TRANSCRIPTIONAL REGULATION IN SPINAL CORD MOTOR NEURON SPECIFICATION AND MOTOR NEURON SUBTYPE DEVELOPMENT

By

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ABSTRACT

Cell fate specification and diversification in the embryonic spinal cord is critical for the development of functional sensory and motor circuits. Two LIM-homeodomain transcription factors, Isl1 and Lhx3, play critical roles in motor neuron specification and later in motor neuron subtype diversification. During motor neuron fate specification Isl1 and Lhx3 are highly expressed and they form the Isl1-Lhx3 transcription complex. The Isl1-Lhx3 complex directs motor neuron specification by activating the transcription of genes important for motor neuron differentiation and motor neuron function. Later in development, the differential expression of Isl1 and Lhx3 directs motor neuron subtype differentiation. Although the roles of Isl1 and Lhx3 during motor neuron specification and motor neuron subtype diversification have been well characterized, the transcriptional pathways that regulate their expression in these cells remain unclear. I investigated the transcriptional regulation of Lhx3, and Isl1, in newly-specified motor neurons by characterizing two putative Lhx3 enhancers, and one Isl1 enhancer. I also investigated the transcriptional regulation of LMO4, a LIM-Only protein that regulates the activity of Lhx3 in motor neurons, by characterizing a putative LMO4 enhancer. I found that each enhancer is activated specifically in embryonic motor neurons, and each enhancer is directly activated by the Isl1-Lhx3 complex. These results show that the Isl1-Lhx3 complex activates a positive transcriptional feedback loop to potentiate and maintain its own expression in newly-specified motor neurons. To examine the transcriptional regulation of Isl1 in motor neuron subtypes, I investigated the activity of the Isl1

enhancer SE2. SE2 is specifically activated in LMCm neurons located in the brachial spinal cord. Hoxc9 is recruited to SE2 and represses SE2 activity in thoracic motor neurons. To test if Hoxc9 regulates the expression of other LMCm genes, I examined the enhancer activity of additional Hoxc9 genomic binding loci. I found that one novel Hoxc9 binding locus, associated with the gene Testin, also activates transcription specifically in LMCm neurons. Thus Hoxc9 broadly represses the transcription of LMCm genes in thoracic motor neurons. Delineating the transcriptional pathways that regulate motor neuron specification and motor neuron diversification is crucial for understanding the cellular and molecular mechanisms that control motor neuron development and motor neuron circuit formation. Disruptions in motor neuron development and motor neuron circuit formation contribute to pediatric motor neuron disorders such as spinal muscular atrophy. Characterizing the genetic pathways that direct motor neuron specification and maturation will be critical for developing effective treatments for pediatric motor neuron diseases and motor neuron degenerative disorders such as amyotrophic lateral sclerosis.

INTRODUCTION

The spinal cord is composed of a diverse array of neurons, each with distinct physical and molecular properties. These neurons form spinal cord circuits that perform many critical functions. Circuits in the dorsal spinal cord receive and process sensory and proprioceptive signals, and then relay this information to the brain (Kandel et al 2013). Ventral spinal cord circuits integrate sensory and proprioceptive information and converge onto motor neurons. Motor neurons project out of the spinal cord, to synapse directly onto muscle cells and control motor output (Kandel et al 2013). In order for these circuits to form and function properly, the spinal cord must generate specific types of spinal cord neurons during embryonic development with high temporal and spatial precision.

Graded Sonic Hedgehog Signaling Patterns the Ventral Spinal Cord

The process of cell fate specification is orchestrated early in embryonic development, beginning with the invagination of the neural plate. As the neural tube forms, the notochord, which is adjacent to the ventral region of this structure, induces neighboring neural tube cells to form the floorplate (Yamada et al., 1991, 1993; Placzek et al., 1993). Both the notochord and the floorplate express high levels of Sonic Hedge Hog (Shh) (Fig 1) (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994; Roelink et al., 1994, 1995; Jessell, 2000a; Novitch et al., 2003; Price and Briscoe, 2004). Cells in these structures process Shh through proteolysis and secrete the amino-terminal cleavage product of the protein (Roelink et al., 1995). As this amino-terminal cleavage

product induces floorplate formation, it is likely that Shh signaling from the notochord induces the formation of floorplate cells (Roelink et al., 1995). Consistent with this model, floorplate cells are also capable of inducing floorplate characteristics in neighboring cells (Placzek et al., 1991). Additionally, blocking Shh activity (with either anti-Shh antibodies *in vitro*, or with Shh mutant mouse embryos) prevents floorplate formation (Chiang et al., 1996; Ericson et al., 1996).

Secretion of Shh protein from the notochord and floorplate establishes a morphogen gradient that directs the formation of progenitor domains in the ventral spinal cord (Jessell, 2000; Price and Briscoe, 2004). In addition to inducing floorplate formation, the notochord also induces motor neuron generation in the ventral spinal cord. Experiments in chick embryos have shown that grafting an extra notochord to an adjacent region of the spinal cord induces the generation of an additional floorplate and additional motor neurons (Yamada et al., 1991). Likewise, when the notochord is removed early in development, embryos fail to generate either floorplate or motor neurons (Yamada et al., 1991). Motor neuron generation can also be induced in neural plate explant cultures, by the addition of media conditioned with notochord tissue (Yamada et al., 1993). These findings indicate that a secreted factor from the notochord induces motor neuron generation, even in the absence of floorplate cells. Adding the amino-terminal cleavage product of Shh in both floorplate explants and in tissue culture experiments recapitulated these findings (Roelink et al., 1995). High concentrations of Shh induce floorplate formation whereas lower concentrations of Shh induce motor neuron generation (Roelink et al., 1995). Similar

experiments using varying concentrations of Shh in neural plate explant cultures also support the model that graded Shh signaling directs the spatial patterning of ventral spinal cord development. The highest concentrations of Shh promoted motor neuron generation; moderate Shh concentrations induced V2-Interneuron (IN) generation; and low Shh levels induced the generation of V1-INs (Ericson et al., 1997).

Graded Shh signaling patterns the ventral neural tube into five distinct progenitor domains through the induction of specific LIM-homeodomain transcription factors (Fig 1). Sequentially increasing concentrations of Shh repress the expression of distinct Class I transcription factors, and activate the expression of specific Class II transcription factors (Goulding et al., 1993; Ericson et al., 1997; Briscoe et al., 2000). This pattern differentially induces the combinatorial expression of transcription factors along the dorso-ventral access of the spinal cord to establish the ventral progenitor domains (Fig 1). Grafting an additional notochord, adjacent to the embryonic neural tube, represses the expression of the Class I transcription factors Paired Box 3 and Paired Box 6 (Pax3 and Pax6) (Goulding et al., 1993). Likewise, Shh represses the transcription of Pax6, Paired Box 7 (Pax7) and Iroquois homeobox 3 (Irx3) in neural plate explant cultures (Ericson et al., 1997; Briscoe et al., 2000). Interestingly Irx3 is only repressed when Shh concentration is four-fold higher than the concentration required to inhibit the expression of Pax7 (Briscoe et al., 2000), consistent with their expression in the embryonic spinal cord. The

expression of Pax7 is restricted to more dorsal regions of the spinal cord, which are exposed to lower concentrations of Shh (Briscoe et al., 2000).

Shh also activates the expression of the Class II transcription factors NK2 Homeobox 2 (Nkx2.2) and NK6 Homeobox 1 (Nkx6.1) in neural plate explant cultures. Similar to Class I transcription factors, different concentrations of Shh are required to modulate the expression of these two genes. Low concentrations of Shh activate the expression of Nkx6.1, and a four-fold increase in Shh concentration is required to activate the expression of Nkx2.2 (Ericson et al., 1997; Briscoe et al., 2000). Again, this induction pattern corresponds to the spatial expression pattern of these two genes in the embryonic spinal cord (Fig 1). Nkx2.2 expression is restricted to the most ventral region of the spinal cord, near the floorplate and the notochord, where Shh concentration is high (Briscoe et al., 2000), whereas Nkx6.1 expression extends to more dorsal regions of the spinal cord, where the concentration of Shh is lower (Briscoe et al., 2000). Together, these findings show that different concentrations of Shh induce and repress the expression of LIM-homeodomain transcription factors corresponding to the expression profiles of these proteins *in vivo*.

The combinatorial expression of Class I and Class II transcription factors establishes discrete progenitor domains in the ventral spinal cord (Fig 1). These progenitor domains have sharp boundaries, which are established through cross-repressive interactions between specific Class I and Class II transcription factors. For example, forced expression of the Class I transcription factor Pax6 in chick neural tube inhibits the expression of the Class II transcription factor Nkx2.2, and

ectopic expression of Nkx2.2 inhibits the expression of Pax6 (Briscoe et al., 2000). This cross-repressive relationship establishes the boundary between the p3 progenitor domain and the motor neuron progenitor (pMN) domain (Fig 1). Likewise, ectopic expression of the Class I transcription factor Developing Brain Homeobox 2 (Dbx2) has been shown to inhibit the expression of the Class II transcription factor Nkx6.1, and forced expression of Nkx6.1 inhibits Dbx2 expression (Briscoe et al., 2000) (Fig 1). This cross-repressive relationship establishes the boundary between the p2 progenitor domain and the p1 progenitor domain (Fig 1). Although the cross-repressive interactions of these transcription factors is potent and critical for the establishment of ventral progenitor domains, it is unclear whether these factors directly regulate their counterpart's expression, or if the repression is mediated indirectly through induction, or repression of additional factors.

LIM-Homeodomain Transcription Factors Direct Fate Specification in Ventral Spinal Cord Neurons

The establishment of progenitor domains in the ventral spinal cord corresponds to the generation of distinct neuronal subtypes, with each progenitor domain generating a specific type of neuron, with a distinct gene expression profile (Fig 2A). The p0 domain generates Even Skipped Homeobox 1/2 (Evx1/2) -positive V0-Interneurons. The p1 domain generates Engrailed 1 (En1) positive V1-Interneurons. The p2 domain generates Visual System Homeobox 2 (Chx10) / LIM-Homeobox-3 (Lhx3) double-positive V2-Interneurons. The pMN domain

generates Islet-1 (Isl1) / Lhx3 / Motor Neuron and Pancrease Homeobox 1 (Hb9) expressing motor neurons and the p3 domain generates Single-minded Family bHLH Transcription Factor 1 (Sim1)-positive V3-Interneurons. Consistent with this model, different concentrations of Shh induce the generation of distinct ventral spinal cord neurons in neural plate explant cultures (Ericson et al., 1997). High concentrations of Shh induce motor neuron generation, moderate levels of Shh induce V2-Interneuron generation and low Shh concentrations promote the generation of V1-Interneurons (Ericson et al., 1997). These findings show that graded Shh signaling, in addition to establishing progenitor domains in the ventral spinal cord, also directs the formation of distinct neural subtypes. This is most likely through the induction of appropriate LIM-homeodomain transcription factors, which is supported by experiments that disrupt the expression of these factors in the embryonic spinal cord (Ericson et al., 1997; Briscoe et al., 2000).

Pax6 mutant mouse embryos fail to generate En1-positive V1-Interneurons or Chx10-positive V2-Interneurons, and they generate fewer Isl1/2-positive motor neurons (Fig 2B) (Ericson et al., 1997). Pax6 mutation is also accompanied by a dorsal expansion of both the Nkx2.2-expressing p3 domain and the Sim1-positive V3 neurons (Fig 2B) (Ericson et al., 1997). Likewise, Nkx2.2 overexpression promotes the generation of V3 neurons and represses motor neuron generation, at least in part, by inhibiting the expression of the motor neuron-specifying gene Mnr2 (Fig 2C) (Briscoe et al., 1999, 2000). Irx3 has also been shown to repress motor neuron generation by inhibiting Mnr2 expression in the p2 domain (Briscoe et al., 2000). These results indicate that

Pax6, Nkx2.2 and Irx3 play critical roles in establishing ventral progenitor domains and in regulating the generation of neuronal subtypes in the spinal cord.

Motor Neuron Specification is Directed by Olig2 and Mnr2

The pMN domain initially expresses a combination of LIM-homeodomain transcription factors, including Nkx6.1 and Pax6 (Briscoe et al., 2000). In addition to these factors, the pMN domain also specifically expresses Oligodendrocyte Lineage Transcription Factor 2 (Olig2) (Fig 1) (Novitch et al., 2001; Lee et al., 2005b). Experiments examining Olig2 knock out mouse embryos have shown that Olig2 expression is required to establish and maintain the pMN domain and, consequently, to generate motor neurons (Lu et al., 2002). Olig2 promotes the formation of the pMN domain by inhibiting the expression of Irx3 (Fig 3) (Novitch et al., 2001). Olig2 also promotes the generation and differentiation of motor neurons by inducing the expression of Neurogenin 2 (Ngn2), Lhx3 and Mnr2 (Fig 3) (Novitch et al., 2001). Olig2 promotes pMN formation via transcriptional repression. Thus Olig2 mediated induction of Ngn2, Lhx3 and Mnr2 is most likely accomplished indirectly through repression of genes that inhibit their expression (Novitch et al., 2001). Interestingly, Olig2 also represses the transcription of the motor neuron gene Hb9, and down-regulation of Olig2 expression is required for motor neuron differentiation (Fig 3) (Lee et al., 2005b). As Hb9 is expressed later in motor neuron development, decreased Olig2 expression likely releases Hb9 from transcriptional repression as motor neurons exit the cell cycle and terminally differentiate.

Mnr2 is a homeodomain transcription factor that is specifically expressed in differentiating motor neurons (Tanabe et al., 1998). It is initially expressed in pMN cells during their final division cycle (Tanabe et al., 1998). Its expression is induced by Nkx6.1 and inhibited by Irx3 and Nxk2.2 in neighboring p2 and p3 cells, respectively (Fig 3) (Briscoe et al., 2000). Mnr2 is sufficient to direct motor neuron specification as ectopic expression of Mnr2 in the dorsal chick spinal cord induces the expression of the motor neuron genes Isl1, Lhx3 and Hb9. Mnr2 also activates its own expression through positive transcriptional autoregulation (Fig 3) (Tanabe et al., 1998). Forced expression of Mnr2 in the dorsal spinal cord is also sufficient to induce the expression of the mature motor neuron marker Choline Acetyltransferase (ChAT) and to direct axons to the ventral horn (Tanabe et al., 1998). As ectopic expression of Mnr2 does not alter the expression of Class I or Class II transcription factors, its ability to promote motor neuron specification is independent of progenitor patterning. Instead, Mnr2 acts as a downstream determinant of motor neuron cell fate (Tanabe et al., 1998).

The Isl1-Lhx3 Complex Directs Motor Neuron Fate Specification and Maturation by Activating the Transcription of Motor Neuron Genes

As motor neurons exit the cell cycle and terminally differentiate, they induce the expression of two LIM-homeodomain transcription factors Isl1 and Lhx3 (Fig 3) (Tanabe et al., 1998; Thaler et al., 1999, 2002). Isl1 is expressed in motor neurons and in the dorsal spinal cord, and Lhx3 is expressed in motor neurons and V2-Interneurons (Tanabe et al., 1998; Thaler et al., 2002; Agalliu et al.,

2009; Song et al., 2009). Motor neurons are the only cells that co-express both Isl1 and Lhx3, suggesting a model in which the combinatorial expression of Isl1 and Lhx3 promotes motor neuron specification (Tanabe et al., 1998; Thaler et al., 2002; Agalliu et al., 2009) . Subsequent experiments have shown that, when co-expressed in the presence of Nuclear LIM Interacting Protein (NLI), Isl1 and Lhx3 interact with each other and NLI to form the Isl1-Lhx3 transcription complex (Fig 4) (Pfaff et al., 1996; Thaler et al., 2002). The Isl1-Lhx3 complex is composed of two Isl1 proteins, two Lhx3 proteins and two NLI proteins (Thaler et al., 2002). As NLI is widely expressed in post mitotic embryonic spinal cord cells, any post-mitotic spinal cord cells expressing both Isl1 and Lhx3 will generate the Isl1-Lhx3 complex (Thaler et al., 2002; Song et al., 2009).

Neither Isl1 nor Lhx3 is sufficient to promote motor neuron specification in the dorsal spinal cord. However, when both Isl1 and Lhx3 are expressed in chick dorsal spinal cord, they activate the expression of the motor neuron genes, Mnr2, Hb9 and Islet-2 (Isl2) (Thaler et al., 2002). RNA sequencing experiments in a mouse embryonic stem cell model also show that the Isl1-Lhx3 complex induces the transcription of a cohort of motor neuron genes while simultaneously repressing interneuron genes (Lee et al., 2012). Genes induced by the Isl1-Lhx3 complex include motor neuron transcription factors, genes important for the processing of the motor neuron neurotransmitter acetylcholine, and genes that direct axon guidance (Lee et al., 2012). These results strongly support a model in which the Isl1-Lhx3 complex acts to promote motor neuron fate specification and motor neuron maturation in the spinal cord.

In the absence of Isl1, Lhx3 and NLI interact to form the V2-tetramer transcription complex, composed of 2 Lhx3 proteins and 2 NLI proteins (Fig 4) (Thaler et al., 2002; Lee et al., 2008). Unlike the motor neuron hexamer, the Lhx3 complex suppresses motor neuron generation and promotes V2-Interneuron specification. Specifically, ectopic expression of Lhx3 in the embryonic spinal cord induces the expression of the V2-Interneuorn specific gene Chx10 (Tanabe et al., 1998; Thaler et al., 2002; Lee et al., 2008). Chx10 is a transcriptional repressor that promotes V2a-Interneron fate specification and inhibits the expression of motor neuron genes, such as Hb9, Isl1, ChAT and Vesicular Acetylcholine Transporter (VAChT) (Clovis et al., 2016). Because motor neurons express both Lhx3 and NLI, they have the potential to form the Lhx3 complex and induce the expression of Chx10, but this is prevented by the expression Lim Only Protein 4 (LMO4). LMO4 is expressed in embryonic motor neurons and it competes with Lhx3 to bind NLI, thereby inhibiting the formation of the Lhx3 complex in motor neurons (Fig 4) (Lee et al., 2008). In LMO4 knockout mouse embryos, Chx10 is ectopically expressed in motor neurons and this effect is substantially amplified when Hb9 is also deleted (Lee et al., 2008). Hb9 directly suppresses Chx10 transcription by binding a regulatory element upstream of the *Chx10* coding region (Lee et al., 2008).

Interestingly, despite having a high affinity for NLI binding, LMO4 does not block the formation of the Isl1-Lhx3 complex in embryonic motor neurons (Lee et al., 2008; Song et al., 2009). In Isl1 knock-down models, embryos with decreased Isl1 expression have fewer motor neurons and more V2-Interneurons

(Song et al., 2009). This finding suggests that, when expression levels of both Isl1 and Lhx3 are high, Isl1-Lhx3 complex formation is favored. However, when Isl1 levels are low and Lhx3 levels are high, Lhx3 complex formation is favored. The expression level of LMO4 also appears to regulate the formation and activity of LIM-homeodomain complexes. When LMO4, Isl1 and Lhx3 are ectopically expressed in the dorsal spinal cord, the high levels of LMO4 block the formation of the Isl1-Lhx3 complex, and prevent the induction of the motor neuron gene Mnr2 (Song et al., 2009). Together, these findings suggest that the relative expression levels of Isl1 and LMO4 in embryonic motor neurons are critical for promoting the formation and function of the Isl1-Lhx3 complex.

Motor Neuron Subtypes Have Distinct Gene Expression Profiles and Muscle Targets

Shortly after motor neurons terminally differentiate, these cells migrate laterally and continue to refine their gene expression profiles, to further specify into distinct motor neuron subtypes (Tsuchida et al., 1994; Jessell, 2000). The specification of motor neuron subtypes is critical for motor neuron circuit formation as each subtype occupies a specific region of the spinal cord and projects to a specific muscle target (Fig 5 & Fig 6). Because different regions along the rostro-caudal axis of the spinal cord are correspondingly located adjacent to distinct muscle groups, different regions of the spinal cord contain corresponding groups of motor neuron subtypes (Fig 6). Furthermore, each motor neuron subtype occupies a discrete region of the ventral horn, generating

motor neuron subtype columns along the rostro-caudal axis of the spinal cord (Fig 5) (Cruce, 1974; Landmesser et al., 1975; Lamb, 1976; Landmesser, 1978; Tsuchida et al., 1994; Jessell, 2000).

Medial Motor Column (MMC) neurons are located in the medial portion of the ventral horn and are further divided into medial and lateral subtypes. Medial MMC (MMCm) neurons are located in the medial most region of the ventral horn and form a continuous column through the brachial, thoracic and lumbar spinal cord. MMCm neurons project specifically to dorsal axial muscles (Fig 5 & Fig 6) (Tsuchida et al., 1994; Jessell, 2000). Lateral MMC (MMCl) neurons are located ventrally and slightly lateral to MMCm neurons. MMCl neurons are found in the thoracic spinal cord but they are absent from the brachial and lumbar spinal cord. MMCl neurons project to intercostal muscles (Tsuchida et al., 1994; Jessell, 2000). Lateral Motor Column (LMC) neurons are also further specified into lateral and medial groups (LMCl and LMCm, respectively) (Fig 5 & Fig 6). Both LMCm neurons and LMCl neurons are located in the brachial and the lumbar spinal cord but are absent from the thoracic spinal cord (Tsuchida et al., 1994). Additionally, both LMCm neurons and LMCl neurons project axons to limb muscles. LMCm neurons project axons specifically to ventral limb muscles and LMCl neurons project axons specifically to dorsal limb muscles (Cruce, 1974; Landmesser et al., 1975; Lamb, 1976; Landmesser, 1978). Preganglionic (PGC) neurons are located in the thoracic spinal cord, and project axons specifically to sympathetic ganglia (Fig 5 & Fig 6) (Tsuchida et al., 1994; Jessell, 2000).

