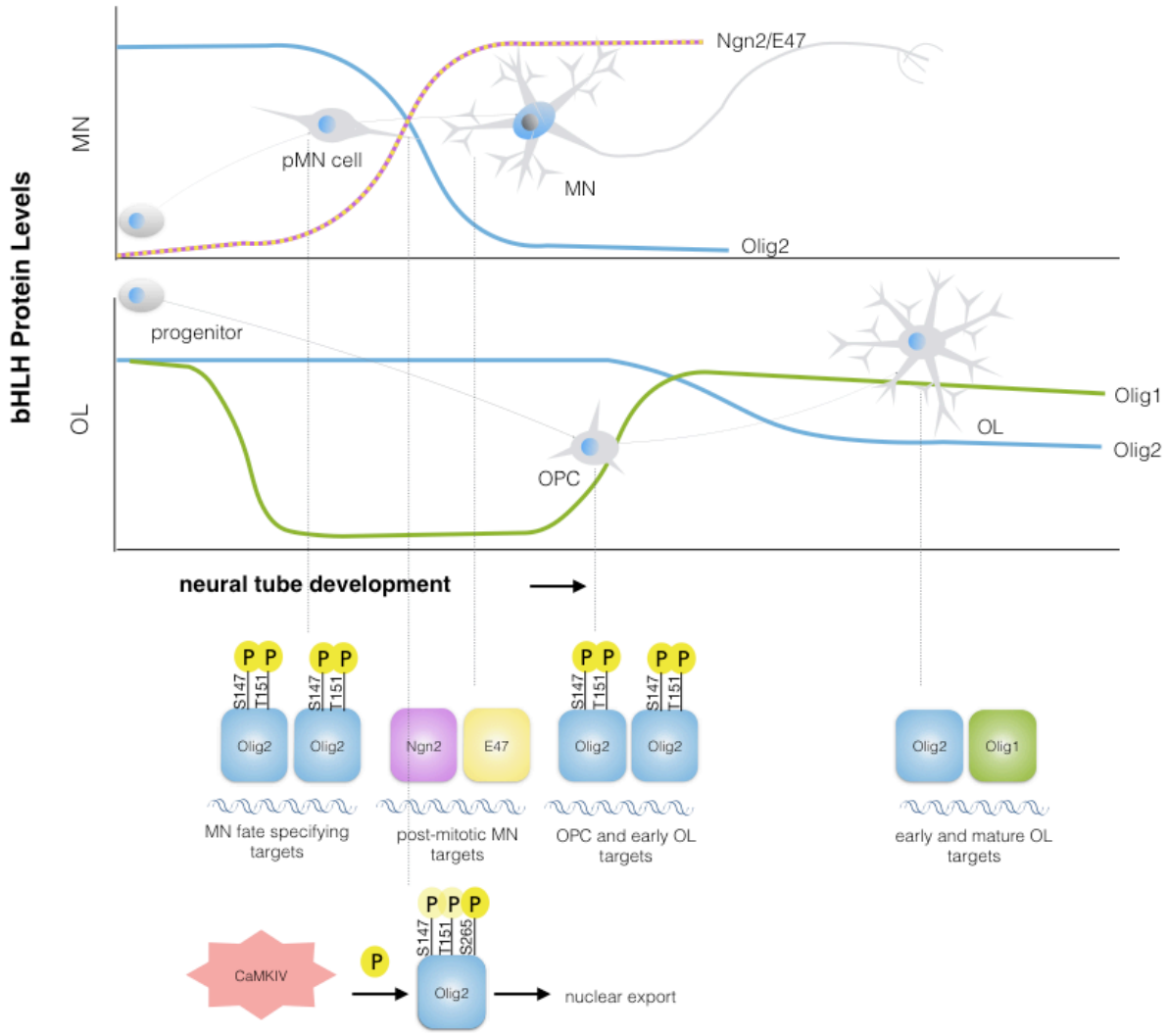


Figure 1. Integrated model.
A summary of the roles both phosphorylation and dimerization play in the specification of motor neurons and oligodendrocytes.

Implications

Although this work focuses on the roles of Olig2 in the developing spinal cord, Olig2 plays similar roles in the developing telencephalon as well. During telencephalon development, Olig2 generates oligodendrocytes and is involved in cholinergic neuron production. Therefore, the findings in this study may be more broadly applicable to CNS development. Further investigation is needed to determine whether Olig2 activity is similarly regulated during cortical development.

Beyond providing insight into the molecular basis for the disparate activities of Olig2 during development, understanding the factors that regulate Olig2 has important clinical ramifications. Olig2 is either causative or implicated in a number of human disease states and disorders, such as Down Syndrome, glioma, and reactive gliosis. Additionally, a number of diseases involve the loss or dysfunction of Olig2 derived cell types. Disorders like spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) result in the selective loss of motor neurons, whereas MS disrupts myelination. Treatment of these disorders will likely rely on our knowledge of the mechanisms that govern the specification and maintenance of these cell types. Therefore, understanding the factors that influence Olig2 activity in different cellular contexts could prove useful in addressing human conditions in the future. Small molecule inhibitors for Olig2 kinases are already of interest as therapeutics for glioma but could potentially be useful in other contexts to modulate the function of Olig2.