In addition to occupying discrete regions of the spinal cord, and having specific muscle targets, motor neuron subtypes also have distinct gene expression profiles. The first genes that were found to be differentially expressed in motor neuron subtypes were LIM-homeodomain transcription factors, as well as genes that encode signaling proteins (Table 1) (Tsuchida et al., 1994; Sharma et al., 2000; Rousso et al., 2008; Roy et al., 2012).

Motor Neuron Subtype Specification

The differential expression of LIM-homeodomain transcription factors in motor neuron subtypes is influenced by signals from surrounding tissue. In zebrafish, motor neurons that are transplanted to new locations adopt the LIM-homeodomain expression profiles that correspond to motor neurons in that position (Appel et al., 1995). Spinal cord grafting experiments in chick embryos produce similar results. Transplanting brachial spinal cord into the thoracic region of an embryo inhibits the specification of brachial LMC neurons and promotes the generation of thoracic MMCl neurons and PGC neurons (Ensini et al., 1998). Likewise, grafting thoracic spinal cord into the brachial region of an embryo inhibits the generation of MMCl and PGC neurons and induces the generation of LMC neurons (Ensini et al., 1998). Grafting paraxial mesoderm tissue to new locations in an embryo has similar effects. Brachial tissue induces the generation of brachial motor neurons and thoracic tissue induces the generation of thoracic motor neurons (Ensini et al., 1998). Together, these results show that motor

neuron subtype specification is directed by positional cues from surrounding tissue, specifically signaling from paraxial mesoderm.

The differential expression of LIM-homeodomain transcription factors in motor neuron subtypes directs their distinct gene expression profiles, cell migration patterns and axonal trajectories. In mouse embryos, the forced expression of Lhx3 in all thoracic motor neurons converts PGC and MMCl neurons into MMCm neuron (Sharma et al., 2000). First, the expression of MMCm-specific gene LIM-Homeobox-4 (Lhx4) is expanded in the ventral horn, indicating a genetic conversion of PGC and MMCl neurons to MMCm neurons (Sharma et al., 2000). Second, PGC neurons fail to migrate dorsally to their normal position in the dorsal-lateral region of the spinal cord (Sharma et al., 2000). Lastly, forced expression of Lhx3 redirects motor axons towards dorsal axial muscles (Sharma et al., 2000).

Although multiple motor neuron subtypes express Isl1, LMCl neurons are the only motor neuron subtype that express LIM-Homeobox 1 (Lim1) (Table 1) (Tsuchida et al., 1994; Kania et al., 2000; Sockanathan et al., 2003; Alaynick et al., 2011). LMCl neurons are generated after LMCm neurons. As they differentiate, LMCl neurons migrate through LMCm neurons, towards the lateral portion of the ventral horn (Sockanathan et al., 2003). During this time, LMCm neurons express Aldehyde Dehydrogenase A2 (Raldh2), an enzyme that generates the signaling molecule retinoic acid (Sockanathan et al., 2003). As LMCl neurons migrate through LMCm neurons, they are exposed to secreted retinoic acid which induces the expression of Lim1 and represses the expression

of Isl1 (Sockanathan et al., 2003). Disruption of retinoic acid signaling through application of retinoic acid receptor (RAR) antagonists *in vitro* inhibits the specification of LMCl neurons (Sockanathan et al., 2003). Misexpression of Raldh2 in the thoracic spinal cord is also sufficient to induce ectopic specification of LMCl neurons in neighboring motor neurons (Sockanathan et al., 2003). These findings show that retinoic acid signaling is critical for specifying LMCl neurons.

Manipulating the expression of Isl1 or Lim1 interferes with LMCl and LMCm specification. Forced expression of Lim1 is sufficient to direct motor neurons to settle into the lateral region of the ventral horn and to project axons into dorsal limb muscles (Kania and Jessell, 2003). Correspondingly, experiments examining chimeric embryos show that LMCl neurons with mutated Lim1 project axons into both dorsal and ventral limb muscle indiscriminately (Kania et al., 2000). These findings strongly support a model in which Lim1 contributes to LMCl specification and directs LMCl axon growth into the dorsal limb. Likewise, when Isl1 is ectopically expressed, motor neurons acquire LMCm characteristics, settling medially in the ventral horn and projecting axons into ventral limb muscles (Kania and Jessell, 2003). Interestingly, Isl1 and Lim1 have a cross-repressive interaction, suggesting that these proteins play critical roles in establishing gene expression profiles in their respective cell types (Kania and Jessell, 2003). The ability of Lim1 to promote axon pathfinding into dorsal limb muscles appears to be mediated by ephrin signaling. Lim1 induces the expression of Eph Receptor A4 (EphA4) in LMCl neurons (Kania and Jessell, 2003). EphA4 directs axons into dorsal limb muscles away from the repulsive

signaling molecule ephrin-A, which is expressed in ventral limb muscles (Kania and Jessell, 2003). However, the mechanisms through which Isl1 promotes LMCm axon pathfinding towards ventral limb muscles remain unclear.

Hox Genes Directs Motor Neuron Subtype Specification Along the Rostro-Caudal Axis of the Spinal Cord

Hox genes are differentially expressed along the rostro-caudal axis of the spinal cord and they direct the specification of motor neuron subtypes (Fig 6) (Liu et al., 2001; Dasen et al., 2003). Hox protein expression is detectable in the embryonic chick spinal cord as early as Hamburger and Hamilton stage (HHst) 16 (Hamburger and Hamilton, 1993; Liu et al., 2001). Hoxc genes are initially expressed in the rostral spinal cord and their expression gradually expands through the caudal spinal cord as the embryo develops (Liu et al., 2001). Homeobox C6 (Hoxc6) is expressed in the brachial spinal cord and Homeobox C9 (Hoxc9) is expressed in the thoracic spinal cord (Fig 6) (Liu et al., 2001; Dasen et al., 2003). Homeobox C5 (Hoxc5) is expressed in the cervical spinal cord, as well as in the rostral brachial spinal cord, partially overlapping with Hoxc6 expression (Liu et al., 2001; Dasen et al., 2003). Likewise, Hoxc8 is expressed in the caudal brachial spinal cord and the rostral thoracic spinal cord, partially overlapping with Hoxc6 and Hoxc9 expression, but not overlapping with Hoxc5 expression (Liu et al., 2001; Dasen et al., 2003). Lastly, Hoxc10 is expressed in the lumbar spinal cord (Fig 6) (Liu et al., 2001; Dasen et al., 2003).

Hoxc genes are highly expressed in embryonic motor neurons, and the expression of Hoxc6 and Hoxc9 corresponds to brachial and thoracic motor columns respectively (Liu et al., 2001; Dasen et al., 2003). Hoxc6 is expressed in Raldh2-positive LMC neurons and Hoxc9 expression coincides with the expression of BMP5, which marks PGC neurons in the thoracic spinal cord (Dasen et al., 2003). Although Hoxc5 and Hoxc8 are co-expressed in cells that also express Hoxc6, Hoxc9 and Hoxc6 expression is mutually exclusive (Dasen et al., 2003). This effect is mediated by a cross-repressive interaction between these two proteins (Dasen et al., 2003; Jung et al., 2010; Lacombe et al., 2013). Like the cross-repressive interactions in ventral spinal cord progenitor domains, this genetic interaction establishes a sharp boundary between brachial and thoracic motor columns.

Like LIM-homeodomain transcription factors, Hoxc gene expression in the spinal cord is induced by signals from surrounding tissues. Experiments utilizing neural plate explant cultures have shown that graded Fibroblast Growth Factor 2 (FGF2) and Growth Differentiation Factor 11 (Gdf11) signaling from Hensen's node regulates Hoxc gene expression during early spinal cord development (Liu et al., 2001). Higher concentrations of these two signaling molecules promote caudal Hoxc gene expression profiles, specifically repression of Hoxc6 and induction of Hoxc9 and Hoxc10 expression (Liu et al., 2001). At the same time, lower concentrations of these two molecules fail to induce the expression of Hoxc9 and Hoxc10, which establishes a rostral Hoxc expression profile (Liu et al., 2001). These findings were confirmed *in vivo* using chick neural tube

electroporation to overexpress Fibroblast Growth Factor 8 (Fgf8) throughout the spinal cord. Ectopic Fgf8 induces the expression of Hoxc9 and Hoxc8, and represses Hoxc6 and Hoxc5 expression (Dasen et al., 2003). Hoxc6 and Hoxc5 expression are not induced by this signaling pathway. Instead, their induction depends on retinoic acid signaling from rostral paraxial mesoderm (Liu et al., 2001). Thus competing morphogen gradients from the rostral and caudal spinal cord activate and repress the expression of specific Hoxc genes to establish their sequential expression along the rostro-caudal axis of the embryonic spinal cord.

Hoxc6 and Hoxc9 are expressed in brachial and thoracic motor columns, respectively, which suggests that these genes may contribute to motor neuron subtype specification (Fig 6) (Liu et al., 2001; Dasen et al., 2003). Indeed, analysis of motor neuron subtype specification and motor circuit formation in Hoxc6 and Hoxc9 knock out mice has shown that these genes play critical roles in directing the specification of brachial and thoracic motor neuron subtype development and maturation. Hoxc6 knockout mice have fewer Forkhead Box 1 (Foxp1) positive LMC neurons (Lacombe et al., 2013). This effect is restricted to the rostral regions of the brachial spinal cord, suggesting that Hox genes expressed in the caudal regions of the brachial spinal cord compensate for loss of Hoxc6 (Lacombe et al., 2013). Indeed, overexpression of Homeobox A6 (Hoxa6), Homeobox A7 (Hoxa7) or Homeobox C8 (Hoxc8) induces the generation of Foxp1 and Raldh2 expressing LMC neurons in the thoracic spinal cord (Lacombe et al., 2013). Additionally, Hoxc6/Hoxa6 double knockout mice show defects in limb innervation (Lacombe et al., 2013). These findings show

that Hoxc6 and Hoxa6 also contribute to later stages of motor neuron subtype development and circuit formation.

Likewise, experiments altering the expression pattern of Hoxc9 have shown that it plays critical roles in directing both spinal cord Hox gene expression and in specification and maturation of thoracic motor neuron subtypes. Hoxc9 knockout mice have expanded expression of rostral Hoxc genes, including Hoxc6, into the thoracic spinal cord (Jung et al., 2010). These mice also lose thoracic motor neuron subtypes, and instead generate motor neurons with brachial LMC neuron gene expression profiles (Jung et al., 2010). This phenotype includes loss of Neuronal Nitric Oxide Synthase 1 (nNOS) positive PGC neurons and loss of Isl1/Hb9 double-positive MMCl neurons (Jung et al., 2010). Hoxc9 knock out embryos also have severely diminished axonal projections to the sympathetic ganglia, which are the main target cells of PGC neurons (Jung et al., 2010). Forced expression of Hoxc9 in the brachial spinal cord has corresponding effects. Hoxc9 represses the expression of rostral Hox genes, including Hoxc6 (Jung et al., 2010). It also reduces the number of Raldh2 positive, Foxp1 positive LMC neurons and induces the specification of Hb9/Isl1 double positive MMCl neurons in the brachial spinal cord (Jung et al., 2010).

Foxp1 Acts as a Critical Hox Co-Factor to Specify LMC and PGC Neurons

Although Hox genes play critical roles in specifying region-specific motor neuron subtype specification along the rostro-caudal axis of the embryonic spinal cord, their activity cannot account for the specification of distinct motor neuron

subtypes within the same rostro-caudal section of the spinal cord. Differential expression of Hox co-factors could facilitate this aspect of Hox activity.

Studies investigating the activity of Foxp1 have shown that it acts as a Hox cofactor to regulate the activity of Hox genes in distinct motor neuron subtypes. Foxp1 is specifically expressed in LMC neurons and PGC neurons, with LMC neurons expressing higher levels of Foxp1 protein (Table 1) (Dasen et al., 2008; Rousso et al., 2008). Foxp1 plays critical roles in motor neuron subtype specification, as altering its expression in the embryonic spinal cord severely disrupts this process (Dasen et al., 2008; Rousso et al., 2008). Loss of function Foxp1 mutant embryos have reduced Raldh2, Lim1 and nNOS expression, indicating impaired LMC, LMCl, and PGC specification, respectively (Dasen et al., 2008; Rousso et al., 2008). Loss of LMC and PGC neurons is also accompanied by an expansion of Lhx3-positive MMCm neurons and Isl1/Hb9 double-positive MMCl neurons (Dasen et al., 2008; Rousso et al., 2008b). Accordingly, Foxp1 mutant embryos also have impaired axon pathfinding in projections to limb muscles and sympathetic ganglia (Dasen et al., 2008; Rousso et al., 2008). Forced expression of Foxp1 also disrupts motor neuron subtype specification, by reducing the number of Lhx3-positive MMCm neurons and the number of Hb9/Isl1 double positive MMCl neurons (Rousso et al., 2008). As both Hox genes and Foxp1 play critical roles in motor neuron subtype specification, it is likely that these genes cooperate to regulate this complex process. Foxp1 acts as a critical co-factor for Hox genes at different levels of the spinal cord, with high

levels of Foxp1 expression promoting LMC neuron specification and low levels of Foxp1 expression promoting PGC specification.

Motor Neuron Development is Directed by the Precise Temporal and Spatial Regulation of Gene Expression

A great deal of progress has been made characterizing the developmental processes that contribute to establishing progenitor domains in the ventral spinal cord, and many genes have been identified that are critical for motor neuron specification, such as Isl1, Lhx3 and LMO4. Likewise, recent work investigating motor neuron subtype specification and development has identified numerous factors that contribute to this complex process, including Hox gene expression, Hox co-factor activity and retinoic acid signaling. Although the function of LIM-Homeodomain proteins in embryonic motor neurons has been well characterized, the mechanisms that regulate their precise temporal and spatial expression patterns in newly specified motor neurons and in mature motor neuron subtypes remain unclear.

In this dissertation, I investigate the activity and regulation of motor neuron specific enhancers associated with *Isl1*, *Lhx3* and *Lmo4*, as well as an LMCm-specific *Isl1*-associated enhancer. These studies reveal that the Isl1-Lhx3 complex directly activates the transcription of its own components, creating an autoregulatory feedback loop to potentiate its own expression during motor neuron specification. The Isl1-Lhx3 complex simultaneously activates the transcription of *Lmo4*, which further facilitates Isl1-Lhx3 complex formation and

motor neuron specification by inhibiting the formation of the Lhx3-complex. My experiments also show that Hoxc9 directly inhibits the activity of LMCm-specific enhancers in the thoracic spinal cord. Identifying and characterizing genetic regulatory elements and target genes of motor neuron transcription factors provides crucial insights into the transcriptional regulation of motor neuron specification and motor neuron subtype development.

FIGURES

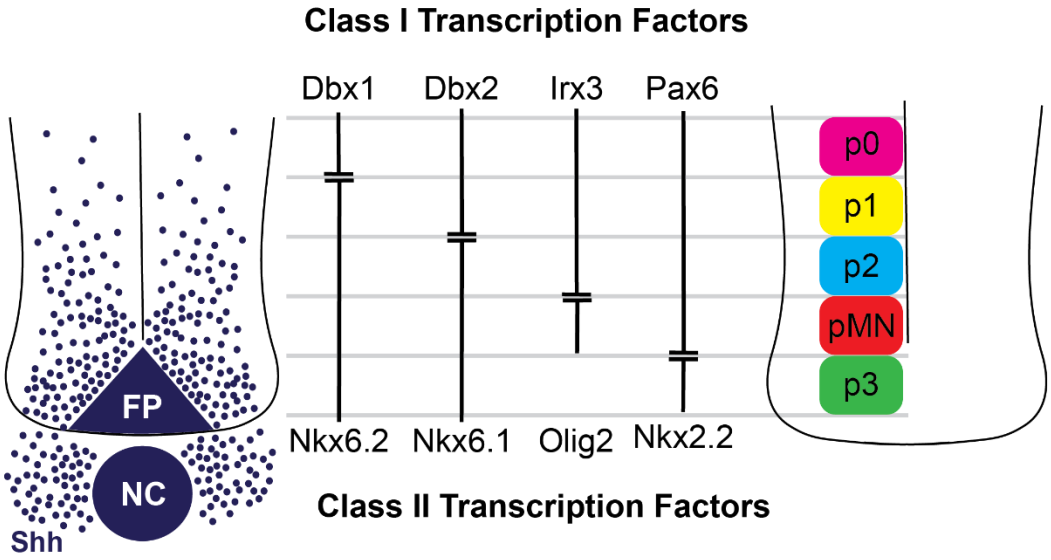

Figure 1: Sonic Hedge Hog Signaling in the Ventral Spinal Cord. Sonic Hedge Hog (Shh) is secreted from the notochord and the floorplate creating a morphogen gradient along the dorsal-ventral axis of the ventral spinal cord. This morphogen gradient induces the expression of Class II transcription factors Nkx6.2, Nkx6.1, Olig2 and Nkx2.2, and represses the expression of Class I transcription factors Developing Brain Homeobox 1 (Dbx1), Dbx2, Irx3 and Pax6. These transcription factors have cross-repressive interactions that creates sharp boundaries of transcription factor expression. These boundaries delineate the ventral progenitor cell domains, the p0-p3 domains and the pMN domain, in the ventral spinal cord.

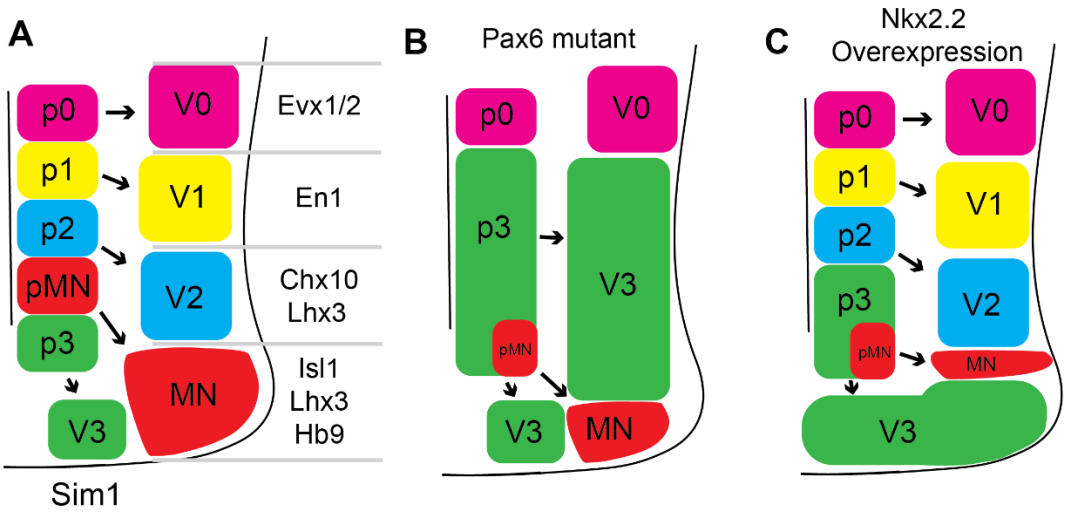

Figure 2: Motor Neuron Specification is Controlled by the Differential Expression of LIM-Homeodomain Transcription Factors. A) Ventral spinal cord progenitor domains generate distinct types of spinal cord neurons that express distinct combinations of LIM-homeodomain transcription factors. B) Pax6 loss-of-function mutant embryos have expanded p3 domains, smaller pMN domains and they lose the p1 and p2 domains. Likewise, they have more V3 neurons, fewer motor neurons, and they lose V1 and V2 interneurons. C) Nkx2.2 overexpression embryos have expanded p3 domains and smaller pMN domains. Likewise, they have more V3 interneurons and fewer motor neurons.

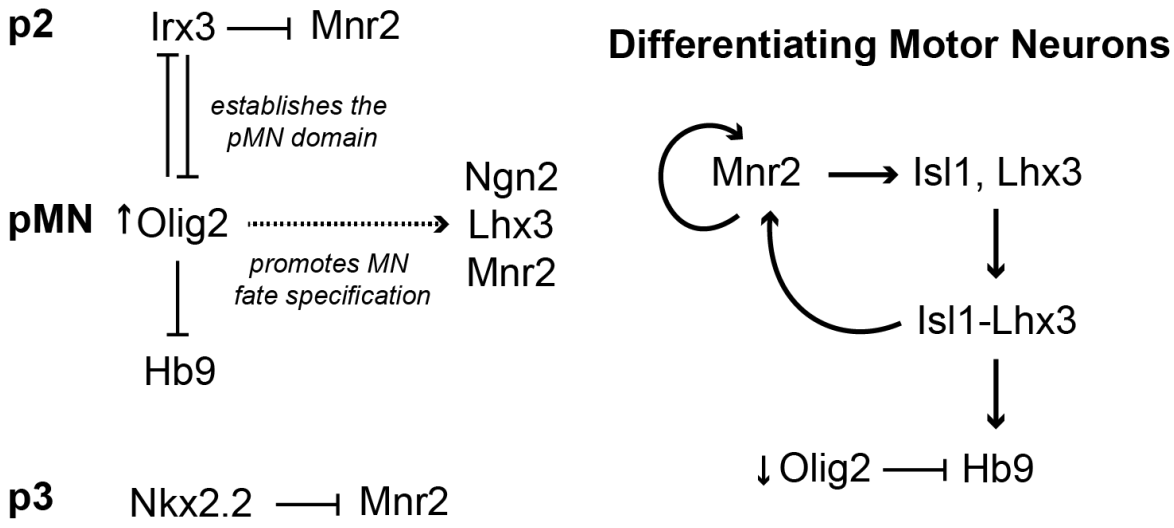

Figure 3: Olig2 Establishes the pMN Domain and Promotes Motor Neuron Specification. Motor neuron progenitor cells (pMN cells) express high levels of Olig2. Olig2 represses the transcription of Irx3 and Hb9 to establish the pMN domain. Olig2 also indirectly activates the transcription of Ngn2, Lhx3 and Mnr2. In differentiating motor neurons Olig2 levels decrease which releases Hb9 from transcriptional repression. Mnr2 activates the expression of Isl1 and Lhx3 and potentiates its own expression through positive transcriptional autoregulation. The Isl1-Lhx3 complex also potentiates the expression of Mnr2 and activates the expression of Hb9.

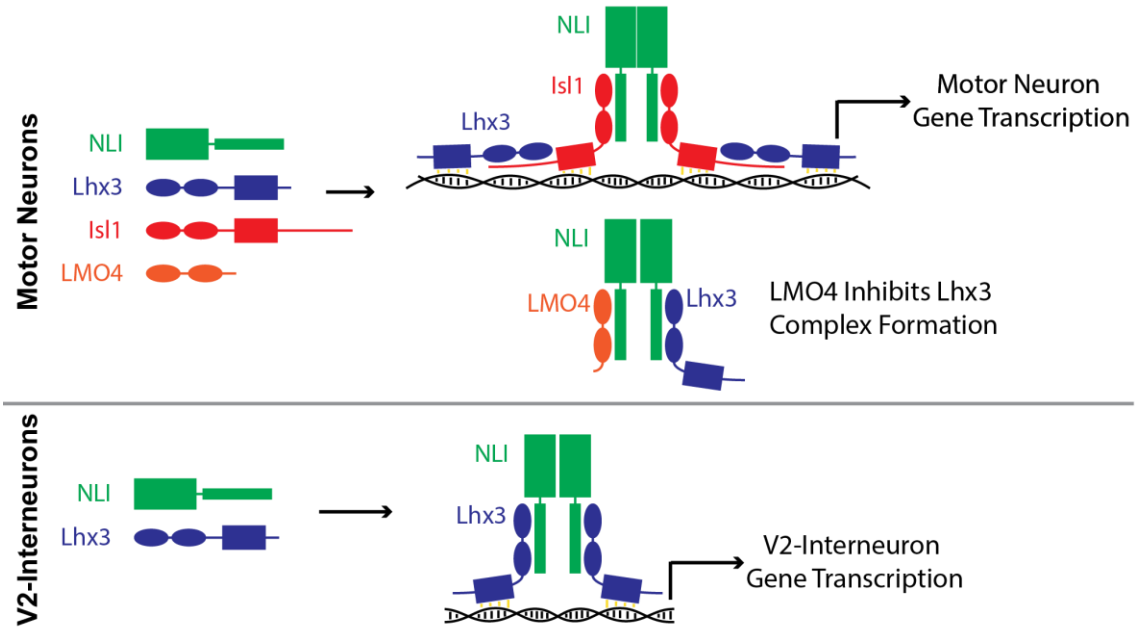

Figure 4: Isl1 and Lhx3 Form the Isl1-Lhx3 Complex in Differentiating Motor Neurons. Motor Neurons express NLI, Isl1, Lhx3 and LMO4. NLI contains a self-dimerization domain (large rectangle) and a LIM-Interacting domain (narrow rectangle). Isl1 and Lhx3 each contain two Lim domains (ovals) and one homeodomain (rectangle). LMO4 contains two Lim domains and no homeodomain. Isl1, Lhx3 and NLI form the Isl1-Lhx3 complex composed of two NLI, two Isl1 and 2 Lhx3 proteins. The Isl1-Lhx3 complex activates the transcription of motor neuron genes. In motor neurons, LMO4 competes with Lhx3 to bind the LIM-Interacting domain of NLI, and thereby inhibits the formation of the Lhx3 complex to prevent the transcription of V2-Interneuron genes. V2-Interneurons express Lhx3 and NLI, which forms the Lhx3 complex composed of 2 Lhx3 and 2 NLI proteins. The Lhx3 complex activates the transcription of V2-Interneuron genes.

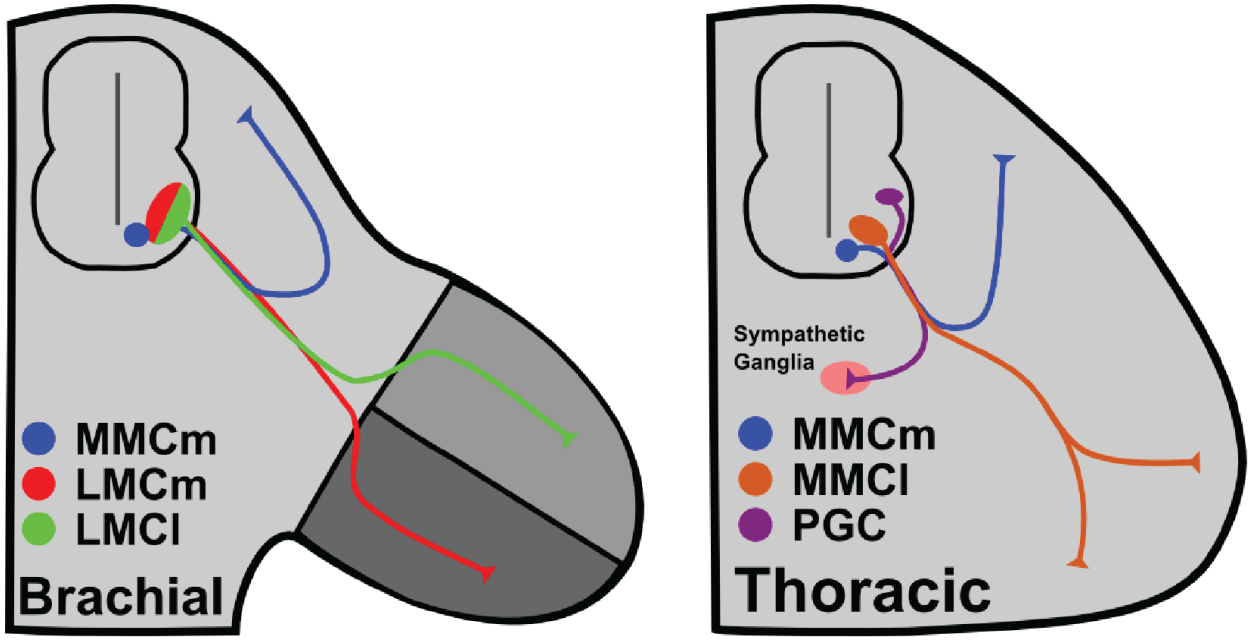

Figure 5: Motor Neuron Columns in the Brachial and Thoracic Spinal Cord. The brachial spinal cord is adjacent to limb muscles and contains MMCm neurons, LMCm neurons and LMCl neurons. MMCm neurons are located medially in the brachial spinal cord and project to dorsal axial muscles. LMCm neurons are located laterally in the brachial spinal cord and project axons to ventral limb muscles. LCMl neurons are located lateral to LMCm neruons. LMCl neurons project axons to dorsal limb muscles. The thoracic spinal cord is adjacent to trunk muscles. It contains MMCm neurons, MMCl neurons and PGC neurons. Like brachial MMCm neruons, MMCm neurons in the thoracic spinal cord are located medially and they project axons to dorsal axial muscles. MMCl neurons are located in the lateral portion of the ventral horn. They project axons to intercostal muscles. PGC neurons are located dorsally and laterally in the spinal cord. They project axons to sympathetic ganglia.

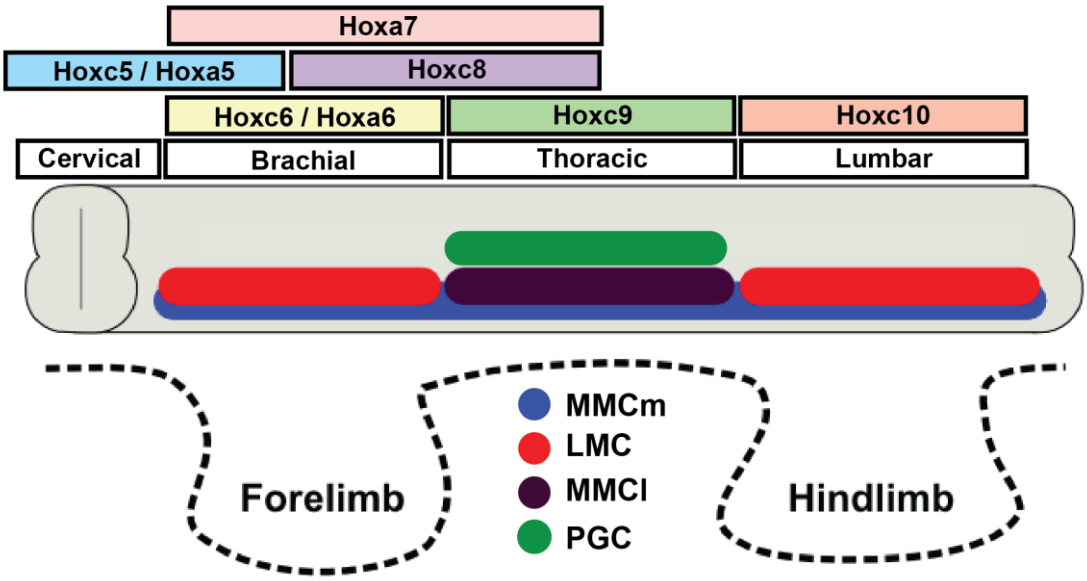

Figure 6: Hox Genes Are Differentially Expressed Along the Rostro-Caudal Axis of the Spinal Cord. Schematic shows the positions of different motor neuron subtypes and the corresponding Hox gene expression profile in that region of the spinal cord. Hox gene expression corresponds to distinct motor neuron subtypes, with Hoxc6 and Hoxa6 corresponding to LMC neurons in the brachial spinal cord, Hoxc9 corresponding to PGC and MMCl neurons in the thoracic spinal cord and Hoxc10 expression corresponding to LMC neurons in the lumbar spinal cord.

Subtype	Gene Expression	Location	Target Cells	References
MMCm	Isl1, Isl2, Lhx3, Lhx4, Hb9, SCIP	Brachial, Thoracic, Lumbar	Dorsal Axial Muscle	Tsuchida et al., 1994; Roy et al., 2012; Rousso et al., 2008
MMCl	Isl1, Isl2, SCIP, Hb9	Thoracic	Intercostal Muscles	Tsuchida et al., 1994; Roy et al., 2012; Rousso et al., 2008
LMCm	Isl1, Isl2, Foxp1, Hb9, Raldh2	Brachial and Lumbar	Ventral Limb Muscles	Tsuchida et al., 1994; Landmesser, 1978; Rousso et al., 2008; Dasen et al., 2008; Dasen et al., 2008; Roy et al., 2012; Sockanathan and Jessell, 1998
LMCl	Lim1, Isl2, Foxp1, Raldh2	Brachial and Lumbar	Dorsal Limb Muscles	Tsuchida et al., 1994; Landmesser, 1978; Rousso et al., 2008; Dasen et al., 2008; Roy et al., 2012; Sockanathan and Jessell, 1998
PGC	Isl1, Isl2, Hb9, Foxp1, nNOS	Thoracic	Sympathetic ganglia	Tsuchida et al., 1994; Rousso et al., 2008; Dasen et al., 2008; Roy et al., 2012;

Table 1: Gene Expression Profiles of Motor Neuron Subtypes. Table shows the genes that are expressed in each motor neuron subtype, plus the location of each motor neuron subtype along the rostro-caudal axis of the spinal cord and the target cells of each motor neuron subtype.

CHAPTER 1

The Isl1-Lhx3 Complex Promotes Motor Neuron Specification by Activating Transcriptional Pathways that Enhance its Own Expression and Formation

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ABSTRACT

Motor neuron progenitor cells rapidly induce high expression of the transcription factors Isl1 and Lhx3 as well as LMO4 as they exit the cell cycle and differentiate. Isl1 and Lhx3 form the Isl1-Lhx3 complex, which induces the transcription of genes that contribute to motor neuron specification and maturation. LMO4 blocks the formation of competing LIM complexes, such as the V2 interneuron-specifying Lhx3-complex, inhibiting the expression of non-motor neuron genes and increasing the pool of free Lhx3 available to incorporate into the Isl1-Lhx3 complex. However, the mechanisms regulating the precise temporal and spatial regulation of Isl1, Lhx3 and LMO4 expression are not well characterized. I used

ChIP-Seq experiments to identify genomic binding loci for the Isl1-Lhx3 complex associated with *Isl1*, *Lhx3* and *Lmo4*. Each of these genomic loci recruited the Isl1-Lhx3 complex to activate the transcription of *Isl1*, *Lhx3* or *Lmo4* in embryonic motor neurons. *De novo* motif analysis and site-directed mutagenesis experiments also revealed that these enhancers utilize several distinct genetic mechanisms to recruit the Isl1-Lhx3 complex and promote transcription of their target genes. Our results support a model in which the Isl1-Lhx3 complex amplifies its own expression through a potent autoregulatory feedback loop and simultaneously enhances the transcription of *Lmo4*. LMO4 then promotes incorporation of Lhx3 into the Isl1-Lhx3 complex. At the same time, high levels of LMO4 likely disrupt the formation of the Isl1-Lhx3 complex, creating a negative feedback loop to stabilize the expression of these three genes.

SIGNIFICANCE STATEMENT

The precise temporal and spatial regulation of transcription factor expression is critical for embryos to generate the appropriate number and variety of motor neurons. This process dictates the formation of motor circuits, which regulate coordinated movement and homeostasis. When motor neuron specification is impaired, it leads to serious medical conditions such as spinal muscular atrophy. Understanding motor neuron development is crucial for effectively treating pediatric motor neuron disorders and neurodegenerative disorders, such as amyotrophic lateral sclerosis. Isl1, Lhx3 and LMO4 are essential for motor neuron development. Here I show that their expression is directly regulated by

the Isl1-Lhx3 complex. Characterizing the pathways that direct the expression of these factors provides key insights into the genetic mechanisms that regulate motor neuron development.

INTRODUCTION

Combinatorial expression of specific transcription factors establishes discrete progenitor domains in the embryonic spinal cord, which each generate distinct types of neurons. The p0-p3 domain generate ventral interneurons and the pMN domain generates motor neurons. While the signaling cascades that establish the pMN domain are well characterized, the mechanisms that promote the initiation and maintenance of motor neuron transcription factor expression remain unclear (Pituello, 1997; Jessell, 2000b; Price and Briscoe, 2004).

Immediately prior to differentiating, pMN cells express two LIM-Homeodomain transcription factors, Islet-1 (Isl1) and LIM-homeobox 3 (Lhx3) (Tanabe et al., 1998; Thaler et al., 2002). Both proteins contain 2 LIM domains, that facilitate protein-protein interactions, as well as a single homeodomain that binds DNA (Matthews et al., 2008). When co-expressed, Isl1 and Lhx3 interact with each other and with Nuclear LIM Interactor (NLI) to form a hexameric transcription complex, called the Isl1-Lhx3 complex, with a 2:2:2 stoichiometry (Thaler et al., 2002). When Lhx3 is expressed in the absence of Isl1, as is the case in developing V2 interneurons, Lhx3 and NLI form a tetrameric complex called the Lhx3 complex containing 2NLI:2Lhx3 (Thaler et al., 2002). The Isl1-Lhx3 complex and Lhx3 complex primarily function through binding to the

hexamer response element (HxRE) and the tetramer response element (TeRE), respectively (Lee et al., 2008). The Isl1-Lhx3 complex activates the transcription of genes that are essential for motor neuron specification such as Hb9, as well as genes that are required for cholinergic neurotransmission such as VaCHT (Thaler et al., 2002; Lee and Pfaff, 2003; Lee et al., 2012). Knockdown of Isl1 or Lhx3 or disruption of Isl1-Lhx3 complex assembly severely impairs motor neuron specification (Pfaff et al., 1996; Sharma et al., 1998; Thaler et al., 2002; Song et al., 2009; Liang et al., 2011).

To efficiently transition from a progenitor state to a terminally differentiated state, pMN cells must rapidly upregulate and maintain expression of the Isl1-Lhx3 complex. Following motor neuron specification and migration, Isl1 expression is maintained in many motor neuron subtypes, but Lhx3 expression is only maintained in Medial Motor Column (MMCm) neurons (Tsuchida et al., 1994; Kania and Jessell, 2003). Despite extensive progress characterizing the spatial and temporal patterns of gene expression in differentiating motor neurons, the genetic mechanisms that direct differentiating motor neurons to induce high levels of Isl1 and Lhx3 transcription during motor neuron specification, and the mechanisms utilized to maintain high levels of Isl1 and Lhx3 expression in MMCm neurons, remain unclear.

I found that the Isl1-Lhx3 complex binds two distinct genomic loci downstream of *Lhx3*, as well as a known Isl1 enhancer (Uemura et al., 2005; Kim et al., 2015). Interestingly, I also found a binding site within the second intron of the LIM Only Protein 4 (LMO4) coding region. LMO4 is expressed in embryonic

motor neurons, and is important for inhibiting the formation of the Lhx3 complex in motor neurons (Lee et al., 2008). Using Green Fluorescent Protein (GFP)-reporter studies and embryonic chick neural tube electroporation, I found that each of these Isl1-Lhx3 binding sites act as motor neuron-specific enhancers and each is activated by the Isl1-Lhx3 complex.

Our results show that the Isl1-Lhx3 complex activates two distinct transcription pathways in parallel to potentiate its own expression and function during motor neuron development. First, a positive autoregulatory loop amplifies the expression of the complex's key components, Isl1 and Lhx3. Second the Isl1-Lhx3 complex activates the expression of LMO4, which promotes Lhx3 incorporation into this complex by blocking the formation of the Lhx3-complex.

METHODS

Chromatin Immunoprecipitation (ChIP)-qPCR assays

ChIP assays were performed using embryonic day 12.5 (E12.5) mouse (ms) embryo spinal cord lysates as described by (Thiebes et al., 2015). Antibodies used for immunoprecipitation were rabbit (rb) anti Isl1/2 (kindly provided by Tom Jessell) (Tsuchida et al., 1994), rb anti Lhx3 (Abcam ab14555), and non-specific rb IgG. Following immunoprecipitation, Quantitative Polymerase Chain Reaction (qPCR) was performed to detect putative enhancers using the following primers: Lhx3-En-A Forward (Fwd): GGTCTGCCTCCCGTAAAACT Reverse (Rev): CACCATCAATGCTTTGTTCAG, Lhx3-En-B Fwd: CAATGCAGGGTGACCTGG Rev: GTGGGATTGACTGGGGTC,

Isl1-En Fwd: CTGCCACTCCACTTAATAACCTAA

Rev: ATGGACACACCAGCTGGATAAATC,

LMO4-En Fwd: ATCACTCGAGGACGTGGGTCCCTTTAAGATCC

Rev: CTGAGTCGACGGATTCTGCCTCCTCTCCTC. All ChIP experiments were repeated independently 3 times. Results shown were obtained from technical triplicates from representative experiments.

In Ovo Electroporation

Electroporation was performed in HHst 12-14 chick embryos, by injecting DNA into the embryonic neural tube (Hamburger and Hamilton, 1993). A square pulse electroporater was used to apply 5 pulses, 25V, 50ms with 1 second between each pulse across the neural tube. Enhancers were cloned into PBS-miniCMV-eGFP or SP72-TATA-eGFP reporter plasmids. Lhx3-Enhancers and the LMO4-En were cloned from the mouse genome, and the Isl1-En was cloned from the human genome. Embryos were injected with 2.5µg/µL of reporter construct and 1.75µg/µL of β-galactosidase (LacZ) or Isl1-Lhx3 expression construct (Fig 12). Embryos were harvested and processed for immunolabeling 3 days post electroporation (DPE) at HHst 25.

Enhancers

Lhx3-En-A (ms) (chr2:26194774-26194788): Fwd: CTAGAGGTAGCCAAGGCC

Rev: TGGAGAGGGCTAGCCAC. Hx-wt: CATTTTAACTAATGG

ΔHx: CGCGGCCGCAGCCGG Te-wt: CTAATTAAA ΔTe: CGGCCGCAA

Lhx3-En-B (ms) (chr2:26186472-26187246): Fwd-CAATGCAGGGTGACCTGG Rev-GTGGGATTGACTGGGGTC. Hx-wt: ATTTGATTAATCA. ΔHx: AGCGGCCGCCTCA.