Appendix

An Investigation of Olig2 in Down Syndrome Motor Deficits

Down Syndrome is the most prevalent congenital defect in the United States. Roughly 1 in 737 live births present with Down Syndrome, and this number is even higher for total pregnancies (Parker et al. 2010). The prevalence of this chromosomal condition makes Down Syndrome an important public health concern. Down Syndrome results from the nondisjunction and triplication of human chromosome 21 and leads to a variety of disabilities in affected persons. This variability depends on whether the entire chromosome is triplicated or just a part. However, some features of Down Syndrome are nearly universal, regardless of which portion of chromosome 21 is triplicated. Dysmorphic facial features and cognitive impairment are the most widely recognizable of these unvarying characteristics. In addition, hypotonia, or poor muscle tone, and motor dysfunction, including delayed gross and fine motor skills, are consistent features of Down Syndrome. Given that the region most invariably triplicated in Down Syndrome contains over 300 genes, identifying which genes are responsible for the key features of Down Syndrome has proved a considerable challenge. Currently, little is known about the specific genes and pathways that are responsible for the phenotypes seen in Down Syndrome. Although some genes that are important in Down Syndrome have been identified, we still lack a complete understanding of which genes are critical in Down Syndrome and could potentially serve as therapeutic targets. This research focuses on one gene, Olig2, which is consistently triplicated in Down Syndrome.

During embryonic development, Olig2 is essential for producing several cell types in the CNS. In the developing spinal cord, Olig2 is responsible for generating motor neurons and oligodendrocytes (Novitch et al. 2001; Mizuguchi et al. 2001; Lu et al. 2002; Zhou et al. 2001; Park et al. 2002). Additionally, Olig2 has been shown to play a crucial role in the production of astrocytes derived from ventral progenitor domains (Setoguchi and Kondo 2004; Agius et al. 2004). Olig2 plays similar cell fate-specifying roles in the developing telencephalon. In the ventral telencephalon, Olig2 is involved in a binary cell fate choice between GABAergic interneuron and oligodendrocyte

production (He et al. 2001; Yung et al. 2002; Petryniak et al. 2007). Olig2 is also required for specification of oligodendrocyte precursors in the forebrain. Furthermore, recent evidence suggests that Olig2 is involved in the generation of basal forebrain neurons that, like spinal motor neurons, are cholinergic (Furusho et al. 2006). Because of these diverse and essential roles of Olig2 in spinal cord and brain development, triplication of the Olig2 gene in Down Syndrome could have profound effects.

Recently, triplication of Olig1/2 were found to be a major cause of the cognitive impairment seen in people with Down Syndrome (Chakrabarti et al. 2010). In a mouse model of Down Syndrome (Ts65Dn), there is an overproduction of specific interneuron populations in both the neocortex and hippocampus, as well as an increased rate of sIPSCs. These interneurons are produced from bipotent progenitors that give rise to both oligodendrocytes and GABAergic interneurons (He et al. 2001; Yung et al. 2002). Although Olig2, Dlx1/2, and Mash1/Ascl1 are all implicated in the cell fate choice of these progenitors (Petryniak et al. 2007), only Olig2 is triplicated in Down Syndrome. For this reason, Chakrabarti et al investigated whether triplication of Olig2 was related to interneuron overproduction in Down Syndrome mice. They found that triplication of Olig2 results in a significant increase in the expression of Olig2 in the developing telencephalon, based on both in situ hybridization and RT-PCR (Chakrabarti et al. 2010). By crossing these Down Syndrome model mice to Olig1/2 heterozygous mice (to generate mice with two copies of Olig1/2), Olig2 expression was rescued to wild-type levels (Chakrabarti et al. 2010). Furthermore, the inhibitory interneuron phenotype, including the overproduction of GABAergic interneurons and increased sIPSCs, are rescued in Ts65Dn Olig1+/-; Olig2+/- mice (Chakrabarti et al. 2010). From these results, the authors conclude that overexpression of Olig2 in Down Syndrome results in overproduction of interneurons and increased spontaneous firing, which, at least in part, account for the cognitive impairments seen in Down Syndrome.

Since Olig2 is involved in spinal cord development and is essential for generating motor neurons, I sought to investigate whether triplicating Olig2 is also responsible for all or part of the motor dysfunction seen in Down Syndrome. The motor dysfunction seen in people with Down Syndrome represents a profound disability, which is evident at birth and persists through adulthood. Infants with Down Syndrome have hypotonia, which is evident at birth. Additionally, delayed motor skill development is observed in people with Down Syndrome. Furthermore, people with Down Syndrome display difficulties with intentional motor tasks, which contributes to difficulty moving and performing tasks. All of these features are present in childhood and persist into adulthood. Thus, Down Syndrome involves a range of motor deficits that are physically limiting. However, the cause of these motor dysfunction characteristics remain unexplained. Given the essential role of Olig2 for motor system development, from progenitor patterning through motor neuron and oligodendrocyte specification, and the involvement of Olig2 in the cognitive impairment seen in Down Syndrome, Olig2 is a strong candidate for the motor dysfunction seen in Down Syndrome.