Isl1-En (human)(chr5:51559189-51559911):
Fwd: CAGATGCACCTACCTCTTAAAG Full-Length Rev: GGACATATGGCTAGAGTGTGG Δ3'315 Rev: CCCTACTCTGTCTGCCACTCC TAAT-210: TTTTAATTAGCT Δ210: TTTCTAGAAGCT TAAT-260: ATATTAAAAT Δ260: ATCTAGAAAT Motif-470: AATTTTAGCATAT Δ470: ACGGTTGGCGCCT

LMO4-En (ms)(ch3:144198960-144199257):
Fwd: GACGTGGGTCCCTTTAAGATCC Rev: GGATTCTGCCTCCTCTCCTC Hx-wt: AATTTTGTTAATTAA ΔHx: AACCATGGTAGGTAA

Immunofluorescent Labeling

Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline solution (PFA/PBS) for 90 minutes, embedded in Optimal Cutting Temperature (OCT) media and cryosectioned at 12µm. Embryo sections were incubated in primary antibody in either 0.1% Fish Gelatin or 0.3% Bovine Serum Albumin (BSA) blocking buffer, overnight at 4°C. Primary antibodies used were goat (gt) anti LacZ (Sigma 1:2000), rb anti LacZ (Cappel 55976 1:2000), ms anti Mnr2

(DSHB 5C10 1:250), and chicken (chk) anti GFP (Aves Labs 1020 1:1000). Sections were imaged using a Zeiss Axio Imager.Z2 microscope.

GFP Quantification

Embryos used for GFP fluorescence analysis were not immunostained for GFP. 750ms exposure time was used for all images that were analyzed for GFP quantification. Integrative pixel density was measured in the ventral horn of the electroporated side of the spinal cord, using ImageJ-win64. 4-12 embryos were analyzed for each reporter construct. And for each embryo, average fluorescence intensity was calculated from analyzing 3-7 sections.

***In Situ* Hybridization**

Electroporated embryos were harvested at 3 days post electroporation (DPE) and fixed in 4% PFA/PBS for 90 minutes. They were embedded in OCT media and cryosectioned at 18μm. cDNA for chick Lhx3, Isl1 and LMO4 3' Untranslated Region (UTR) was cloned into pBluescript vector and these vectors were used to generate digoxigenin-labeled riboprobes. Riboprobes were generated using T7 polymerase PCR. In situ hybridizations were performed as previously described (Lee et al., 2005a)

Luciferase Assays

Assays were performed in cultured P19 embryonic mouse carcinoma cells (McBurney and Rogers, 1982). Cells were cultured in α-minimal essential media

with 7.5% BCS and 2.5% FBS. For luciferase assays, cells were seeded in 48 well plates, and transfected using Lipofectatmine 2000 (Invitrogen). Cells were transfected with reporter constructs, transcription factor expression constructs, a CMV-β-galactosidase construct, to test transfection efficiency, and with empty plasmid to equalize the total amount of DNA for each condition. Luciferase and β-galactosidase activity was measured 48 hours after transfection.

RESULTS

The Isl1-Lhx3 complex binds genomic loci associated with *Isl1*, *Lhx3* and *Lmo4*

To identify novel genomic binding sites for the Isl1-Lhx3 complex in a genome-wide and unbiased manner, we performed chromatin immunoprecipitation (ChIP) experiments in conjunction with high-throughput sequencing (ChIP-Seq). For these experiments we used mouse embryonic stem cells with a doxycycline-inducible Isl1-Lhx3 fusion gene to induce the expression of the Isl1-Lhx3 complex, coupled with a motor neuron differentiation protocol (Fig. 7B) (Lee et al., 2012, 2013). As transcriptional autoregulation is a powerful mechanism utilized by a variety of systems during development, I hypothesized that the Isl1-Lhx3 complex may act to directly regulate its own expression (Johnson et al., 1994; Belaguli et al., 1997; Smith et al., 2000; Aota et al., 2003; Bai et al., 2007; Borromeo et al., 2014). This hypothesis is further supported by the observation that the Isl1-Lhx3 fusion protein induces the transcription of *Isl1*, *Lhx3* and *Lmo4* in the motor neuron embryonic stem cell system (Lee et al., 2012).

I found two Isl1-Lhx3 complex binding loci downstream of the *Lhx3* gene. Lhx3 Enhancer A (Lhx3-En-A) is located approximately 5.1kb downstream of Lhx3 (Fig. 7A). Lhx3 Enhancer B (Lhx3-En-B) is located approximately 19.5kb downstream of the Lhx3 gene (Fig. 7A). I also found an Isl1-Lhx3-complex binding locus within a previously identified Isl1 enhancer (Isl1-En) (Fig. 7A) (Uemura et al., 2005; Kim et al., 2015). Unexpectedly, I found that the Isl1-Lhx3 complex also binds a locus within the second intron of *Lmo4* (LMO4-En) (Fig. 7A). Given the known role of LMO4 in blocking the formation of the Lhx3-complex, this finding suggests an additional regulatory pathway to indirectly facilitate the formation of the Isl1-Lhx3 complex.

The Isl1, Lhx3 and LMO4 enhancers are bound by the Isl1-Lhx3 complex *in vivo*

To investigate if these putative Isl1, Lhx3 and LMO4 enhancers are utilized by the Isl1-Lhx3 complex during embryonic motor neuron development, we assessed the *in vivo* occupancy of each enhancer by the Isl1-Lhx3 complex. To do this, we performed ChIP for endogenous Isl1 or Lhx3, using E12.5 mouse spinal cord lysate, precipitating with either anti-Isl1, anti-Lhx3 antibody or control IgG, followed by quantitative PCR (qPCR) for Lhx3-En-A, Lhx3-En-B, Isl1-En and LMO4-En. Compared to IgG, antibodies against both Isl1 and Lhx3 precipitated significantly more Lhx3-En-A, Lhx3-En-B, Isl1-En, and LMO4-En (Fig. 7C-F). As a negative control, we also performed qPCR for the untranslated genomic locus

Untr-6 (Mali et al., 2008), and saw no enrichment in the amount of *Untr-6* precipitated with antibodies against Isl1 or Lhx3, compared to IgG (Fig. 7G).

These results indicate that, during embryonic motor neuron development, the Isl1-Lhx3 complex specifically binds Lhx3-En-A, Lhx3-En-B, the Isl1-En, and the LMO4-En *in vivo*. This finding raises the possibility that the Isl1-Lhx3 complex directly activates the transcription of *Lhx3*, *Isl1* and *Lmo4* by directly interacting with these putative enhancers.

Lhx3-En-A is activated by the Isl1-Lhx3 complex

To test whether Lhx3-En-A activates transcription in motor neurons I performed chick neural tube electroporation with a GFP-reporter construct containing two copies of Lhx3-En-A upstream of a minimally active TATA-box promoter and EGFP (Lhx3-En-A-GFP, Fig. 8B). Embryos were also electroporated with a ubiquitously expressing LacZ construct to mark electroporated cells. Chick embryos were electroporated at HHst 14 and analyzed 3 days post electroporation (3DPE).

Lhx3-En-A induced modest GFP expression specifically in cells expressing the motor neuron-specific gene Mnr2, a homolog of Hb9 (Fig. 8C & 8G). This result is consistent with the hypothesis that endogenous Isl1-Lhx3 complex activates Lhx3 transcription in motor neurons via Lhx3-En-A. Next, I co-electroporated Lhx3-En-A-GFP reporter with an Isl1-Lhx3 fusion protein vector. When electroporated, this construct activates ectopic expression of Isl1-Lhx3 fusion protein, which complexes with endogenous NLI to form the Isl1-Lhx3

complex (Thaler et al., 2002; Lee et al., 2008; Song et al., 2009). Ectopic expression of the Isl1-Lhx3 complex activated GFP expression throughout the dorsal and ventral spinal cord (Fig. 8). Further, both dorsal GFP^+ cells and GFP^+ cells in the ventral horn also expressed the motor neuron specific marker Mnr2 (Fig. 8O).

Because forced expression of the Isl1-Lhx3 complex initiates ectopic motor neuron fate specification, it was unclear if the Isl1-Lhx3 complex directly activates GFP expression in ectopic motor neurons or if the change in cell-fate specification indirectly activates Lhx3-En-A. To test if the Isl1-Lhx3 complex can directly activate transcription via Lhx3-En-A, even without initiating motor neuron fate specification, I performed luciferase reporter assays in cultured mouse embryonic carcinoma P19 cells. For these experiments, I transfected Lhx3-En-A-LUC reporters with expression vectors for Isl1, Lhx3, Isl1+Lhx3, or with empty vector. I cultured cells for two days and then performed luciferase assays to measure transcription of the luciferase reporter-gene. Transfection of Isl1 plus Lhx3 significantly activated Lhx3-En-A-LUC compared to control LUC reporter containing no enhancer (Fig 8S). Transfection of Isl1 plus Lhx3 also significantly activated Lhx3-En-A compared to transfection of Isl1 or Lhx3 alone (Fig 14A). Combined with the ChIP-qPCR results from mouse embryonic spinal cord (Fig. 7C), these results suggest that the Isl1-Lhx3 complex directly binds Lhx3-En-A to initiate the transcription of *Lhx3*.

Lhx3-En-A activity is mediated by two binding sites for the Isl1-Lhx3 complex

We have previously reported that the Isl1-Lhx3 complex is capable of binding both the HxRE and the TeRE (Lee et al., 2008). I found that Lhx3-En-A contains both a putative HxRE and a putative TeRE (Fig 8A). To test if either or both of these response elements contribute Lhx3-En-A activity, I generated mutated versions of Lhx3-En-A where either the HxRE, the TeRE or both sites are mutated (ΔHx, ΔTe, ΔHxΔTe, respectively) (Fig. 8B). Next, I made GFP-reporter constructs with each of these mutated versions of Lhx3-En-A and performed chick neural tube electroporation with either LacZ or Isl1-Lhx3.

When co-electroporated with LacZ, the Lhx3-En-A-ΔHx reporter construct activated GFP expression in Mnr2-positive motor neurons (Fig 8D). However, neither Lhx3-En-A-ΔTe, or Lhx3-En-A-ΔHxΔTe activated any detectable GFP expression in the spinal cord (Fig. 8E-F). Co-electroporation with Isl1-Lhx3 activated both single mutant constructs throughout the spinal cord, but failed to activate the Lhx3-En-A-ΔHxΔTe double mutant (Fig. 8L-N). These results show that both the HxRE and the TeRE in Lhx3-En-A contribute to its motor neuron enhancer activity. Without the HxRE, Lhx3-En-A is activated by endogenous levels of the Isl1-Lhx3 complex. However, when the TeRE is ablated, the enhancer requires high levels of the Isl1-Lhx3 complex to be activated. And when both sites are mutated, the Isl1-Lhx3 complex cannot activate Lhx3-En-A.

I observed similar results with luciferase assays in P19 cells. In cells transfected with luciferase reporter constructs containing Lhx3-En-A, Lhx3-En-A-

ΔHx, or Lhx3-En-A-ΔTe, transcription is activated by co-transfection with Isl1 plus Lhx3. However, when both response elements are mutated co-transfection with Isl1 plus Lhx3 fails to activate transcription (Fig. 8S).

Lhx3-En-B is activated in embryonic motor neurons

To test if Lhx3-En-B acts as a motor neuron specific enhancer, I electroporated Lhx3-En-B-GFP (Fig. 9B) with either LacZ or Isl1-Lhx3 in embryonic chick neural tube. When co-electroporated with LacZ, Lhx3-En-B activated GFP expression specifically and robustly in Mnr2-positive motor neurons (Fig. 9C). Co-electroporation of Lhx3-En-B-GFP with Isl1-Lhx3 activated GFP expression throughout the spinal cord, specifically in cells expressing ectopic or endogenous Mnr2 (Fig. 9G), Interestingly, when I performed luciferase assays in P19 cells, Lhx3-En-B was not activated by co-transfection of Isl1 plus Lhx3 compared to control reporter construct with no enhancer (Fig. 9K). These results indicate that Lhx3-En-B acts as a strong motor neuron specific enhancer in embryonic motor neurons *in vivo*. However, the Isl1-Lhx3 complex is not sufficient to activate Lhx3-En-B in all cellular contexts. Cultured P19 cells could lack critical co-factors that are required for the Isl1-Lhx3 complex to activate transcription via Lhx3-En-B. However, as the Isl1-Lhx3 complex can activate other motor neuron specific enhancers in these cells, it is more likely that P19 cells express transcriptional repressors that specifically recognize Lhx3-En-B to block Isl1-Lhx3 complex binding or activity.

Lhx3-En-B Activity is mediated by a single HxRE

Lhx3-En-B contains one putative HxRE (Fig 9A). To test if this HxRE contributes to the enhancer activity of Lhx3-En-B, I generated a mutated version of Lhx3-En-B where the HxRE is ablated (Lhx3-En-B-ΔHx) (Fig. 9B). When electroporated with LacZ, Lhx3-En-B-ΔHx did not activate any detectable GFP expression in $MnR2^{+}$ motor neurons (Fig 9D). Co-electroporation of Isl1-Lhx3 fusion protein also failed to activate GFP expression, indicating that the HxRE is critical for the motor neuron specific enhancer activity of Lhx3-En-B in the embryonic spinal cord (Fig 9H).

Motor neuron progenitor cells must rapidly upregulate the transcription of the Isl1-Lhx3 complex to promote terminal differentiation and motor neuron cell fate specification. Immediately following the onset of Isl1 and Lhx3 expression in newly specified motor neurons, Lhx3-En-A and Lhx3-En-B likely contribute to this rapid increase in the transcription of *Lhx3.* This positive feedback-loop facilitates the switch from a non-specified motor neuron progenitor cell to a fully-committed, differentiated motor neuron.

The Isl1-En Is activated by the Isl1-Lhx3 complex via multiple TAAT motifs

As described previously, the Isl1-En activates transcription in newly born motor neurons and in mature MMCm neurons in mouse, zebrafish and chick embryos (Uemura et al., 2005; Kim et al., 2015). I confirmed this finding in chick embryos by electroporating Isl1-En-GFP reporter construct (Fig. 10C & D). It was also reported that the Isl1-En is activated by ectopic expression of the Isl1-Lhx3

complex (Kim et al., 2015). I tested this by electroporating Isl1-En-GFP with Isl1-Lhx3 fusion protein and found that indeed ectopic expression of the Isl1-Lhx3 complex expanded GFP expression to the dorsal spinal cord and GFP expression co-localized with ectopic Mnr2 expression (Fig 10V). These results are consistent with our findings that both Isl1 and Lhx3 bind to the Isl1-En in embryonic stem cells and in the mouse embryonic spinal cord (Fig. 7A & E). The Isl1-En is 720 base pairs long and contains fifteen TAAT sites (Fig 10A). TAAT sequences act as binding sites for homeodomain transcription factors, and both the HxRE and the TeRE contain TAAT sequences. Thirteen of the TAAT sites within the Isl1-En are completely conserved between mouse and human, indicating that they likely play important roles in Isl1-En activity. Of the conserved TAAT sequences, two closely resemble TeRE sequences, located at 210 base pairs and 505 base pairs into the Isl1-En (Fig 10A & B).

Because the Isl1-En contains many conserved TAAT sites, including two putative TeREs, I hypothesized that one or more of these sites could mediate Isl1-En activity. To test this hypothesis, I made a truncated version of the Isl1-En missing 315 base pairs from the 3' end of the enhancer. This short Isl1-En (Isl1-En-Δ3'315) is 406 base pairs long and contains 8 of the 15 TAAT sites, including one of the putative TeREs (210) (Fig. 10A). To test whether Isl1-En-Δ3'315 maintained motor neuron-specific enhancer activity, I made GFP reporter constructs and performed chick neural tube electroporations (Fig 10C). I found that Isl1-En-Δ3'315 activated GFP expression specifically in Mnr2 positive embryonic motor neurons (Fig 10I). But the fluorescence intensity in Isl1-En-

Δ3’315-GFP embryos was significantly lower than experiments with full length Isl1-En (Fig. 10NN). These results support the hypothesis that the seven deleted TAAT sites contribute to the motor neuron-specific enhancer activity of Isl1-En.

To test if individual TAAT sites contribute to Isl1-En activity, I mutated two specific TAAT sites in both the full-length and the truncated Isl1-En, TAAT-210, which resembles a TeRE, and TAAT-260 (Fig. 10A & C). I found that for both the full-length and the truncated enhancer, mutating TAAT-210 (Δ210) significantly reduced GFP expression (Fig. 10 E, J, NN & OO). However, mutating TAAT-260 (Δ260) did not reduce GFP expression compared to wt Isl1-En (Fig 10 F, K, NN & OO). Surprisingly, in the full-length Isl1-En, mutating both sites (Δ210Δ260) reduced GFP expression even further than mutating TAAT-210 alone (Fig 10H & NN). These results indicate that TAAT-210, TAAT-260 and the TAAT motifs located within the 3’315 region, cooperate to recruit the Isl1-Lhx3 complex, and activate motor neuron-specific transcription of *Isl1*.

I also mutated motif-470 (Δ470), which is only present in the full length version of the Isl1-En (Fig. 4 A & C). Previous reports have shown that this site is required for Isl1-En activity in endogenous motor neurons and in ectopic motor neurons induced by overexpression of the Isl1-Lhx3 complex (Kim et al., 2015). However, I found that Isl1-En-Δ470 activated robust GFP expression in both endogenous and ectopic motor neurons (Fig 10 G & Y). In endogenous motor neurons, there was no difference between Isl1-En-Δ470-GFP expression and wt Isl1-En-GFP expression (Fig 4NN). Next I co-electroporated each mutant construct with an Isl1-Lhx3 fusion protein construct to activate ectopic expression

of the Isl1-Lhx3 complex (Fig. 10V-DD). I found that, like the wt enhancer, each mutant enhancer activated robust GFP in the dorsal spinal cord that co-localized with ectopic Mnr2 expression. Because this approach expresses high levels of Isl1-Lhx3 complex, it is more sensitive than Isl1-En-GFP electroporations alone, and is able to detect weaker enhancer activity.

Luciferase assays in cultured P19 cells are consistent with these results. Co-transfection of Isl1 and Lhx3 with wt Isl1-En-LUC reporter construct activates transcription compared to empty-LUC reporter construct (Fig10PP). Likewise, truncating or mutating the Isl1-En has no effect on its enhancer activity (Fig 10PP).

These findings support a model in which the Isl1-Lhx3 complex activates transcription via the Isl1-En, by binding multiple TAAT sites within this enhancer. The TAAT sites in the Isl1-En act redundantly and cooperatively to promote Isl1-En activity and *Isl1* transcription. It is also important to note that different TAAT sites may differentially contribute to enhancer activity, with TAAT-210 contributing substantially to enhancer activity and TAAT-260 contributing less to the activity of the Isl1-En. TAAT-210 may act as a more potent recruiter of the Isl1-Lhx3 complex because it closely resembles a TeRE, which is a known Isl1-Lhx3 complex binding motif.

The LMO4-En is activated in embryonic motor neurons

In addition to rapidly and robustly upregulating its own expression, I hypothesized that the Isl1-Lhx3 complex also activates the transcription of LMO4 in newly-

differentiating embryonic motor neurons. During motor neuron specification, LMO4 blocks the formation of the V2-interneuron specifying Lhx3 complex, and thereby inhibits the expression of V2-specific genes in motor neurons (Lee et al., 2008). High levels of LMO4 expression have also been shown to block the activity of the Isl1-Lhx3 complex (Song et al., 2009). To test if the LMO4-En (Fig. 7A) is activated in embryonic motor neurons, I performed chick neural tube electroporations with an LMO4-En-GFP reporter construct (Fig. 11B). When I electroporated LMO4-En-GFP with LacZ, I found that the LMO4-En activates GFP expression specifically in $Mnr2^{+}$ motor neurons (Fig. 11C). When I co-electroporated Isl1-Lxh3 with LMO4-En-GFP, I saw that the LMO4-En activated GFP expression throughout the spinal cord, specifically in cells expressing endogenous or ectopic Mnr2 (Fig. 11E). Luciferase assays using LMO4-En-LUC with Isl1, Lhx3 or Isl1 plus Lhx3, are consistent with these results (Fig. 11K). Transfection of Isl1 plus Lhx3 significantly activates LMO4-En-LUC expression, compared to control vector containing no enhancer (Fig 11K). And transfection of Isl1 plus Lhx3 activates LMO4-En-LUC expression significantly more than transfection with Isl1 or Lhx3 alone (Fig 14C).

These results indicate that in embryonic motor neurons, the LMO4-En recruits the Isl1-Lhx3 complex to activate the transcription of *Lmo4*. Upregulating the transcription of *Lmo4* in embryonic motor neurons serves two functions. First, LMO4 blocks the formation and activity of the Lhx3 complex. This action inhibits the transcription of V2-IN specific genes in motor neurons, and increases the pool of free Lhx3 available to incorporate into the Isl1-Lhx3 complex. Second,

high levels of LMO4 compete with Isl1 for NLI binding, which blocks the formation and activity of the Isl1-Lhx3 complex. Inhibiting Isl1-Lhx3 complex activity creates a negative feedback loop that counteracts the positive transcriptional autoregulation of the Isl1-Lhx3 complex, and thereby stabilizes the expression levels of Isl1, Lhx3 and LMO4 in embryonic motor neurons (Fig 15).

LMO4-En activity Is mediated by a single HxRE

The LMO4-En contains a HxRE and a TeRE (Fig 11A). To test if the HxRE contributes to the activity of the LMO4-En, I generated a mutated version of the LMO4-En where the HxRE sequence is ablated (ΔHx) (Fig. 11B). Chick neural tube electroporations with LMO4-En-ΔHx-GFP did not activate any detectable GFP expression in the embryonic spinal cord, despite robust electroporation efficiency, seen with LacZ expression (Fig. 11D, Fig 12N). Co-electroporation of Isl1-Lhx3 fusion protein with LMO4-En-ΔHx-GFP also failed to activated GFP expression (Fig. 11F). Likewise, LMO4-En-ΔHx-LUC was not activated by co-transfection with Isl1 plus Lhx3 in P19 cells (Fig. 5K). These results indicate that the HxRE is required for the Isl1-Lhx3 complex to activate transcription via the LMO4-En.