In order to investigate whether triplicating the Olig2 gene in Down Syndrome results in altered motor system development and subsequent motor system dysfunction, I examined spinal cord development and motor neuron production using a mouse model of Down Syndrome. Although many previous Down Syndrome mouse studies have used the mouse line Ts65Dn, I chose to use a relatively new mouse line called Tc1. Because the genes found on human chromosome 21 lie on mouse chromosomes 16 and 17, Ts65Dn mice were created by reciprocal translocation and result in mice that are trisomic for a segment of chromosome 16 and 17. Unlike Ts65Dn, Tc1 mice contain a freely segregating portion of human chromosome 21, which contains the genes most frequently triplicated in Down Syndrome (O'Doherty et al. 2005). Previous studies have shown that the Tc1 mouse model exhibits key features of Down Syndrome, including motor dysfunction (Costa et al. 1999; Galante et al. 2009). Furthermore, Tc1 mice do not have additional features found in

Ts65Dn mice—male sterility and recessive retinal degeneration that results in blindness—that are not associated with Down Syndrome. Together, these features led us to consider Tc1 a more appropriate and complete model of Down Syndrome for our studies.

Using these mice, I compared motor neuron and oligodendrocyte production in trisomic mice to wild-type littermates. As others have noted, Tc1 mice had significantly smaller spinal cords than wild type littermates at e15.5, based on cross-section area. Additionally, consistent with the finding that Down Syndrome model mice have more Olig2 in the cortex as assessed by RT-PCR (Chakrabarti et al. 2010), there is also a significant increase in Olig2+ cells in Tc1 mouse spinal cords based on immunohistochemistry. Despite their small spinal cord area, these mice had significantly more Hb9+motor neurons. This finding is particularly interesting considering that the functional ramifications of this result are unknown. Although this increase in motor neurons is consistent with chick neural tube electroporation data in which Olig2 over-expression results in ectopic motor neuron production (Novitch et al. 2001), to the best of our knowledge, no human disorders have been shown to involve an over-production of spinal motor neurons. Physiological studies are needed to determine whether over-production of motor neurons results in an imbalance of excitatory and inhibitory signals in the brain similar to the effect of interneuron over-production in the cortex. Additionally, Tc1;Olig2+/- mice need to be examined to

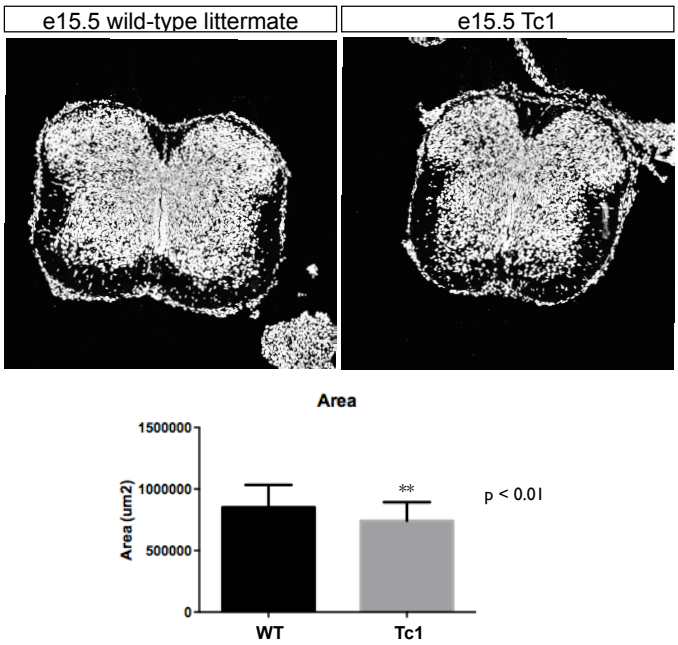


Figure1. The cross-sectional area of the Tc1 mouse spinal cord is significantly smaller than that of wild-type littermates at e15.5

assess whether rescuing the gene dosage of Olig2 rescues motor neuron number. Unfortunately, no mice of this genotype were obtained in four litters thus far. In addition to examining motor neurons, I also stained with interneuron markers. This preliminary analysis indicates that there is no statistically significant difference in the number of ventral interneurons produced in mutant animals. This observation is surprising because Olig2 over-expression in chick neural tube results in dorsal ectopic motor neuron expansion at the expense of more dorsal interneuron populations (Novitch et al. 2001). Further investigation is needed to determine whether any other cell subtypes are affected by Olig2 over-expression. Lastly, because Olig2 is required for oligodendrocyte specification, I assessed whether oligodendrocyte production was altered by immunohistochemistry. Initial analysis indicates that there may be more oligodendrocytes. However, this assessment is based on the increase in Olig2+ cells outside the pMN domain, which are likely oligodendrocyte precursors cells. Other oligodendrocyte markers were examined, including Sox10, PDGFRa and MBP, but will need to be quantified in order to determine whether the increase in Olig2+ OPCs translates to an increase in mature oligodendrocytes in Tc1 spinal cords. Together, these data provide preliminary evidence that Olig2 may play a role in Down Syndrome motor deficits through aberrant motor neuron generation and, possibly, oligodendrocyte production.

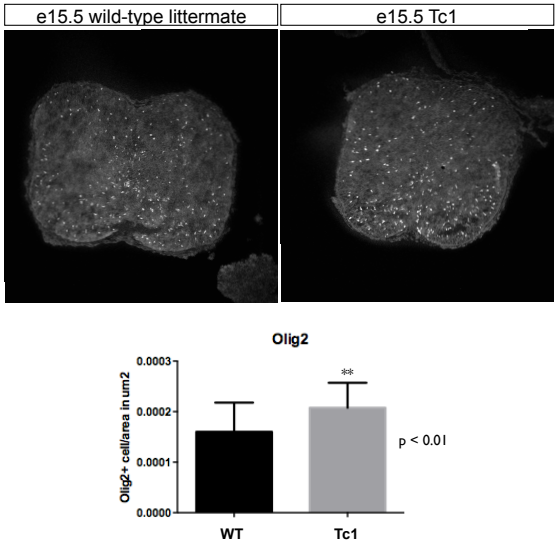


Figure 2. Tc1 mice have significantly more spinal cord Olig2+ cells.