The Isl1-Lhx3 complex activates the transcription of endogenous Lhx3, Isl1 and LMO4

Next I tested if the Isl1-Lhx3 complex activates the transcription of *Isl1*, *Lhx3* and *Lmo4* in the embryonic spinal cord. To distinguish endogenous Isl1 and Lhx3

transcript from the transcript of overexpressed Isl1 and Lhx3, or Isl1-Lhx3 fusion protein, I ectopically expressed mouse Isl1 and rat Lhx3 in the embryonic chick spinal cord through neural tube electroporation. Next, I harvested embryos at 3DPE and performed *in situ* hybridizations with chicken-specific probes designed to recognize the 3' untranslated region (UTR) of chick *Isl1*, *Lhx3* or *Lmo4*. Because the Isl1, Lhx3 and Isl1-Lhx3 expression constructs lack 3' UTR sequences, these probes exclusively detected endogenous chick transcripts.

Embryos that were electroporated with Isl1 alone showed no change in the expression of endogenous Isl1, Lhx3 or LMO4 compared to the unelectroporated side of the spinal cord (Fig. 13A-C). Lhx3 electroporation slightly increased the transcription of LMO4, but did not affect expression of endogenous Isl1 or Lhx3 (Fig. 13D-F). Embryos that were electroporated with Isl1-Lhx3 showed robust increases in transcription of Isl1, Lhx3, and LMO4 throughout the spinal cord (Fig. 13G-I).

DISCUSSION

A great deal of progress has been made characterizing the activity and expression patterns of Isl1, Lhx3, and LMO4 in embryonic motor neurons (Thaler et al., 2002; Lee et al., 2008, 2012, 2013; Rousso et al., 2008; Song et al., 2009; Roy et al., 2012). However, the mechanisms that activate the transcription of these factors in differentiating motor neurons, and the pathways that regulate their expression in specific motor neuron subtypes remain unclear. Our results show that the Isl1-Lhx3 complex binds genomic loci associated with *Lhx3*, *Isl1*

and *Lmo4*, both in a motor neuron embryonic stem cell system and in the embryonic spinal cord. Each of these loci acts as a motor neuron specific enhancer and is robustly activated by the Isl1-Lhx3 complex. Additionally, I show that the Isl1-Lhx3 complex activates the transcription of endogenous *Isl1*, *Lhx3* and *Lmo4.*

These findings support a model in which positive transcriptional autoregulation of the Isl1-Lhx3 complex contributes to the rapid induction of Isl1, Lhx3 and LMO4 expression in differentiating motor neurons. Simultaneously, high levels of LMO4 expression likely inhibit Isl1-Lhx3 complex formation and function, which creates a negative feedback loop to prevent excessive Isl1, Lhx3 and LMO4 expression (Fig 15). These autoregulatory transcriptional pathways also contribute to maintaining the expression of Isl1 and Lhx3 in mature MMCm neurons.

Expression of the Isl1-Lhx3 complex

Onecut transcription factors including Hnf6 and Onecut-2 activate the transcription of Isl1 in early motor neurons and regulate the expression of Isl1 in multiple motor neuron subtypes (Roy et al., 2012). However, in the absence of Hnf6 and Onecut-2, newly generated motor neurons still maintain low levels of Isl1 expression, and normal numbers of $Hb9^{+}$ motor neurons are generated, indicating that there are additional pathways contributing to the onset of Isl1 expression (Roy et al., 2012). Likewise, the activation of Lhx3 and LMO4 expression in differentiating motor neurons is critical for motor neuron

specification and little is known regarding the specific mechanisms that activate and regulate the expression of these two factors (Sharma et al., 1998; Lee et al., 2008).

Here I propose that, early in motor neuron specification, Isl1 and Lhx3 are released from transcriptional repression via reduced levels of Olig2 expression (Lee et al., 2005b). This de-repression allows for modest transcriptional activation and expression of these two factors. The resulting low levels of the Isl1-Lhx3 complex in turn, activate a positive transcriptional feedback loop that rapidly induces high levels of Isl1 and Lhx3 expression, while concurrently activating LMO4 transcription (Fig. 15). When LMO4 expression is high, it competes with Isl1-Lhx3 for NLI binding (Song et al., 2009), generating a negative feedback loop to regulate the transcription of *Isl1*, *Lhx3* and *LMO4* (Fig. 15). Transcriptional feedback, both positive and negative, has been shown to contribute to the temporal regulation of gene expression in a variety of cellular contexts. Both direct and indirect examples of transcriptional autoregulation have been observed. In particular, transcriptional autoregulation is prominent in development and cell specification (Johnson et al., 1994; Belaguli et al., 1997; Smith et al., 2000; Aota et al., 2003; Bai et al., 2007; Borromeo et al., 2014). Positive autoregulation of the Isl1-Lhx3 complex is an efficient mechanism to ensure the rapid transition from a pluripotent, progenitor cell state to a post-mitotic, differentiated motor neuron. It facilitates rapid induction of the Isl1-Lhx3 complex and thereby, quickly induces the expression of genes essential for motor neuron differentiation such as Hb9 and LMO4 (Arber et al., 1999).

Recruitment of the Isl1-Lhx3 complex

While each enhancer in this study is activated by the Isl1-Lhx3 complex, the genetic mechanisms utilized to recruit this transcription complex vary widely. Both Lhx3 enhancers and the LMO4 enhancer contain sequences closely resembling the HxRE, while Lhx3-En-A and the LMO4-En also contain a TeRE (Lee et al., 2008). In Lhx3-En-A, the HxRE and the TeRE cooperate to mediate transcriptional activation, and transcriptional activation is only lost completely when both sites are ablated. In contrast to this finding, the HxREs in Lhx3-En-B and the LMO4-En are critical for the activity of these enhancers, and the TeRE in the LMO4-En does not compensate for the loss of the HxRE. TeREs serve as high affinity binding sites for the V2-IN specifying Lhx3 complex and, in embryonic motor neurons, Hb9 recognizes and binds TeREs to inhibit the transcription of Lhx3 complex target genes (Lee et al., 2008). Our results indicate that the TeREs found in Lhx3-En-A and the LMO4-En may not be recognized by Hb9, raising an interesting possibility that Hb9 binds only a subset of high affinity TeREs. Future genome-wide analysis of Hb9 binding sites in developing motor neurons will shed critical insights into this issue.

Unlike the Lhx3 and LMO4 enhancers, the Isl1-En does not contain any HxREs. Instead, I found fifteen TAAT motifs within the Isl1-En, including two sites that closely resemble TeREs. Through truncation and mutation experiments, I found that activity of the Isl1-En is mediated by cooperative action of multiple TAAT motifs, where reducing the number of TAAT motifs within the enhancer

reduces its transcriptional activity. While our results suggest that the motifs resembling TeREs contribute substantially to Isl1-En activity, even reporter constructs without these motifs were activated by ectopic expression of Isl1-Lhx3. These results indicate that the remaining TAAT sites within the Isl1-En act to recruit Isl1-Lhx3 to activate transcription. Because TAAT is a widely recognized binding motif, utilized by homeodomain and homeobox proteins, it is intriguing that the Isl1-En is specifically activated by the Isl1-Lhx3 complex, but not activated by other homeodomain transcription factors in the spinal cord. Further study of this enhancer could reveal interesting genetic mechanisms for transcriptional specificity in the absence of optimized consensus binding sequences.

Isl1, Lhx3 and LMO4 expression in motor neuron subtypes

In addition to facilitating Isl1 and Lhx3 transcription during motor neuron specification, the Isl1-En and Lhx3-Enhancers likely act to maintain high levels of Isl1 and Lhx3 expression in mature MMCm neurons. Following motor neuron specification, many motor neuron subtypes downregulate the expression of *Isl1* or *Lhx3*. LMCl neurons do not express *Isl1*, and *Lhx3* expression is only maintained in MMCm neurons (Tsuchida et al., 1994; Rousso et al., 2008). To halt the expression of Isl1 or Lhx3, motor neurons must disrupt the positive transcriptional feedback loop generated by these proteins. Transcriptional repressor proteins or translational repressing pathways, such as the expression of specific micro-RNAs, are efficient mechanisms to downregulate the expression

of Isl1 or Lhx3. Indeed, as LMCl neurons are specified, the onset of Lhx1 expression has been shown to repress Isl1 expression (Kania and Jessell, 2003).

While a great deal of work has been done to characterize the genetic mechanisms that activate the expression of specific transcription factors and signaling molecules during motor neuron subtype development, the pathways utilized to repress individual genes, specifically Lhx3, are not well understood. These repressive pathways are critical for motor neuron subtype development, as forced expression of Lhx3 has been shown to convert motor neurons to an MMCm fate (Sharma et al., 2000). It will therefore be important to identify the mechanisms utilized to downregulate Lhx3 expression in specific motor neuron subtypes, in order to build a comprehensive model of transcriptional regulation in motor neuron development.

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FIGURES

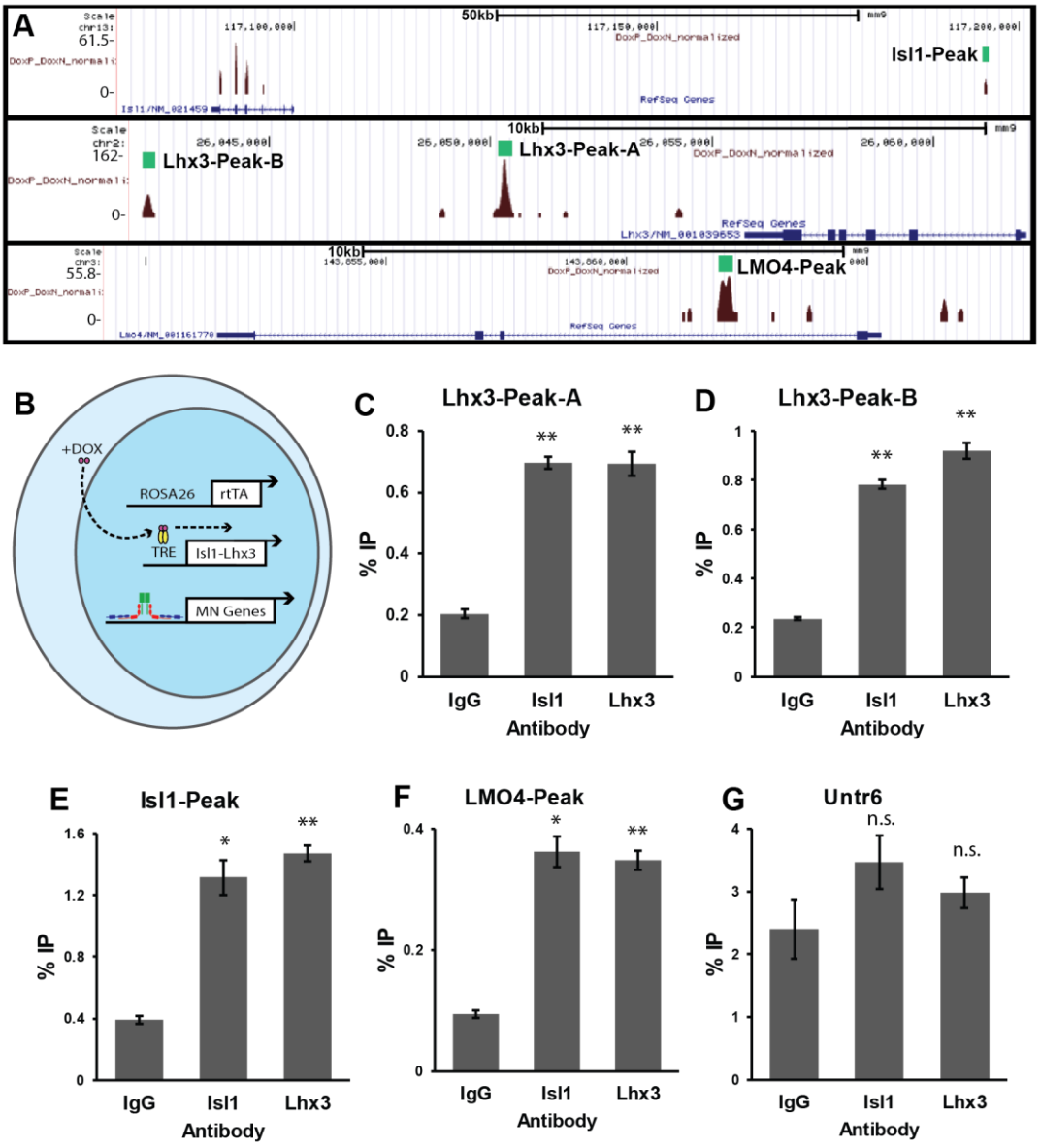


Figure 7: The Isl1-Lhx3 complex binds genomic loci associated with *Isl1*, *Lhx3* and *Lmo4*. (A) Isl1-Lhx3 complex binding sites, identified via ChIP-Seq, in association with *Lhx3*, *Isl1* and *Lmo4.* (B) Embryonic stem cells with a Doxycycline-inducible Isl1-Lhx3 fusion protein to induce motor neuron differentiation with high levels of Isl1-Lhx3 complex expression, were used for Isl1-Lhx3 complex ChIP-Seq. rtTA: reverse tetracycline transactivator, TRE:

tetracycline response element, DOX: doxycycline (C-G) E12.5 mouse spinal cord ChIP performed with Isl1 or Lhx3 antibodies. qPCR was performed for Lhx3-En-A, Lhx3-En-B, Isl1-En, LMO4-En, or Untr-6 negative control region. * $p<0.05$, ** $p<0.01$ compared to non-specific IgG. n=3 technical replicates. Two-tailed t-test assuming equal variance. Error bars represent the standard error of the mean.

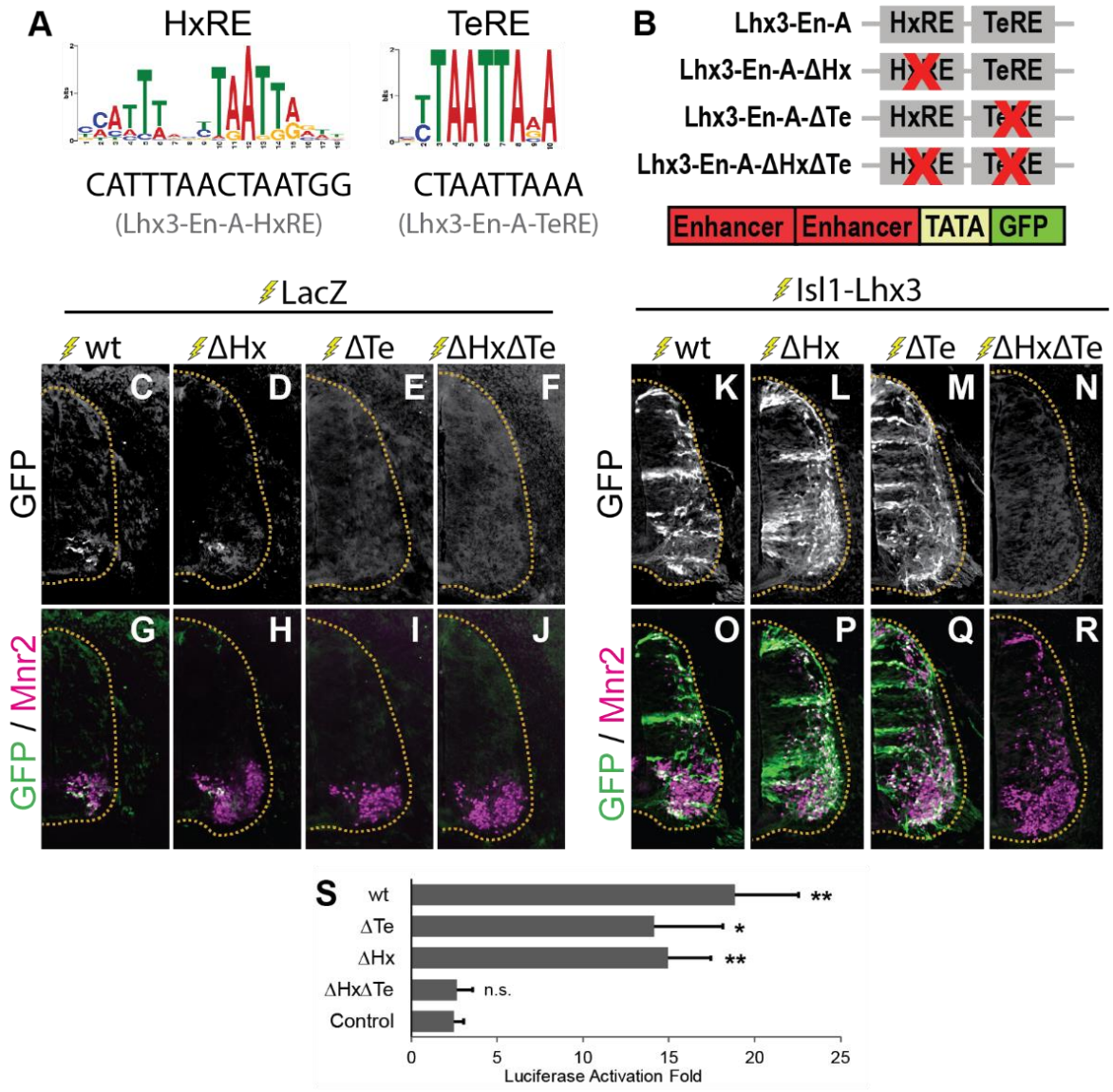


Figure 8: Lhx3-En-A is activated by the Isl1-Lhx3 complex. (A) HxRE and TeRE sequences identified by ChIP-Seq *de novo* motif analysis, and the HxRE and TeRE sequences in Lhx3-En-A. (B) Lhx3-En-A HxRE and TeRE mutants used for GFP-reporter experiments. For wt and mutant HxRE and TeRE sequences see methods section. (C-J) GFP-reporter experiments for Lhx3-En-A variants, embryos were electroporated with Lhx3-En-A-GFP reporter constructs plus ubiquitously expressing LacZ to mark electroporated cells. Sections were

immunostained for GFP and for Mnr2 to mark motor neurons. (K-R) Embryos electroporated with Lhx3-En-A-GFP reporter construct plus Isl1-Lhx3 fusion protein construct. Sections were immunostained for GFP and Mnr2.
(S) Luciferase assays testing Lhx3-En-A wt and mutants. Luciferase assays were performed in cultured P19 cells. Results show the luciferase activation fold upon the addition of Isl1 plus Lhx3, compared to empty vector (no transcription factors). n = 5 experiments. Two-tailed t-test assuming equal variance, comparing each reporter construct to control reporter (no enhancer). *$p<0.05$, **$p<0.01$, n.s: not significant. Error bars represent the standard error of the mean.

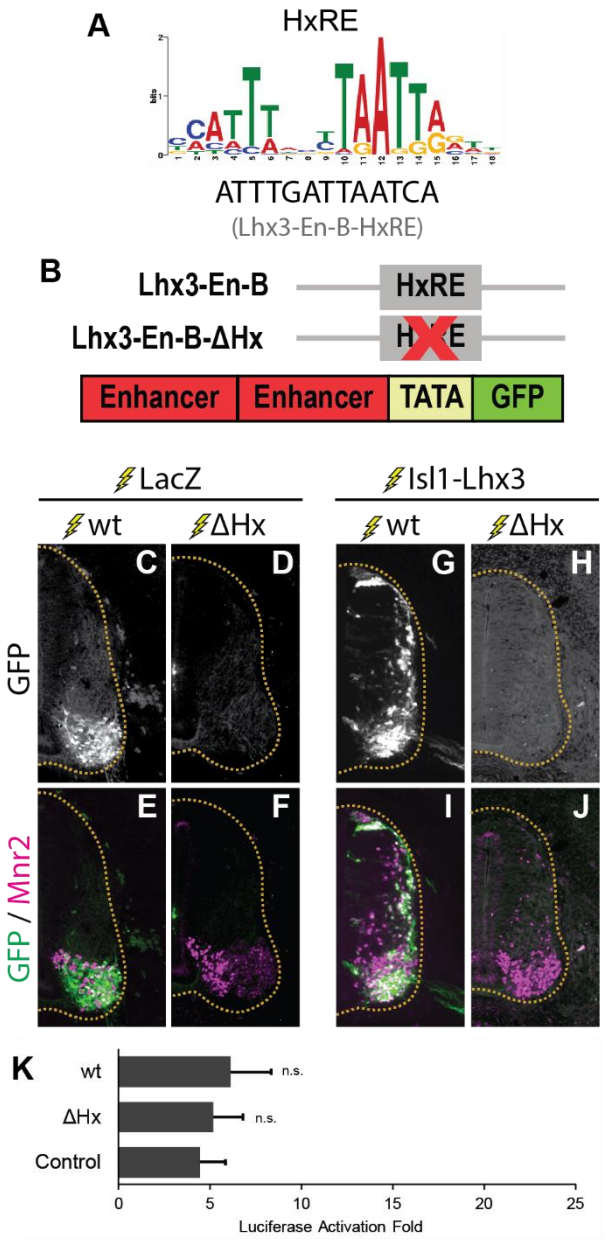
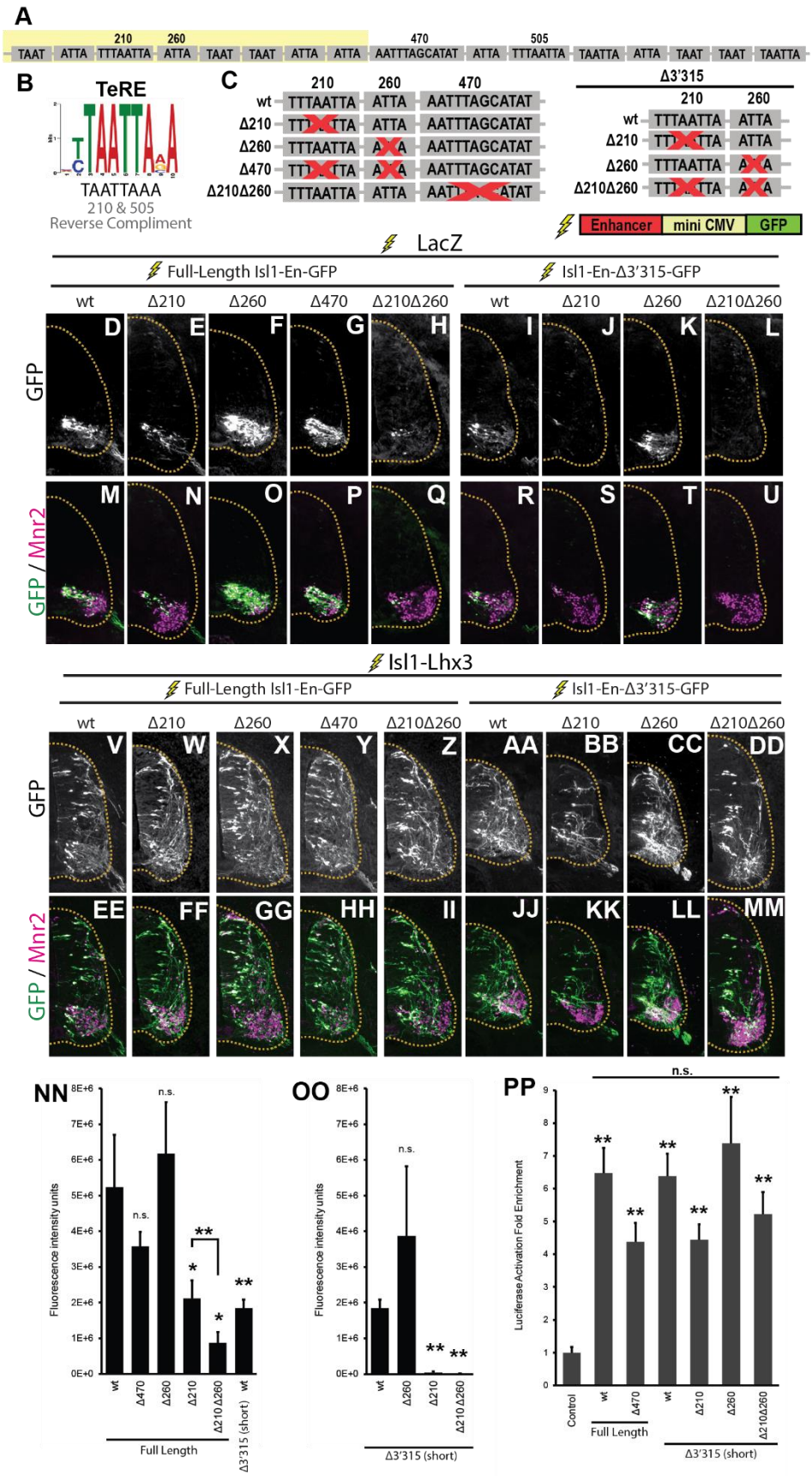


Figure 9: Lhx3-En-B is activated by the Isl1-Lhx3 complex. (A) HxRE sequence identified by ChIP-Seq *de novo* motif analysis, and the HxRE sequence in Lhx3-En-B. (B) Lhx3-En-B wt and HxRE mutant used for GFP-reporter experiments. For wt and mutant HxRE sequences see methods section. (C-F) GFP-reporter experiments for Lhx3-En-B variants, embryos were electroporated with Lhx3-En-B-GFP reporter constructs plus ubiquitously

expressing LacZ to mark electroporated cells. Sections were immunostained for GFP and Mnr2 to mark motor neurons. (G-J) Embryos electroporated with Lhx3-En-B-GFP wt or ΔHx reporter construct plus Isl1-Lhx3 fusion protein construct. Sections immunostained for GFP and Mnr2. (K) Luciferase assays testing Lhx3-En-B wt and HxRE mutant. Luciferase assays performed in cultured P19 cells. Results show the luciferase activation fold upon the addition of Isl1 plus Lhx3, compared to empty vector (no transcription factor). n = 4 experiments. Two-tailed t-test assuming equal variance, comparing each reporter construct to control reporter (no enhancer) n.s: not significant. Error bars represent the standard error of the mean.


A
210
260
470
505
TAAT ATTA TTTAATTA ATTA TAAT TAAT ATTA ATTA AATTTAGCATAT ATTA TTTAATTA TAATTA ATTA TAAT TAAT TAATTA
B
TeRE
TAATTAAA
210 & 505
Reverse Compliment
C
210
260
470
wt TTTAATTA ATTA AATTTAGCATAT
Δ210 TTTATTA ATTA AATTTAGCATAT
Δ260 TTTAATTA ATTA AATTTAGCATAT
Δ470 TTTATTA ATTA AATTTAGCATAT
Δ210Δ260 TTTAATTA ATTA AATTTAGCATAT
Δ3'315
210
260
wt TTTAATTA ATTA
Δ210 TTTATTA ATTA
Δ260 TTTAATTA ATTA
Δ210Δ260 TTTATTA ATTA
Enhancer
mini CMV
GFP
LacZ
Full-Length Isl1-En-GFP
Isl1-En-Δ3'315-GFP
wt Δ210 Δ260 Δ470 Δ210Δ260 wt Δ210 Δ260 Δ210Δ260
GFP
D E F G H I J K L
GFP / Mnr2
M N O P Q R S T U
Isl1-Lhx3
Full-Length Isl1-En-GFP
Isl1-En-Δ3'315-GFP
wt Δ210 Δ260 Δ470 Δ210Δ260 wt Δ210 Δ260 Δ210Δ260
GFP
V W X Y Z AA BB CC DD
GFP / Mnr2
EE FF GG HH II JJ KK LL MM
NN
Fluorescence intensity units
8E+6
7E+6
6E+6
5E+6
4E+6
3E+6
2E+6
1E+6
0E+0
n.s.
n.s.
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wt Δ470 Δ260 Δ210 Δ210Δ260 wt
Full Length
Δ3'315 (short)
OO
Fluorescence intensity units
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wt Δ260 Δ210 Δ210Δ260
Δ3'315 (short)
PP
Luciferase Activation Fold Enrichment
n.s.
**
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**
Control
wt Δ470
Full Length
wt Δ210 Δ260 Δ210Δ260
Δ3'315 (short)

Figure 10: The Isl1-En is activated by the Isl1-Lhx3 complex. (A) TAAT sequences and putative Isl1-Lhx3 complex binding sequence (site 470) within the Isl1-En. Yellow shading indicates the sequences located within the shortened Δ3'315-Isl1-En (B) TeRE sequence identified by ChIP-Seq *de novo* motif analysis, and the TeRE sequences in the Isl1-En (210 and 505) (C) Isl1-En mutants used for GFP-reporter experiments. For wt and mutant sequences see methods section. (D-U) GFP-reporter experiments for Isl1-En variants, embryos were electroporated with Isl1-En-GFP reporter constructs plus ubiquitously expressing LacZ to mark electroporated cells. Sections were immunostained for Mnr2 to mark motor neurons. (V-MM) Embryos electroporated with an Isl1-En-GFP reporter construct plus Isl1-Lhx3 fusion protein construct. Sections immunostained for GFP and Mnr2. (NN & OO) GFP fluorescence intensity for embryos electroporated with Isl1-En-GFP reporter constructs + LacZ. n = 4-12 embryos per condition. Two-tailed t-test assuming equal variance comparing each mutant reporter construct to full length wt-Isl1-En-GFP reporter (NN) or wt-Δ3'315-Isl1-En (OO), *p<0.05, **p<0.01, n.s: not significant. Error bars represent the standard error of the mean. (PP) Luciferase assays testing Isl1-En-wt and Isl1-En mutants. Luciferase assays performed in cultured P19 cells. Results show the luciferase activation fold upon the addition of Isl1 plus Lhx3, compared to empty vector (no transcription factor). n = 5-9 experiments per luciferase reporter construct. Two-tailed t-test assuming equal variance, comparing each reporter construct to control reporter (no enhancer), **p<0.01, n.s: not significant. Single Factor ANOVA, excluding control reporter, showed no significant

difference between any of the Isl1-En reporters, $p>0.05$. Error bars represent the standard error of the mean.

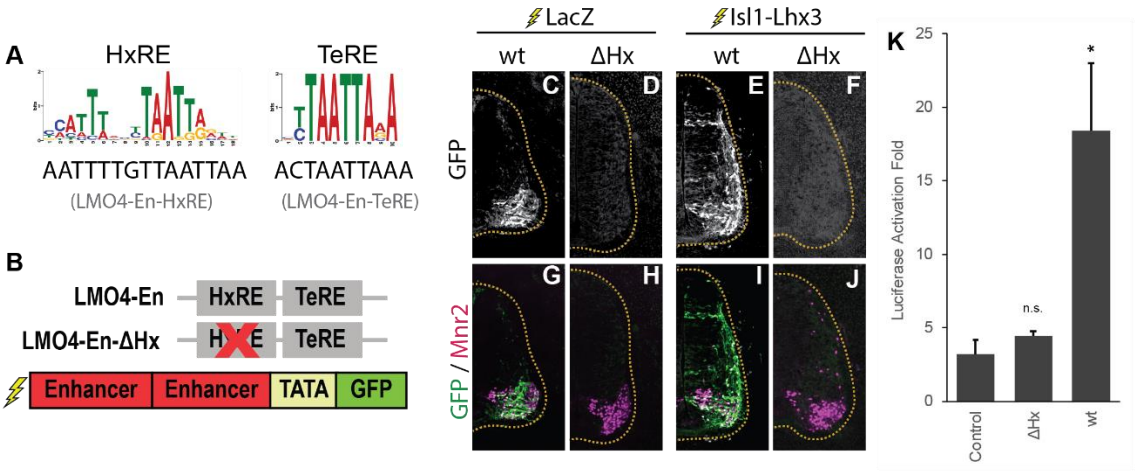


Figure 11: The LMO4-En is activated by the Isl1-Lhx3 complex. (A) HxRE and TeRE sequences identified by ChIP-Seq *de novo* motif analysis, and the HxRE and TeRE sequences in the LMO4-En. (B) LMO4-En HxRE mutant used for GFP-reporter experiments. For wt and mutant HxRE sequences see methods section. (C-D, G-H) GFP-reporter experiments for LMO4-En variants, embryos were electroporated with LMO4-En-GFP reporter constructs plus ubiquitously expressing LacZ to mark electroporated cells. Sections immunostained for GFP and Mnr2 to mark motor neurons. (E-F, I-J) Embryos electroporated with LMO4-En-GFP reporter construct plus Isl1-Lhx3 fusion protein construct. Sections immunostained for GFP and Mnr2. (K) Luciferase assays testing LMO4-En-wt and mutants. Luciferase assays performed in cultured P19 cells. Results show the luciferase activation fold upon the addition of Isl1 plus Lhx3, compared to empty vector (no transcription factor). n = 5 experiments. Two-tailed t-test assuming equal variance, comparing each reporter construct to control reporter (no enhancer), *p<0.05, n.s: not significant. Error bars represent the standard error of the mean.

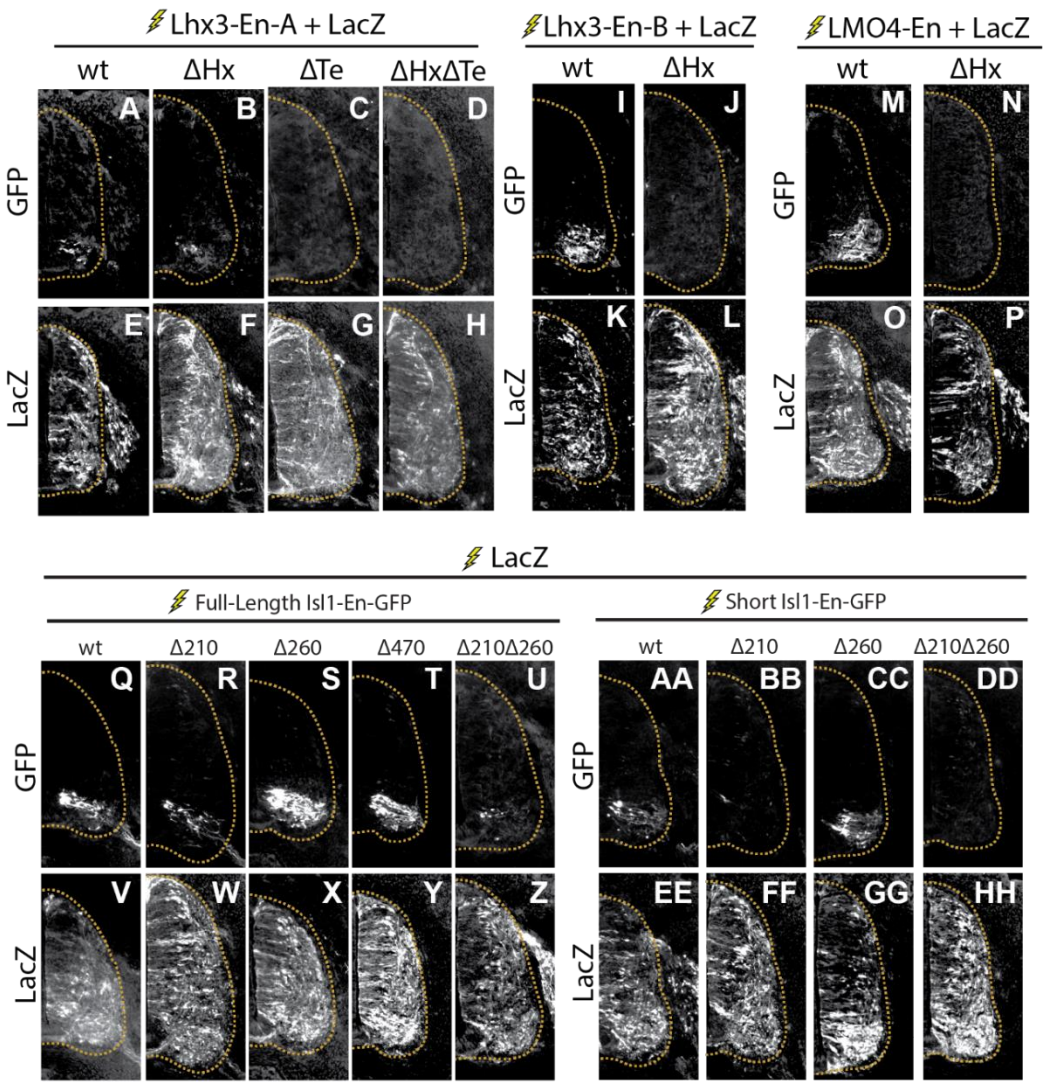


Figure 12: LacZ expression in GFP-reporter experiments. Embryos electroporated with GFP reporter constructs containing putative enhancers or mutated putative enhancers, a minimally active TATA (Lhx3-En-A, Lhx3-En-B, and LMO4-En) or a minimally active CMV promoter (Isl1-En-GFP). Embryos are also electroporated with a ubiquitously expressed LacZ construct to mark

electroporated tissue. Embryos are immunostained for LacZ and embryos A-P are also immunostained for GFP.

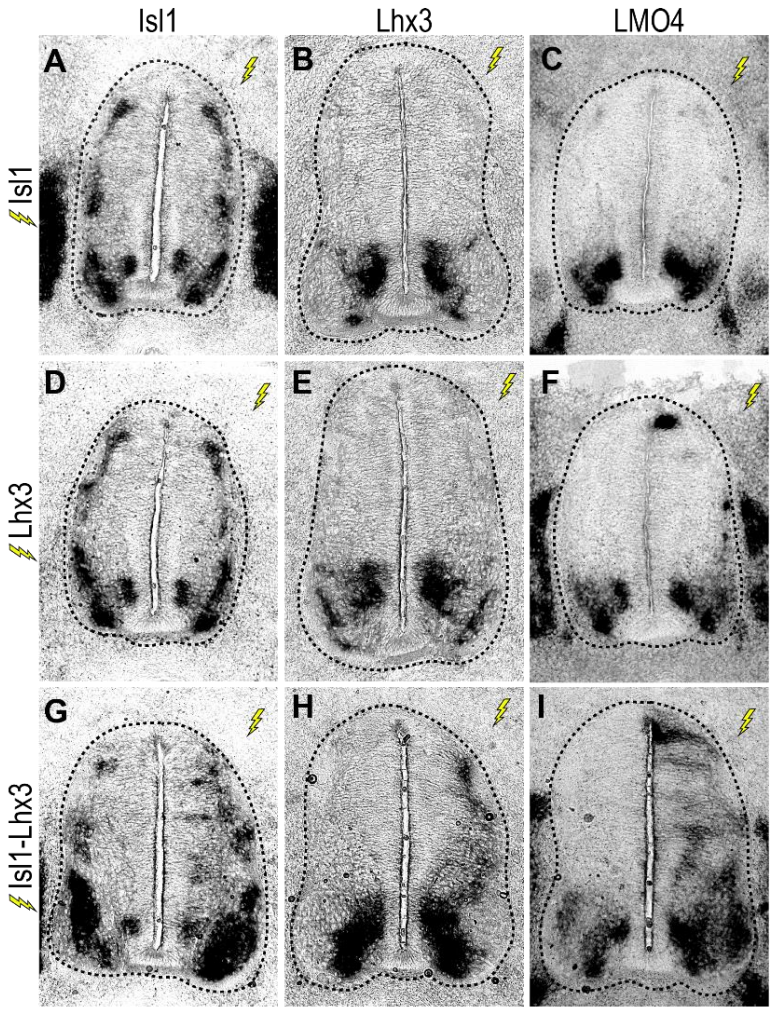
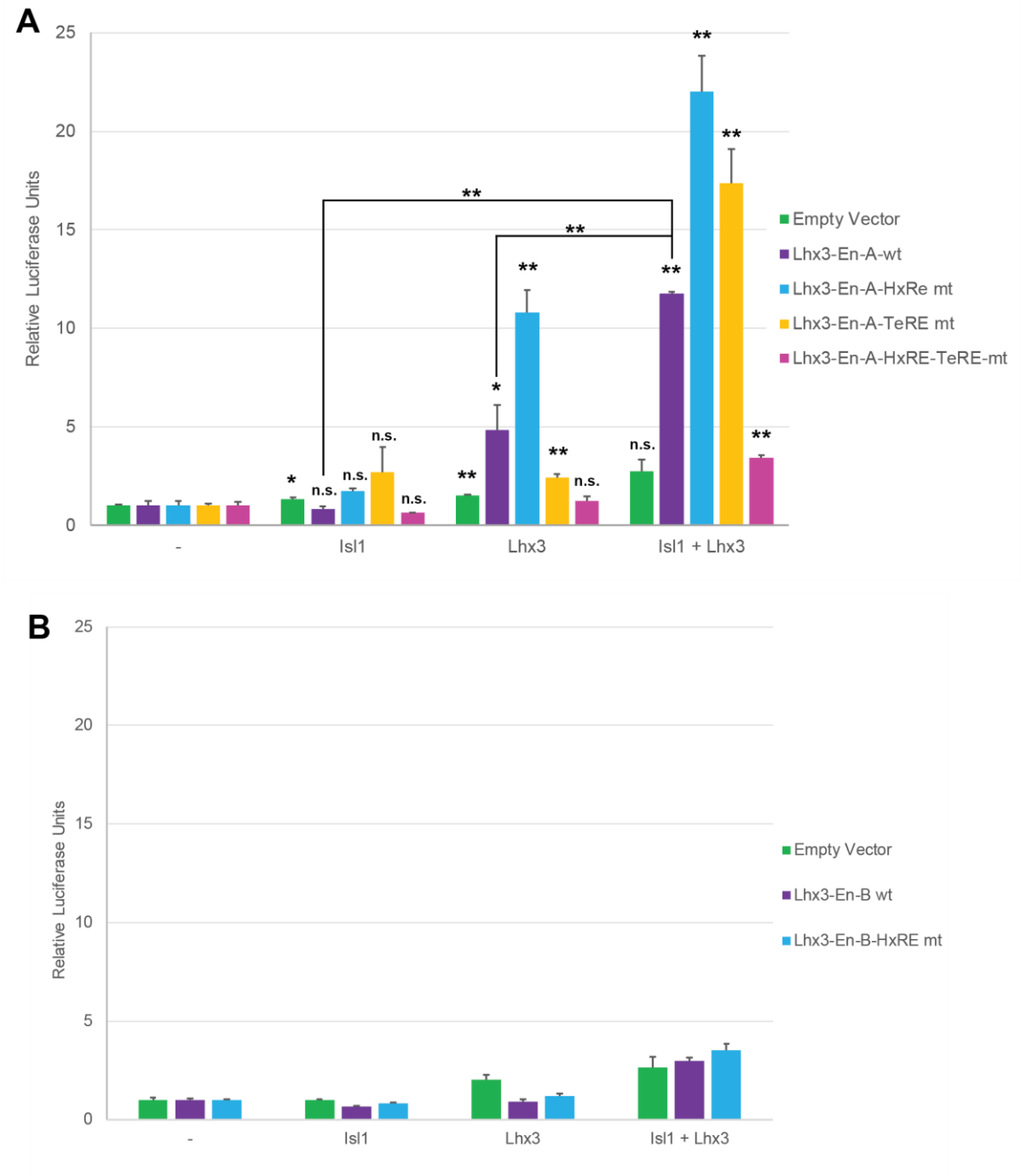


Figure 13: The Isl1-Lhx3 complex activates transcription of endogenous *Isl1*, *Lhx3* and *Lmo4*. Embryos electroporated with Isl1, Lhx3 or Isl1-Lhx3. *In situ* hybridization shows the transcription of endogenous *Isl1*, *Lhx3* and *Lmo4*. Lightning bolts indicate the electroporated side of the embryo (right side), compared to the unelectroproated, control side (left side).