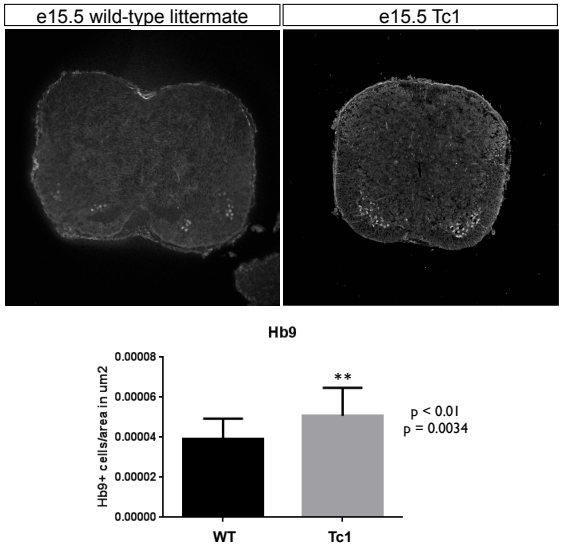


Figure 3. There are more Hb9+ motor neurons per area in e15.5 Tc1 spinal cords compared to wild-type littermates.

REFERENCES

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

Amoutzias GD, Robertson DL, Oliver SG, Bornberg-Bauer E. 2004. Convergent evolution of gene networks by single-gene duplications in higher eukaryotes. *EMBO Rep* **5**: 274–279.

Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S. 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* **23**: 659–674.

Bachoo RM, Kim RS, Ligon KL, Maher E a, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH, et al. 2004. Molecular diversity of astrocytes with implications for neurological disorders. *Proc Natl Acad Sci U S A* **101**: 8384–8389.

Bertrand N, Castro DS, Guillemot F. 2002. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* **3**: 517–530.

Briscoe J, Pierani a, Jessell TM, Ericson J. 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**: 435–445.

Bronchain OJ, Pollet N, Ymlahi-Ouazzani Q, Dhorne-Pollet S, Helbling JC, Lecarpentier JE, Percheron K, Wegnez M. 2007. The olig family: Phylogenetic analysis and early gene expression in Xenopus tropicalis. *Dev Genes Evol* **217**: 485–497.

Brookfield JFY. 2003. Gene duplications: the gradual evolution of functional divergence. *Curr Biol* **13**: R229–R230.

Buffo A, Vosko MR, Ertürk D, Hamann GF, Jucker M, Rowitch D, Götz M. 2005. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *Proc Natl Acad Sci U S A* **102**: 18183–18188.

Cai J, Chen Y, Cai W-H, Hurlock EC, Wu H, Kernie SG, Parada LF, Lu QR. 2007. A crucial role for Olig2 in white matter astrocyte development. *Development* **134**: 1887–1899.

Cai J, Qi Y, Hu X, Tan M, Liu Z, Zhang J, Li Q, Sander M, Qiu M. 2005. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron* **45**: 41–53.

Cassiani-Ingoni R, Coksaygan T, Xue H, Reichert-Scrivner S a., Wiendl H, Rao MS, Magnus T. 2006. Cytoplasmic translocation of Olig2 in adult glial progenitors marks the

generation of reactive astrocytes following autoimmune inflammation. *Exp Neurol* **201**: 349–358.

Castanon I, Von Stetina S, Kass J, Baylies MK. 2001. Dimerization partners determine the activity of the Twist bHLH protein during Drosophila mesoderm development. *Development* **128**: 3145–3159.

Chakrabarti L, Best TK, Cramer NP, Carney RSE, Isaac JTR, Galdzicki Z, Haydar TF. 2010. Olig1 and Olig2 triplication causes developmental brain defects in Down syndrome. *Nat Neurosci* **13**: 927–934. http://dx.doi.org/10.1038/nn.2600.

Chen JA, Huang YP, Mazzoni EO, Tan GC, Zavadil J, Wichterle H. 2011. Mir-17-3p Controls Spinal Neural Progenitor Patterning by Regulating Olig2/Irx3 Cross-Repressive Loop. *Neuron* **69**: 721–735. http://dx.doi.org/10.1016/j.neuron.2011.01.014.

Chen Y, Miles DK, Hoang T, Shi J, Hurlock E, Kernie SG, Lu QR. 2008. The basic helix-loop-helix transcription factor olig2 is critical for reactive astrocyte proliferation after cortical injury. *J Neurosci* **28**: 10983–10989.

Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E. 2006. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. *Stem Cells* **24**: 1001–1010.

Costa a C, Walsh K, Davisson MT. 1999. Motor dysfunction in a mouse model for Down syndrome. *Physiol Behav* **68**: 211–20. http://www.ncbi.nlm.nih.gov/pubmed/10627083.

Dessaud E, Ribes V, Balaskas N, Yang LL, Pierani A, Kicheva A, Novitch BG, Briscoe J, Sasai N. 2010. Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog. *PLoS Biol* **8**.

Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, Mynett A, Novitch BG, Briscoe J. 2007. Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* **450**: 717–720.

Dilworth FJ, Seaver KJ, Fishburn AL, Htet SL, Tapscott SJ. 2004. In vitro transcription system delineates the distinct roles of the coactivators pCAF and p300 during MyoD/E47-dependent transactivation. *Proc Natl Acad Sci U S A* **101**: 11593–11598.

Ding L, Takebayashi H, Watanabe K, Ohtsuki T, Tanaka KF, Nabeshima YI, Chisaka O, Ikenaka K, Ono K. 2005. Short-term lineage analysis of dorsally derived Olig3 cells in the developing spinal cord. *Dev Dyn* **234**: 622–632.

Dugas JC, Emery B. 2013. Purification of oligodendrocyte precursor cells from rat cortices

by immunopanning. *Cold Spring Harb Protoc* **2013**: 745–758.

Dugas JC, Tai YC, Speed TP, Ngai J, Barres B a. 2006. Functional genomic analysis of oligodendrocyte differentiation. *J Neurosci* **26**: 10967–10983.

Ellenberger T, Fass D, Arnaud M, Harrison SC. 1994. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev* **8**: 970–980.

Ericson J, Rashbass P, Schedl a., Brenner-Morton S, Kawakami a., Van Heyningen V, Jessell TM, Briscoe J. 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**: 169–180.

Filippi A, Tiso N, Deflorian G, Zecchin E, Bortolussi M, Argenton F. 2005. The basic helix-loop-helix olig3 establishes the neural plate boundary of the trunk and is necessary for development of the dorsal spinal cord. *Proc Natl Acad Sci U S A* **102**: 4377–4382.

Fogarty M, Richardson WD, Kessaris N. 2005. A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development* **132**: 1951–1959.

Fu H, Qi Y, Tan M, Cai J, Takebayashi H, Nakafuku M, Richardson W, Qiu M. 2002. Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation. *Development* **129**: 681–693.

Fukuda S, Kondo T, Takebayashi H, Taga T. 2004. Negative regulatory effect of an oligodendrocytic bHLH factor OLIG2 on the astrocytic differentiation pathway. *Cell Death Differ* **11**: 196–202.

Furusho M, Ono K, Takebayashi H, Masahira N, Kagawa T, Ikeda K, Ikenaka K. 2006. Involvement of the Olig2 transcription factor in cholinergic neuron development of the basal forebrain. *Dev Biol* **293**: 348–357.

Gabay L, Lowell S, Rubin LL, Anderson DJ. 2003. Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. *Neuron* **40**: 485–499.

Galante M, Jani H, Vanes L, Daniel H, Fisher EMC, Tybulewicz VLJ, Bliss TVP, Morice E. 2009. Impairments in motor coordination without major changes in cerebellar plasticity in the Tc1 mouse model of Down syndrome. *Hum Mol Genet* **18**: 1449–1463.

Goldfarb AN, Lewandowska K, Pennell C a. 1998. Identification of a highly conserved module in E proteins required for in vivo helix-loop-helix dimerization. *J Biol Chem* **273**:

2866–2873.

Gyuris J, Golemis E, Chertkov H, Brent R. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**: 791–803.

He W, Ingraham C, Rising L, Goderie S, Temple S. 2001. Multipotent stem cells from the mouse basal forebrain contribute GABAergic neurons and oligodendrocytes to the cerebral cortex during embryogenesis. *J Neurosci* **21**: 8854–8862.

Hoang-Xuan K, Aguirre-Cruz L, Mokhtari K, Marie Y, Sanson M. 2002. OLIG-1 and 2 gene expression and oligodendroglial tumours. *Neuropathol Appl Neurobiol* **28**: 89–94.

Hochstim C, Deneen B, Lukaszewicz A, Zhou Q, Anderson DJ. 2008. Identification of Positionally Distinct Astrocyte Subtypes whose Identities Are Specified by a Homeodomain Code. *Cell* **133**: 510–522.

Holmberg J, Hansson E, Malewicz M, Sandberg M, Perlmann T, Lendahl U, Muhr J. 2008. SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. *Development* **135**: 1843–1851.

Hu JG, Shen L, Wang R, Wang QY, Zhang C, Xi J, Ma SF, Zhou JS, Lü HZ. 2012. Effects of Olig2-Overexpressing Neural Stem Cells and Myelin Basic Protein-Activated T Cells on Recovery from Spinal Cord Injury. *Neurotherapeutics* **9**: 422–445.

Imayoshi I, Isomura A, Harima Y, Kawaguchi K, Kori H, Miyachi H, Fujiwara T, Ishidate F, Kageyama R. 2013. Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* **342**: 1203–8. http://www.ncbi.nlm.nih.gov/pubmed/24179156.

Küspert M, Hammer A, Bösl MR, Wegner M. 2011. Olig2 regulates Sox10 expression in oligodendrocyte precursors through an evolutionary conserved distal enhancer. *Nucleic Acids Res* **39**: 1280–1293.

Lassar a B, Davis RL, Wright WE, Kadesch T, Murre C, Voronova a, Baltimore D, Weintraub H. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**: 305–315.

Lee S, Lee B, Joshi K, Pfaff SL, Lee JW, Lee SK. 2008. A Regulatory Network to Segregate the Identity of Neuronal Subtypes. *Dev Cell* **14**: 877–889.