A
Relative Luciferase Units
25
20
15
10
5
0
-
Isl1
Lhx3
Isl1 + Lhx3
Empty Vector
Lhx3-En-A-wt
Lhx3-En-A-HxRe mt
Lhx3-En-A-TeRE mt
Lhx3-En-A-HxRE-TeRE-mt
B
Relative Luciferase Units
25
20
15
10
5
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Isl1
Lhx3
Isl1 + Lhx3
Empty Vector
Lhx3-En-B wt
Lhx3-En-B-HxRE mt

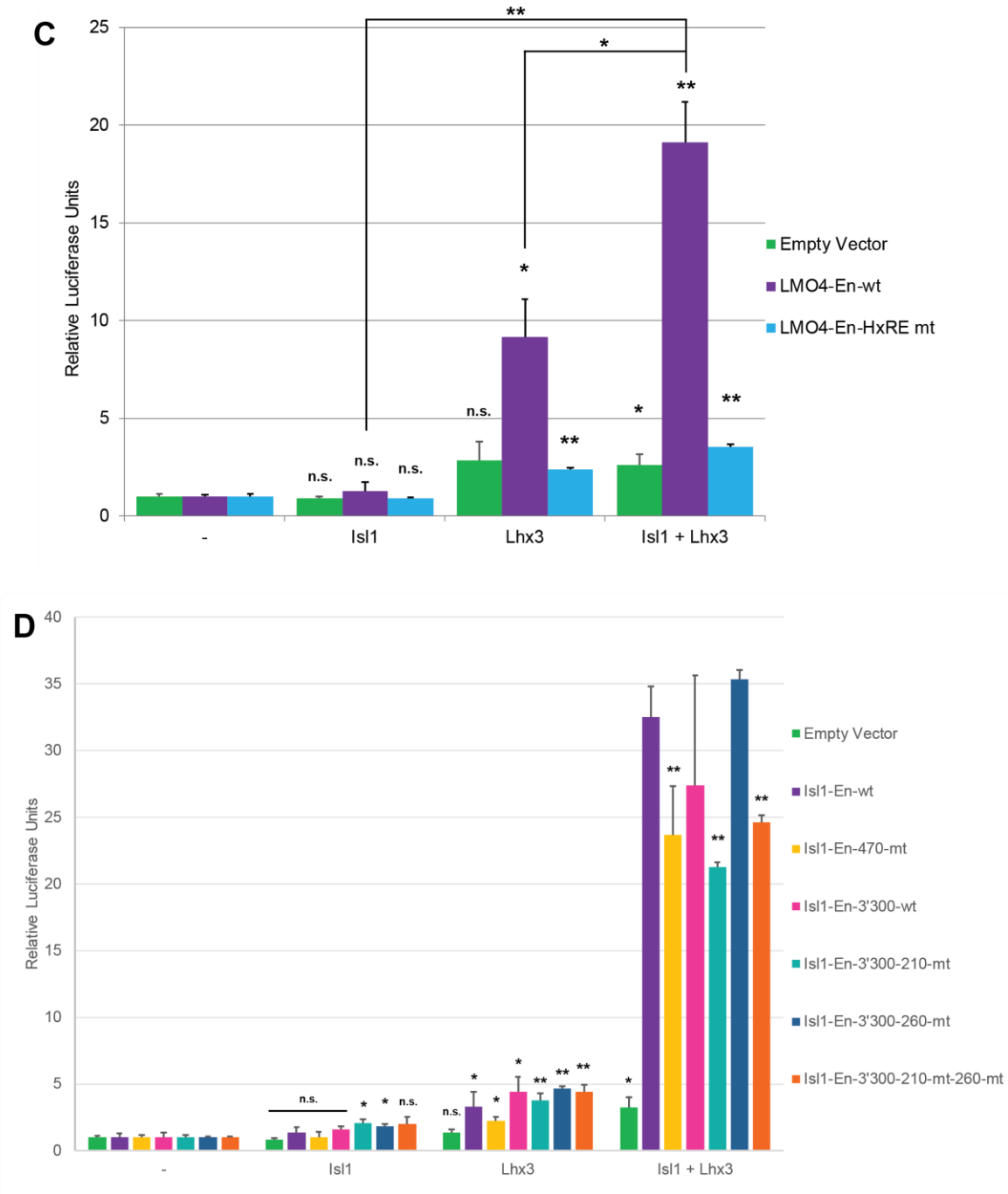

Figure 14: The Isl1-Lhx3 Complex Activates Lhx3-En-A, Lhx3-En-B, the LMO4-En and the Isl1-En. Luciferase assays testing the effects of Isl1, Lhx3 or Isl1 and Lhx3 on the transcriptional activation of wild type and mutant enhancers

(A) Lhx3-En-A (B) Lhx3-En-B (C) LMO4-En (D) Isl1-En. Two-tailed t-tests assuming equal variance were used to compare luciferase measurements from samples transfected without transcription factors to samples transfected with transcription factors, and to compare samples as shown. *$p<0.05$, **$p<0.01$ n.s: not significant. Error bars show the standard error of the mean.

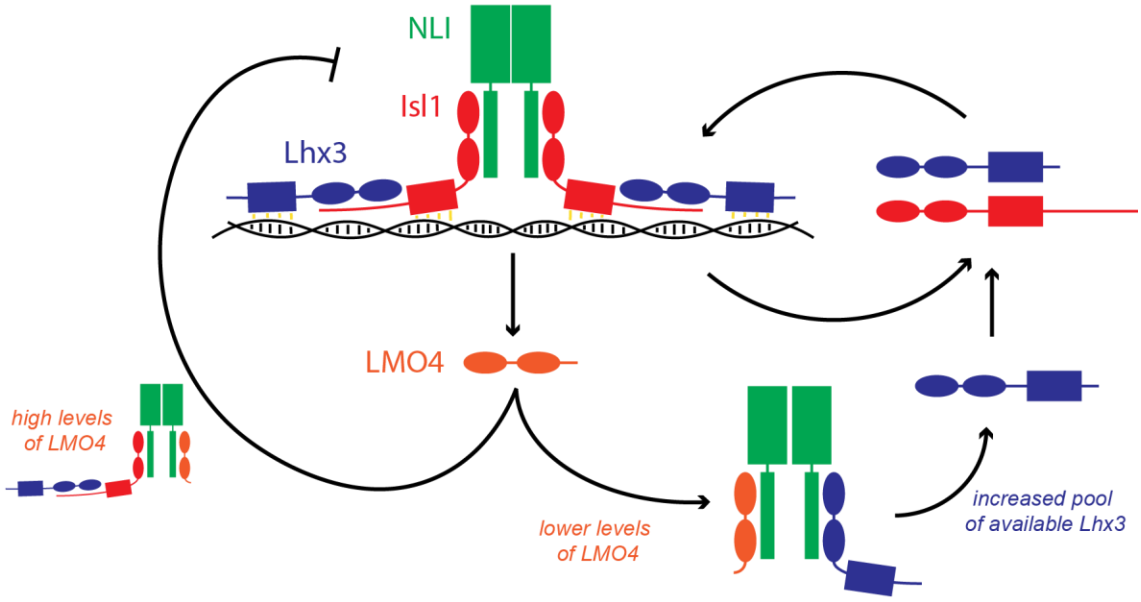

Figure 15: Activity of the Isl1-Lhx3 complex in embryonic motor neurons. Isl1 and Lhx3 each contain two LIM domains (ovals) that facilitate protein-protein interactions, and one homeodomain (rectangles) that bind DNA (yellow lines). NLI contains a LIM-Interacting domain (narrow rectangles) and a self-dimerization domain (wide rectangles). LMO4 contains two LIM domains but no homeodomains, so it cannot bind DNA. The Isl1-Lhx3 complex activates a positive autoregulatory transcriptional loop to enhance the expression of its components. Concurrently, the Isl1-Lhx3 complex also promotes the expression of LMO4 to inhibit Lhx3-complex formation and activity. Inhibiting formation of the Lhx3-complex leads to more free Lhx3, thereby promoting Lhx3 incorporation into the Isl1-Lhx3 complex. Ovals represent LIM domains and rectangles represent homeodomains.

CHAPTER 2

Characterization of an Isl1 Enhancer Reveals that Hoxc9 Directly and Specifically Represses Transcription of LMCm Genes in Thoracic Motor Neurons

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ABSTRACT

Spinal cord motor neuron circuits control coordinated movements and regulate vital homeostatic functions. The generation and maturation of motor neuron subtypes during embryonic development is essential for motor neuron circuit formation, and this process is directed through precise temporal and spatial regulation of LIM-homeodomain transcription factor expression. The expression of the LIM-homeodomain transcription factor Isl1 regulates cell fate specification, cell migration, gene expression and axonal pathfinding in multiple motor neuron subtypes including LMCm neurons. While the cellular and molecular processes that regulate motor neuron subtype specification have become a topic of intense research, the intricate transcriptional pathways that direct the expression of

essential homeodomain transcription factors in these cells remain unclear. In this study, I investigate the transcriptional regulation of the Isl1 enhancer SE2. Although Isl1 is expressed in multiple motor neuron subtypes, SE2 is specifically activated in LMCm neurons, making it an interesting candidate to study motor neuron subtype-specific transcription. I found that SE2 is recruited to and repressed by Hoxc9, which is expressed in thoracic motor neurons, and SE2 is activated by both Hoxc6 and RAR. I also found that both Hoxc6 and Hoxc9 interact with RAR, however the co-expression of either Hox gene with RAR fails to activate SE2. These findings suggest that rather than directly activating SE2, Hoxc6 and RAR likely regulate SE2 activity indirectly by directing cell fate specification in motor neuron subtypes.

INTRODUCTION

Motor neuron axons exit the spinal cord to interact with peripheral tissues and regulate coordinated movement and other critical biological functions. Spinal cord motor neuron circuits are composed of many distinct motor neuron subtypes, which each have unique gene expression profiles and muscle targets (Jessell, 2000; Price and Briscoe, 2004; Stifani, 2014). The generation and diversification of motor neuron subtypes during embryonic development is critical for motor neuron circuit formation and function. While the cellular and molecular mechanisms that regulate the specification and maturation of motor neuron subtypes are becoming more clear, the transcriptional mechanisms that directly control gene expression in these cell types remain unresolved.

Motor neuron subtypes occupy distinct medial-lateral segments of the ventral horn and form columns that span different regions of the rostro-caudal axis of the spinal cord. Medial Motor Column (MMC) neurons are located in the medial region of the ventral horn, and are further divided into medial and lateral subdivisions (MMCm, and MMCl, respectively) (Fig. 5 & 6). MMCm neurons project to dorsal axial muscles, and form a continuous column that stretches from the brachial spinal cord to the lumbar spinal cord. MMCl neurons project to intercostal muscles, and are located specifically in the thoracic spinal cord, but are absent from the brachial and lumbar spinal cord. Preganglionic (PGC) neurons project to sympathetic ganglia and are located in the thoracic spinal cord. Lateral Motor Column (LMC) neurons project to limb muscles, and are located in the brachial and lumbar spinal cord. Like MMC neurons, LMC neurons are also further subdivided into lateral and medial groups (LMCl and LMCm, respectively). LMCm neurons express Isl1, and project to ventral limb muscles. LMCl neurons lack Isl1 expression and project axons to dorsal limb muscles.

Gene expression in newly born motor neurons is uniform, with all motor neurons expressing high levels of Isl1, Lhx3, and Hb9 as they exit the cell cycle and migrate laterally (Tanabe et al., 1998; Thaler et al., 2002). However, as motor neurons settle into their positions in the ventral horn and diversify into mature motor neuron subtypes, they alter their gene expression profiles (Tsuchida et al., 1994; Rousso et al., 2008). While all motor neuron subtypes except LMCl neurons maintain Isl1 and Hb9 expression, MMCm neurons are the only motor neurons to maintain Lhx3 expression (Tsuchida et al., 1994). Individual motor

neuron subtypes also upregulate the expression of specific transcriptional regulator proteins and signaling molecules. LMC neurons and PGC neurons express Foxp1 (Dasen et al., 2008; Rousso et al., 2008). LMC neurons express Raldh2, PGC neurons express nNOS, and LMCl neurons express Lim1 (Tsuchida et al., 1994; Wetts and Vaughn, 1994; Sockanathan and Jessell, 1998; Thaler et al., 2004). These gene expression profiles are critical for motor neuron subtype specification and maturation as altering gene expression in motor neuron subtypes has been shown to disrupt motor axon pathfinding (Kania et al., 2000; Sharma et al., 2000; Kania and Jessell, 2003; Dasen et al., 2008; Rousso et al., 2008; Jung et al., 2010; Roy et al., 2012; Lacombe et al., 2013).

Studies investigating the regulation of motor neuron subtype development have revealed that Hox genes specify motor neuron subtypes by directing gene expression and axonal pathfinding in these cells (Dasen et al., 2003; Jung et al., 2010; Lacombe et al., 2013). Hox genes are differentially expressed along the rostro-caudal axis of the spinal cord, and their expression corresponds to brachial, thoracic and lumbar motor columns (Liu et al., 2001; Dasen et al., 2003). Hoxc6 expression corresponds with brachial LMC neurons. It represses Hoxc9 expression in the brachial spinal cord and specifies LMC neurons (Dasen et al., 2003; Lacombe et al., 2013). Likewise, in the thoracic spinal cord, Hoxc9 represses Hoxc6 expression and specifies MMCl and PGC neurons (Dasen et al., 2003; Jung et al., 2010) . Although it is clear that Hox genes play important roles in motor neuron subtype specification, the genetic mechanisms through which this process is mediated remain unclear.

Hox genes are uniformly expressed in motor neurons along the medial-lateral axis of the ventral horn (Liu et al., 2001). As there are multiple motor neuron subtypes within a single rostro-caudal section of the spinal cord, the activity of Hox genes in different motor neuron subtypes is likely regulated by the activity of Hox cofactors that are differentially expressed in distinct motor neuron populations. Foxp1, which is expressed in LMC and PGC neurons, has been shown to act as a cofactor for Hoxc6 and Hoxc9 to promote the specification of LMC and PGC neurons, respectively (Dasen et al., 2008; Rousso et al., 2008).

Retinoic acid signaling from LMCm neurons has also been shown to direct the specification of LMCl neurons (Sockanathan and Jessell, 1998). LMCm neurons are born first, and they express Raldh2, which synthesizes retinoic acid. As LMCl neurons migrate towards the lateral ventral horn they pass through mature LMCm neurons, and are exposed to secreted retinoic acid (Sockanathan and Jessell, 1998). LMCl neurons respond to retinoic acid signaling by downregulating the expression of Isl1 and upregulating the expression of Lim1 in a non-cell-autonomous manner (Sockanathan and Jessell, 1998). As both retinoic acid signaling and the activity of Hox genes are important for the specification of LMC neurons, I hypothesized that these Hox genes and RARs cooperate to regulate the transcription of LMCm and LMCl genes.

To test this hypothesis, I investigated the activity of Specific Enhancer 2 (SE2). SE2 was originally identified as a highly conserved Isl1 enhancer, and it is specifically activated in LMCm neurons in both mouse and chick (Uemura et al., 2005; Kim et al., 2015). Using GFP reporter studies in chick neural tube, I found

that Hoxc6 or constitutively active RAR activates SE2 in embryonic motor neurons and that Hoxc9 represses SE2. Interestingly, RAR and Hoxc6 activation of SE2 was restricted to embryonic motor neurons, indicating that motor neurons specifically express additional factors required for SE2 activation. I also found that RAR precipitates with both Hoxc6 and Hoxc9, indicating that these proteins interact.

Together our findings support a model in which RAR and Hox genes cooperate to regulate SE2 activity in motor neuron subtypes. I also identified a novel Hoxc9 genomic binding locus that acts as an LMCm-specific enhancer in the chick spinal cord. These results suggest that the transcriptional strategies utilized by Hox genes and RAR to regulate SE2 are widely applicable to LMCm genes.

METHODS

In Ovo Electroporation

Electroporation was performed in HHst 12-14 chick embryos, by injecting DNA into the embryonic neural tube. A square pulse electroporater was used to apply 5 pulses, 25V, 50ms with 1 second between each pulse across the neural tube. SE2 was cloned into PBS-miniCMV-eGFP reporter plasmid. Embryos were injected with 2.5μg/μL of reporter construct and 1.75μg/μL of LacZ, a constitutively active RAR expression construct containing RAR with a VP-16 transcriptional activation domain (VP-16-RAR), Hoxc6 or Hoxc9 expression construct.

Half Mount Experiments

Half mount experiments were performed with embryos electroporated with GFP-reporter constructs. Visceral tissue was removed from fixed embryos and embryos were bisected down their midline to separate the electroporated half of the embryo from the unelectroporated half of the embryo. The electroporated half of the embryos was imaged from the medial side to examine GFP expression in the spinal cord and in peripheral motor neuron axons.

Immunofluorescence labeling

Electroporated embryos were harvested and processed for immunolabeling 3DPE at HHst 25. Embryos were fixed in 4% PFA/PBS for 90 minutes, embedded in OCT and cryosectioned at 12µm. Embryo sections were incubated in primary antibody in either 0.1% Fish Gelatin or 0.3% BSA blocking buffer, overnight at 4°C. Primary antibodies used were gt anti LacZ (Sigma 1:2000), rb anti LacZ (Cappel 55976 1:2000), ms anti Mnr2 (DSHB 5C10 1:250), chk anti GFP (Aves Labs 1020 1:1000), ms anti HA (Covance MMS-101R), rb anti Hoxc6 (Aviva ARP38484_P050 1:2500), ms anti Hoxc9 (DSHB 5B5-2 1:250). Sections were imaged using a Zeiss Axio Imager.Z2 microscope.

Coimmunoprecipitation

Immunoprecipitation experiments were performed in HEK293 cells. Cells were transfected using calcium phosphate, with expression constructs for Flag-tagged

RAR, and HA-Hoxc6, HA-Hoxc9 or HA-Hoxc10. RAR complexes were then precipitated using mouse anti Flag antibodies (Sigma F3165) or non-specific control IgG. Western blots for HA epitope were performed to test for the presence of each Hox gene in the precipitated RAR complexes. Antibodies used for western blot were rb anti HA (Bethyl A190-108A 1:3000).

Luciferase Assays

Assays were performed in cultured HEK293 cells. Cells were seeded in 48 well plates, and transfected using calcium phosphate. Cells were transfected with SE2-Luciferase reporter constructs, transcription factor expression constructs, a CMV-β-galactosidase construct, to test transfection efficiency, and with empty plasmid to equalize the total amount of DNA for each condition. Luciferase and β-galactosidase activity was measured 48 hours after transfection. Luciferase readings were normalized to β-galactosidase measurements. Results show the average of technical duplicates.

RESULTS

SE2 is activated in LMCm neurons

To test if SE2 is activated in embryonic LMC neurons, I electroporated a reporter construct containing human SE2 upstream of a minimally active cytomegalovirus promoter (SE2-GFP). Because this promoter is insufficient to activate transcription by itself, only cells that activate transcription via SE2 express GFP. Embryos were electroporated at HHst 14 (Hamburger and Hamilton, 1993) and

harvested either 3DPE, for cryostat microtome sectioning and immunostaining, or 4DPE for half mount dissections and GFP imaging.

Half mount embryos electroporated with SE2-GFP express GFP specifically in the brachial spinal cord, and GFP labels axons that project to forelimb muscles, but not axons projecting to dorsal axial muscles (Fig 16A). This expression pattern shows that SE2 is specifically activated in LMC neurons but not in MMCm neurons. GFP expression is also largely excluded from the thoracic spinal cord, indicating that SE2 is not activated in MMCl or PGC neurons (Fig 16A). In brachial tissue sections, GFP is expressed in the medial-lateral region of the spinal cord, where LMCm neurons are located (Fig 17A). Tissue sections also show very few GFP-positive cells in the thoracic spinal cord (Fig 17B). Usually fewer than 2 cells express GFP, and GFP-positive cells in the thoracic spinal cord are always located in the ventral horn. Interestingly, GFP expression was not detected in the lumbar LMC neurons (Fig 16A). This expression pattern is likely the result of low electroporation efficiency in the lumbar spinal cord, instead of a fundamental difference in transcriptional regulation in brachial vs thoracic LMC neurons. These findings confirm previously reported findings that SE2 acts as an LMCm specific enhancer (Uemura et al., 2005; Kim et al., 2015).