Lee S-K, Jurata LW, Funahashi J, Ruiz EC, Pfaff SL. 2004. Analysis of embryonic motoneuron gene regulation: derepression of general activators function in concert with

enhancer factors. *Development* **131**: 3295–3306.

Lee SK, Lee B, Ruiz EC, Pfaff SL. 2005. Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. *Genes Dev* **19**: 282–294.

Lee SK, Na SY, Kim HJ, Soh J, Choi HS, Lee JW. 1998. Identification of critical residues for heterodimerization within the ligand-binding domain of retinoid X receptor. *Mol Endocrinol* **12**: 325–332.

Lee SK, Pfaff SL. 2003. Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. *Neuron* **38**: 731–745.

Li H, Paes de Faria J, Andrew P, Nitarska J, Richardson WD. 2011. Phosphorylation Regulates OLIG2 Cofactor Choice and the Motor Neuron-Oligodendrocyte Fate Switch. *Neuron* **69**: 918–929. http://dx.doi.org/10.1016/j.neuron.2011.01.030.

Li XJ, Hu BY, Jones S a., Zhang YS, LaVaute T, Du ZW, Zhang SC. 2008. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells* **26**: 886–893. http://onlinelibrary.wiley.com/doi/10.1634/stemcells.2007-0620/full.

Ligon KL, Alberta J a, Kho AT, Weiss J, Kwaan MR, Nutt CL, Louis DN, Stiles CD, Rowitch DH. 2004. The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. *J Neuropathol Exp Neurol* **63**: 499–509.

Ligon KL, Huillard E, Mehta S, Kesari S, Liu H, Alberta J a., Bachoo RM, Kane M, Louis DN, DePinho R a., et al. 2007. Olig2-Regulated Lineage-Restricted Pathway Controls Replication Competence in Neural Stem Cells and Malignant Glioma. *Neuron* **53**: 503–517.

Liu Z, Hu X, Cai J, Liu B, Peng X, Wegner M, Qiu M. 2007. Induction of oligodendrocyte differentiation by Olig2 and Sox10: Evidence for reciprocal interactions and dosage-dependent mechanisms. *Dev Biol* **302**: 683–693.

Lluís F, Ballestar E, Suelves M, Esteller M, Muñoz-Cánoves P. 2005. E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. *EMBO J* **24**: 974–984.

Lu J, Lian G, Zhou H, Esposito G, Steardo L, Delli-Bovi LC, Hecht JL, Lu QR, Sheen V. 2012. OLIG2 over-expression impairs proliferation of human down syndrome neural progenitors. *Hum Mol Genet* **21**: 2330–2340.

Lu QR, Park JK, Noll E, Chan J a, Alberta J, Yuk D, Alzamora MG, Louis DN, Stiles CD,

Rowitch DH, et al. 2001. Oligodendrocyte lineage genes (OLIG) as molecular markers for human glial brain tumors. *Proc Natl Acad Sci U S A* **98**: 10851–10856.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* **109**: 75–86.

Lu QR, Yuk D, Alberta J a, Zhu Z, Pawlitzky I, Chan J, McMahon a P, Stiles CD, Rowitch DH. 2000. Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* **25**: 317–329.

Lynch M, Katju V. 2004. The altered evolutionary trajectories of gene duplicates. *Trends Genet* **20**: 544–549.

Ma PCM, Rould M a., Weintraub H, Pabo CO. 1994. Crystal structure of MyoD bHLH domain-DNA complex: Perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**: 451–459.

Ma YC, Song MR, Park JP, Henry Ho HY, Hu L, Kurtev M V., Zieg J, Ma Q, Pfaff SL, Greenberg ME. 2008. Regulation of Motor Neuron Specification by Phosphorylation of Neurogenin 2. *Neuron* **58**: 65–77.

Marie Y, Sanson M, Mokhtari K, Leuraud P, Kujas M, Delattre JY, Poirier J, Zalc B, Hoang-Xuan K. 2001. OLIG2 as a specific marker of oligodendroglial tumour cells. *Lancet* **358**: 298–300.

Marshall C a G, Novitch BG, Goldman JE. 2005. Olig2 directs astrocyte and oligodendrocyte formation in postnatal subventricular zone cells. *J Neurosci* **25**: 7289–7298.

Masahira N, Takebayashi H, Ono K, Watanabe K, Ding L, Furusho M, Ogawa Y, Nabeshima YI, Alvarez-Buylla A, Shimizu K, et al. 2006. Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. *Dev Biol* **293**: 358–369.

Mazzoni EO, Mahony S, Iacovino M, Morrison C a, Mountoufaris G, Closser M, Whyte W a, Young R a, Kyba M, Gifford DK, et al. 2011. Embryonic stem cell–based mapping of developmental transcriptional programs. *Nat Methods* **8**: 1056–1058.

Mehta S, Huillard E, Kesari S, Maire CL, Golebiowski D, Harrington EP, Alberta J a., Kane MF, Theisen M, Ligon KL, et al. 2011. The Central Nervous System-Restricted Transcription Factor Olig2 Opposes p53 Responses to Genotoxic Damage in Neural Progenitors and Malignant Glioma. *Cancer Cell* **19**: 359–371.

http://dx.doi.org/10.1016/j.ccr.2011.01.035.

Meijer DH, Kane MF, Mehta S, Liu H, Harrington E, Taylor CM, Stiles CD, Rowitch DH. 2012. Separated at birth? The functional and molecular divergence of OLIG1 and OLIG2. *Nat Rev Neurosci* **13**: 819–31. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3733228&tool=pmcentrez&rendertype=abstract.

Meijer DH, Sun Y, Liu T, Kane MF, Alberta J a, Adelmant G, Kupp R, Marto J a, Rowitch DH, Nakatani Y, et al. 2014. An amino terminal phosphorylation motif regulates intranuclear compartmentalization of olig2 in neural progenitor cells. *J Neurosci* **34**: 8507–18. http://www.ncbi.nlm.nih.gov/pubmed/24948806.

Miyoshi G, Butt SJB, Takebayashi H, Fishell G. 2007. Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. *J Neurosci* **27**: 7786–7798.

Mizuguchi R, Sugimori M, Takebayashi H, Kosako H, Nagao M, Yoshida S, Nabeshima YI, Shimamura K, Nakafuku M. 2001. Combinatorial roles of Olig2 and Neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**: 757–771.

Müller T, Anlag K, Wildner H, Britsch S, Treier M, Birchmeier C. 2005. The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev* **19**: 733–743.

Muroyama Y, Fujiwara Y, Orkin SH, Rowitch DH. 2005. Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature* **438**: 360–363.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera C V, Buskin JN, Hauschka SD, Lassar a B. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**: 537–544.

Murre C, Voronova a, Baltimore D. 1991. B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. *Mol Cell Biol* **11**: 1156–1160.

Nelson JD, Denisenko O, Bomsztyk K. 2006. Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat Protoc* **1**: 179–185.

Neuhold L a., Wold B. 1993. HLH forced dimers: Tethering MyoD to E47 generates a dominant positive myogenic factor insulated from negative regulation by Id. *Cell* **74**: 1033–1042.

Niu J, Mei F, Wang L, Liu S, Tian Y, Mo W, Li H, Lu QR, Xiao L. 2012. Phosphorylated olig1 localizes to the cytosol of oligodendrocytes and promotes membrane expansion and maturation. *Glia* **60**: 1427–1436.

Novitch BG, Chen AI, Jessell TM. 2001. Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31**: 773–789.

Novitch BG, Wichterle H, Jessell TM, Sockanathan S. 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40**: 81–95.

Park H-C, Mehta A, Richardson JS, Appel B. 2002. Olig2 Is Required for Zebrafish Primary Motor Neuron and Oligodendrocyte Development. *Dev Biol* **248**: 356–368.

Parker SE, Mai CT, Canfield M a., Rickard R, Wang Y, Meyer RE, Anderson P, Mason C a., Collins JS, Kirby RS, et al. 2010. Updated national birth prevalence estimates for selected birth defects in the United States, 2004-2006. *Birth Defects Res Part A - Clin Mol Teratol* **88**: 1008–1016.

Petryniak M a., Potter GB, Rowitch DH, Rubenstein JLR. 2007. Dlx1 and Dlx2 Control Neuronal versus Oligodendroglial Cell Fate Acquisition in the Developing Forebrain. *Neuron* **55**: 417–433.

Preusser M, Budka H, Rössler K, Hainfellner J a. 2007. OLIG2 is a useful immunohistochemical marker in differential diagnosis of clear cell primary CNS neoplasms. *Histopathology* **50**: 365–370.

Qi Y, Cai J, Wu Y, Wu R, Lee J, Fu H, Rao M, Sussel L, Rubenstein J, Qiu M. 2001. Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. *Development* **128**: 2723–2733.

Rabadán M a, Cayuso J, Le Dréau G, Cruz C, Barzi M, Pons S, Briscoe J, Martí E. 2012. Jagged2 controls the generation of motor neuron and oligodendrocyte progenitors in the ventral spinal cord. *Cell Death Differ* **19**: 209–219.

Riemenschneider MJ, Koy TH, Reifenberger G. 2004. Expression of oligodendrocyte lineage genes in oligodendroglial and astrocytic gliomas. *Acta Neuropathol* **107**: 277–282.

Rowitch DH, Lu QR, Kessaris N, Richardson WD. 2002. An "oligarchy" rules neural development. *Trends Neurosci* **25**: 417–422.

Samanta J, Kessler J a. 2004. Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. *Development* **131**: 4131–

4142.

Satoh J, Asahina N, Kitano S, Kino Y. 2015. A Comprehensive Profile of ChIP-Seq-Based Olig2 Target Genes in Motor Neuron Progenitor Cells Suggests the Possible Involvement of Olig2 in the Pathogenesis of Amyotrophic Lateral Sclerosis. 1–14.

Schweiger R, Linial M. 2010. Cooperativity within proximal phosphorylation sites is revealed from large-scale proteomics data. *Biol Direct* **5**: 6.

Setoguchi T, Kondo T. 2004. Nuclear export of OLIG2 in neural stem cells is essential for ciliary neurotrophic factor-induced astrocyte differentiation. *J Cell Biol* **166**: 963–968.

Sims R, Hollingworth P, Moskvina V, Dowzell K, O'Donovan MC, Powell J, Lovestone S, Brayne C, Rubinsztein D, Owen MJ, et al. 2009. Evidence that variation in the oligodendrocyte lineage transcription factor 2 (OLIG2) gene is associated with psychosis in Alzheimer's disease. *Neurosci Lett* **461**: 54–59.

Sun T, Dong H, Wu L, Kane M, Rowitch DH, Stiles CD. 2003. Cross-repressive interaction of the Olig2 and Nkx2.2 transcription factors in developing neural tube associated with formation of a specific physical complex. *J Neurosci* **23**: 9547–9556.

Sun T, Echelard Y, Lu R, Yuk D in, Kaing S, Stiles CD, Rowitch DH. 2001. Olig bHLH proteins interact with homeodomain proteins to regulate cell fate acquisition in progenitors of the ventral neural tube. *Curr Biol* **11**: 1413–1420.

Sun Y, Meijer DH, Alberta J a., Mehta S, Kane MF, Tien AC, Fu H, Petryniak M a., Potter GB, Liu Z, et al. 2011. Phosphorylation State of Olig2 Regulates Proliferation of Neural Progenitors. *Neuron* **69**: 906–917. http://dx.doi.org/10.1016/j.neuron.2011.02.005.

Tabu K, Ohba Y, Suzuki T, Makino Y, Kimura T, Ohnishi A, Sakai M, Watanabe T, Tanaka S, Sawa H. 2007. Oligodendrocyte lineage transcription factor 2 inhibits the motility of a human glial tumor cell line by activating RhoA. *Mol Cancer Res* **5**: 1099–1109.

Takebayashi H, Nabeshima Y, Yoshida S, Chisaka O, Ikenaka K, Nabeshima YI. 2002a. The basic helix-loop-helix factor Olig2 is essential for the development of motoneuron and oligodendrocyte lineages. *Curr Biol* **12**: 1157–1163.

Takebayashi H, Ohtsuki T, Uchida T, Kawamoto S, Okubo K, Ikenaka K, Takeichi M, Chisaka O, Nabeshima YI. 2002b. Non-overlapping expression of Olig3 and Olig2 in the embryonic neural tube. *Mech Dev* **113**: 169–174.

Takebayashi H, Yoshida S, Sugimori M, Kosako H, Kominami R, Nakafuku M, Nabeshima YI. 2000. Dynamic expression of basic helix-loop-helix Olig family members:

Implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. *Mech Dev* **99**: 143–148.

Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL. 1999. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* **23**: 675–687.

Thaler JP, Lee SK, Jurata LW, Gill GN, Pfaff SL. 2002. LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* **110**: 237–249.

Vallstedt A, Klos JM, Ericson J. 2005. Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron* **45**: 55–67.

Vervoort M, Ledent V. 2001. The evolution of the neural basic Helix-Loop-Helix proteins. *ScientificWorldJournal* **1**: 396–426.

Wagner A. 2001. Birth and death of duplicated genes in completely sequenced eukaryotes. *Trends Genet* **17**: 237–239.

Wagner A. 1994. Evolution of gene networks by gene duplications: a mathematical model and its implications on genome organization. *Proc Natl Acad Sci U S A* **91**: 4387–91. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=43790&tool=pmcentrez&rendertype=abstract.

Wichterle H, Wichterle H, Lieberam I, Lieberam I, Porter J a, Porter J a, Jessell TM, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* **110**: 385–97. http://www.ncbi.nlm.nih.gov/pubmed/12176325.

Wu S, Wu Y, Capecchi MR. 2006. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. *Development* **133**: 581–590.

Xin M, Yue T, Ma Z, Wu F, Gow A, Lu QR. 2005. Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. *J Neurosci* **25**: 1354–1365.

Yang Z, MacQuarrie KL, Analau E, Tyler AE, Dilworth FJ, Cao Y, Diede SJ, Tapseott SJ. 2009. MyoD and E-protein heterodimers switch rhabdomyosarcoma cells from an arrested myoblast phase to a differentiated state. *Genes Dev* **23**: 694–707.

Yokoo H, Nobusawa S, Takebayashi H, Ikenaka K, Isoda K, Kamiya M, Sasaki A, Hirato J, Nakazato Y. 2004. Anti-human Olig2 antibody as a useful immunohistochemical marker of normal oligodendrocytes and gliomas. *Am J Pathol* **164**: 1717–1725.

http://dx.doi.org/10.1016/S0002-9440(10)63730-3.

Yu Y, Chen Y, Kim B, Wang H, Zhao C, He X, Liu L, Liu W, Wu LMN, Mao M, et al. 2013. Olig2 targets chromatin remodelers to enhancers to initiate oligodendrocyte differentiation. *Cell* **152**: 248–261. http://dx.doi.org/10.1016/j.cell.2012.12.006.

Yung S-Y, Gokhan S, Jurcsak J, Molero AE, Abrajano JJ, Mehler MF. 2002. Differential modulation of BMP signaling promotes the elaboration of cerebral cortical GABAergic neurons or oligodendrocytes from a common sonic hedgehog-responsive ventral forebrain progenitor species. *Proc Natl Acad Sci U S A* **99**: 16273–16278.

Zhou Q, Anderson DJ. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* **109**: 61–73.

Zhou Q, Choi G, Anderson DJ. 2001. The bHLH transcription factor Olig2 Promotes oligodendrocyte differentiation in collaboration with Nkx2.2. *Neuron* **31**: 791–807.

Zhou Q, Wang S, Anderson DJ. 2000. Identification of a Novel Family of Oligodendrocyte Lineage-Specific Basic Helix–Loop–Helix Transcription Factors. *Neuron* **25**: 331–343.