Hoxc6 and RAR activate SE2 in embryonic motor neurons

Both retinoic acid signaling and Hoxc6 activity promote LMC neuron specification during spinal cord development (Sockanathan et al., 2003; Lacombe et al., 2013). Although retinoic acid signaling is essential for the specification of LMCl

neurons, whether it also contributes to LMCm neuron specification remains unclear (Sockanathan and Jessell, 1998). To test if RAR and Hoxc6 activate SE2, I co-electroporated SE2-GFP with either an HA-tagged Hoxc6 expression construct or a constitutively active RAR expression construct, which contains a VP-16 transcriptional-activation domain (VP-16 RAR). In half mount embryos and in spinal cord sections, I found that both VP-16 RAR and Hoxc6 activate GFP expression in the thoracic spinal cord (Fig 16 B & C, Fig 17 D & F). In addition to LMC axons, half mount embryos co-electroporated with either Hoxc6 or VP-16 RAR also express GFP in axons projecting to intercostal muscles (Fig 16 B & C). Interestingly neither RAR nor Hoxc6 activates GFP expression in the dorsal spinal cord, (Fig 17 C-F).

These results show that both Hoxc6 and RAR are sufficient to expand SE2 activation to thoracic motor neurons. However, neither is sufficient to activate SE2 in non-motor neurons. This activation pattern suggests that to activate SE2, RAR and Hoxc6 require additional factors that are expressed in embryonic motor neurons, but are absent in other ventral and dorsal spinal cord cells.

Hoxc9 represses SE2

Opposing the activity of Hoxc6 and RAR, Hoxc9 represses the generation of LMC neurons and induces the specification of thoracic motor neuron subtypes (Jung et al., 2010). To test if Hoxc9 represses SE2 activity, I co-electroporated HA-Hoxc9 and SE2-GFP. In half mount embryos and in spinal cord sections, Hoxc9 represses GFP expression. In half mount embryos, there is no detectable

GFP expression in the spinal cord or in motor neuron axons (Fig 16D). In brachial and thoracic spinal cord sections, GFP expression is substantially reduced compared to SE2-GFP electroporations without Hoxc9 (Fig 17 G & H). High levels of Hoxc9 expression activate GFP expression throughout the spinal cord (Fig 18). However, lower levels of Hoxc9 expression that more closely resemble endogenous Hoxc9 protein levels consistently repressed SE2 activity (Fig 17 G & H). These results strongly suggest that *in vivo*, Hoxc9 represses SE2 activity.

Hoxc6 and Hoxc9 both interact with RAR

Because Hoxc6 and VP-16 RAR both activate SE2, I hypothesized that Hoxc6 and RAR cooperate to activate SE2. To test if Hoxc6 and RAR interact, I expressed HA-tagged Hoxc6 and Flag-tagged RAR in cultured HEK293 cells and performed coimmunoprecipitation experiments. Hoxc6 was significantly enriched when I precipitated Flag-RAR, compared to non-specific IgG controls, indicating that Hoxc6 interacts with RAR (Fig 19). Using the same experimental approach, I found that RAR also interacts with Hoxc9, but not with Hoxc10 (Fig 19). To test if retinoic acid (RA) affects RARs interactions with Hoxc6, Hoxc9 or Hoxc10 I performed experiments with and without RA. I found that RA does not affect RAR interactions with Hoxc6, Hoxc9 or Hoxc10. These results suggest that, in LMCm neurons Hoxc6 and RAR form a transcriptional complex and cooperate to activate the transcription of SE2. Interestingly RAR and Hoxc9 also form a transcriptional complex. This interaction could facilitate the repression of LMCm-specific gene transcription.

Co-expression of RAR and Hoxc6 does not activate SE2

Neither VP-16 RAR nor Hoxc6 is sufficient to activate SE2 outside of embryonic motor neurons. To test if the co-expression of Hoxc6 and VP-16 RAR activates SE2, I co-electroporated Hoxc6, VP-16 RAR and SE2-GFP. Surprisingly, I found that co-expression of these genes induced minimal GFP expression in the brachial and thoracic spinal cord that was greatly attenuated compared to embryos that were co-electroporated with Hoxc6 or VP-16 RAR alone (Fig 20 A & B). Interestingly, co-electroporation of Hoxc9 and VP-16 RAR with SE2-GFP also minimally activated GFP expression in the brachial and thoracic spinal cord (Fig 20 C & D). Although Hoxc9 normally inhibits the activation of SE2, co-electroporation with a constitutively active RAR construct may override Hoxc9's repressive activity and thereby minimally activate SE2.

As neither VP-16 RAR, Hoxc6 nor both are sufficient to activate SE2 outside of embryonic motor neurons, I hypothesized that Hox complexes require a motor neuron specific co-factor to regulate the transcriptional activity of SE2. Foxp1 is expressed in LMCm, LMCl and PGC neurons and acts as a Hox co-factor during motor neuron subtype development (Dasen et al., 2008; Rousso et al., 2008). To test if Foxp1 cooperates with Hoxc6, or Hoxc9 to regulate SE2, I performed luciferase assays in cultured HEK293 cells. I found that, in this system, SE2 is not activated by VP-16 RAR, or Foxp1. SE2 is activated by Hoxc6 or Hoxc9, however, this activation was attenuated when cells were co-transfected with VP-16 RAR or Foxp1 (Fig 21).

Hoxc9 genomic binding locus acts as an LMCm-specific enhancer

As Hoxc9 and Hoxc6 regulate SE2 activity, I hypothesized that Hoxc9 and Hoxc6 could regulate the activity of other LMCm enhancers to direct the expression of LMCm-specific genes during motor neuron development. To test if Hoxc9 regulates the activity of LMCm-specific enhancers I examined Hoxc9 genomic binding loci, and RNA-sequencing data sets obtained from embryonic chick spinal cord segments (Mazzoni et al., 2011). Analysis of Hoxc9 ChIP-Seq data revealed that Hoxc9 binds SE2. First I compared brachial and thoracic RNA-seq data sets to identify transcripts enriched in the brachial spinal cord. Because LMC neurons are present in the brachial spinal cord but not in the thoracic spinal cord, I hypothesized that brachial-enriched transcripts correspond to LMC-enriched genes. Next, we analyzed data from a Hoxc9 ChIP-Seq experiment in an embryonic stem cell model system (Mazzoni et al., 2011). This approach allowed me to identify brachial-enriched genes with at least one nearby Hoxc9 genomic binding locus. Lastly, I checked the expression pattern of these genes using *in situ* hybridizations of P4 mouse spinal cord sections from the Allen Institute Mouse Spinal Cord Atlas.

I identified seven Hoxc9 genomic binding loci that are associated with brachial-enriched transcripts and are also expressed in brachial MNs. I cloned two copies of each Hoxc9 binding locus into GFP reporter constructs and performed chick neural tube electroporation to test enhancer activity in the embryonic spinal cord. Of these Hoxc9 binding loci, the Testin-Enhancer (Tes-Enhancer), was specifically activated in LMCm neurons in the brachial spinal

cord (Fig 22). The activation pattern of the Tes-Enhancer closely resembles the activation pattern of SE2. As both enhancers are bound by Hoxc9, these results suggest that the Tes-Enhancer and SE2 are likely regulated through parallel transcriptional mechanisms.

DISCUSSION

The differential expression of LIM-homeodomain transcription factors in embryonic motor neuron subtypes is critical for motor neuron subtype specification, migration, axon pathfinding, and circuit formation (Kania et al., 2000; Sharma et al., 2000; Kania and Jessell, 2003; Roy et al., 2012). The temporal and spatial regulation of Isl1 expression in motor neuron subtypes directs motor neuron subtype specification and maturation (Roy et al., 2012). Isl1 expression in LMCm neurons promotes axon pathfinding into ventral limb muscles, and when Isl1 expression is impaired, these cells aberrantly project axons into dorsal limb muscles (Roy et al., 2012). When Isl1 is ectopically expressed in LMCl neurons, these cells repress Lim1 expression and misproject axons into ventral limb muscles (Kania and Jessell, 2003).

Research investigating the transcriptional pathways that regulate the differential expression of Isl1 in distinct motor neuron subtypes has begun to elucidate the complex genetic mechanisms that underlie this process. While Isl1 is expressed in many motor neuron subtypes, SE2 is exclusively activated in LMCm neurons (Uemura et al., 2005; Kim et al., 2015). Previous reports show that SE2 is activated by the Onecut transcription factors Onecut-1 (Oc-1) and

Onecut-2 (Oc-2), and these factors are required for SE2 activation in the embryonic spinal cord (Roy et al., 2012; Kim et al., 2015). Oc-1 and Oc-2 also activate Isl1 expression in LMCm neurons (Roy et al., 2012). Interestingly, our findings show that Hoxc9 binds and represses SE2 in the thoracic spinal cord, and that SE2 can be activated by Hoxc6 or RAR (Fig 23).

Hoxc9 Represses SE2

Ectopic expression of Hoxc9 in the brachial spinal cord markedly represses SE2 activity, and ChIP-Seq experiments in embryonic stem cells have shown that Hoxc9 is recruited to this genomic locus (Mazzoni et al., 2011). These findings strongly suggest that in thoracic motor neuron subtypes Hoxc9 binds SE2 to repress the transcription of Isl1. Alternatively, Oc-1 also binds SE2 and activates the transcription of Isl1 in motor neurons throughout the spinal cord (Roy et al., 2012). Thus, in thoracic motor neurons, Hoxc9 and Oc-1 interact with a single enhancer and have opposing functions (Fig 23). As Isl1 is expressed in thoracic motor neuron subtypes, the transcriptional activation of Oc-1 must overcome the repressive activity of Hoxc9 (Tsuchida et al., 1994; Rousso et al., 2008). This could be accomplished by Oc-1 out-competing Hoxc9 for binding to SE2. If that is true, then relative levels of Oc-1 and Hoxc9 expression in thoracic motor neurons will be important for precisely regulating the expression of Isl1.

The finding that Hoxc9 represses SE2, and accordingly Isl1 transcription, in thoracic motor neurons is surprising. Why would thoracic motor neurons employ transcriptional pathways to repress essential LIM-homeodomain

transcription factors? As Hoxc9 is expressed outside of motor neurons (Liu et al., 2001), it could be acting to repress SE2, and Isl1 transcription, in non-motor neuron cells, where Oc-1 is expressed at lower levels. Our findings that ectopic expression of Hoxc9 in the brachial spinal cord repress SE2 support this hypothesis, because it shows that a high level of Hoxc9 expression is sufficient to overpower Oc-1 activity in LMCm neurons.

It is also surprising that SE2 activation does not reflect Isl1 expression in the thoracic spinal cord. Oc-1 is expressed in the thoracic spinal cord and Oc-1 expression in the thoracic spinal cord regulates Isl1 expression in MMCl neurons and PGC neurons (Francius and Clotman, 2010; Roy et al., 2012). While SE2 appears sufficient to recapitulate Isl1 expression in LMC neurons, there must be additional transcriptional pathways regulating the expression of Isl1 in thoracic motor neurons. These pathways likely include additional genomic regulatory elements, such as Isl1 enhancers. These elements could also be regulated by Oc-1, or by other transcription factors.

Hoxc6 and RAR activate SE2

Unlike Hoxc9, I found that ectopic expression of either Hoxc6 or VP-16 RAR activates SE2 in thoracic motor neurons. Because RAR interacts with both Hoxc6 and with Hoxc9 I hypothesized that RAR could be cooperating with these Hox genes to activate or repress SE2. However, co-electroporation of Hoxc6 with VP-16 RAR did not activate SE2 in the dorsal spinal cord. A more likely explanation for the ability of Hoxc6 to activate SE2 in thoracic motor neurons is that it

represses Hoxc9 expression (Dasen et al., 2003; Lacombe et al., 2013). When Hoxc9 expression is reduced, SE2 is de-repressed and GFP expression is activated. Further experiments testing whether Hoxc6 is recruited to SE2 will provide additional insight into whether its activation of this enhancer is direct or if it is mediated indirectly through repression of Hoxc9. The role of RAR in SE2 activation is less clear. Again, testing whether RAR is recruited to SE2 will be informative in uncovering whether or not RAR activates SE2 directly or if this effect is mediated indirectly through a change in motor neuron fate specification.

While Hoxc6 and RAR both expand SE2 activation to thoracic motor neurons, neither activates SE2 in LMCl neurons. This finding suggests that LMCl neurons express factors that specifically repress SE2. Lim1 is specifically expressed in LMCl neurons, and Lim1 represses Isl1 expression in these cells (Kania and Jessell, 2003). Lim1 could be directly repressing Isl1 transcription through SE2. Further studies investigating the effect of Lim1 on SE2 activation and whether Lim1 is recruited to this genomic locus, will provide critical insight into the transcriptional mechanisms that regulate Isl1 expression in LMCl neurons.

Hoxc9 Represses LMCm Enhancers

Our finding that a previously uncharacterized Hoxc9 binding locus acts as an LMCm-specific enhancer in the embryonic spinal cord shows that Hoxc9 regulates the transcription of a myriad of LMCm genes in thoracic motor neuron subtypes. Because the thoracic spinal cord does not generate LMCm neurons, it

is critical for thoracic motor neurons to repress the expression of LMC genes. Our study suggests that Hoxc9 recognizes enhancers associated with LMCm-specific genes, and represses their transcription in thoracic motor neurons. It will be informative to investigate the genomic binding of Hoxc6, and to compare Hoxc6 genomic binding loci to Hoxc9 genomic binding loci. Loci bound by both Hoxc6 and Hoxc9 will be good candidates for enhancer studies in the spinal cord, as these sites are likely to be differentially activated in brachial and thoracic motor neurons.

ACKNOWLEDGEMENTS

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FIGURES

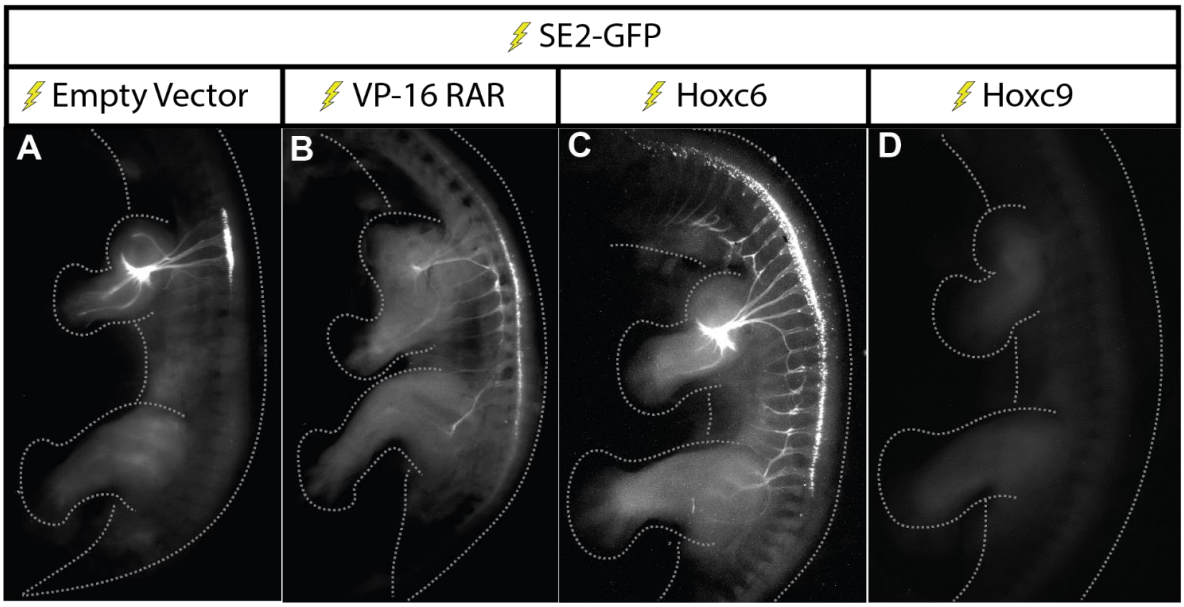

Figure 16: SE2 Activity in the Embryonic Spinal Cord

Embryos were electroporated with SE2-GFP reporter plasmids, plus either empty vector, or expression constructs for VP-16 RAR, Hoxc6, or Hoxc9. Images show GFP expression in the electroporated half of embryos.

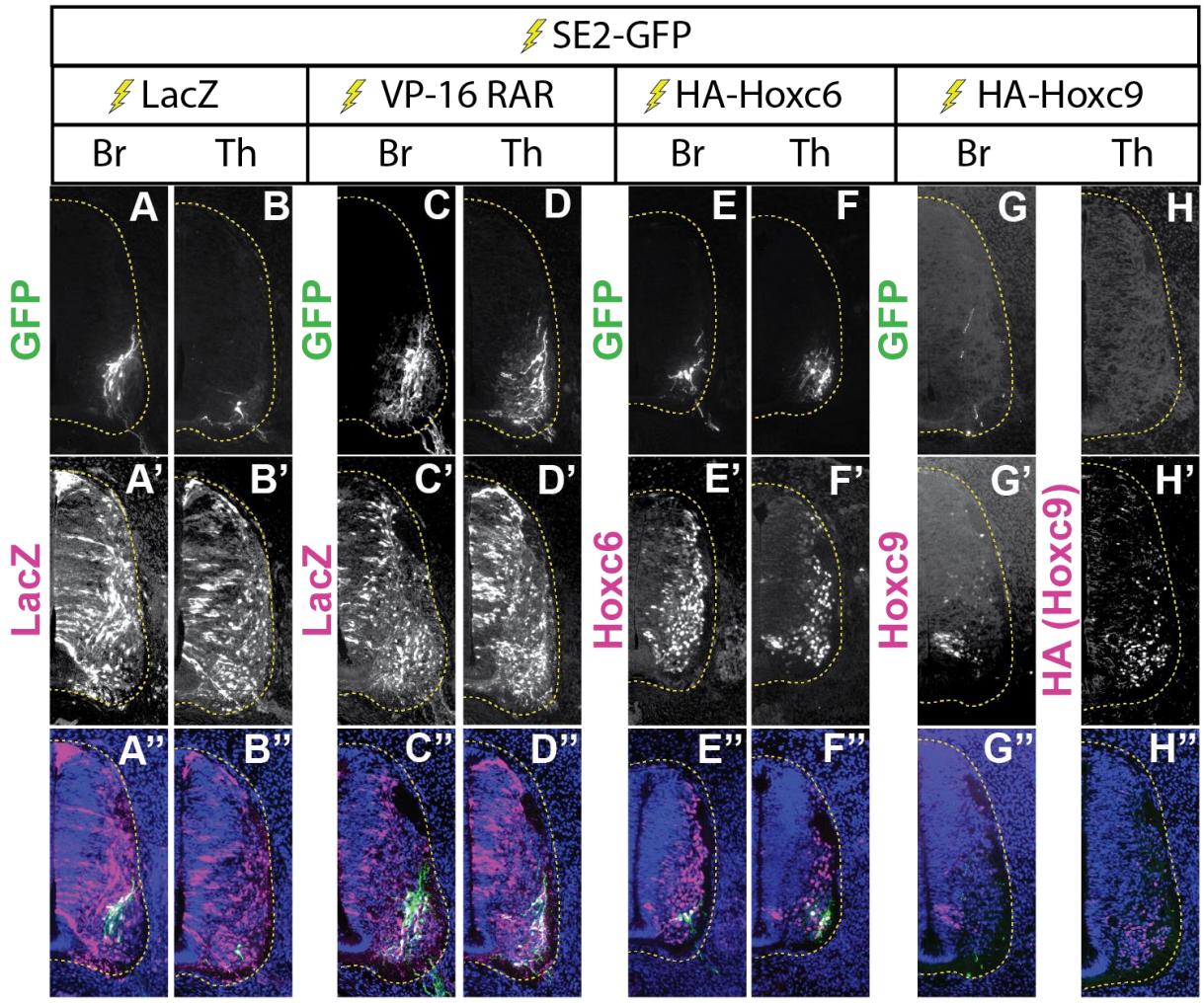

Figure 17: SE2 Activation Pattern in the Ventral Horn

Embryos were electroporated with (A) SE2-GFP and LacZ. (B) SE2-GFP, VP-16 RAR and LacZ (C) SE2-GFP and HA-Hoxc6, or (D) SE2-GFP and HA-Hoxc9. Images show cryostat microtome spinal cord sections from either brachial (Br) or thoracic (Th) spinal cord regions. Embryos were immunostained for GFP and LacZ, Hoxc6 HA or Hoxc9.

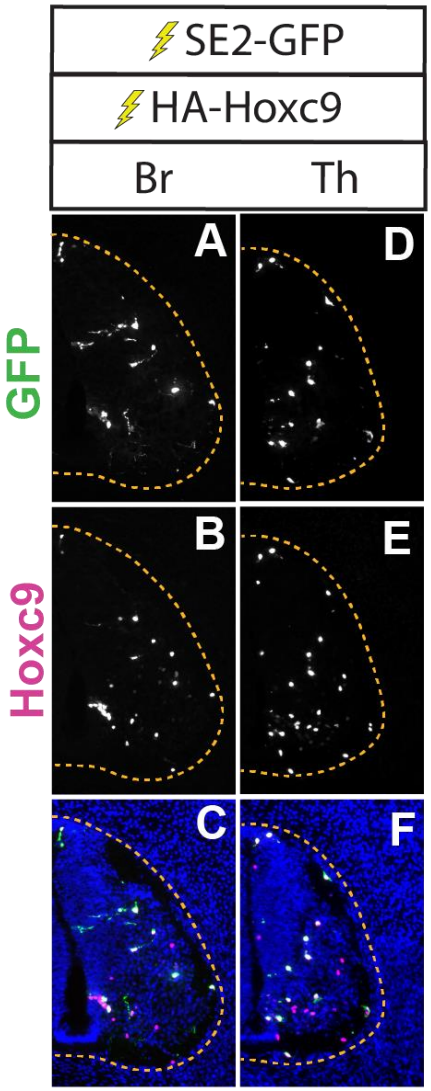

Figure 18: High Levels of Hoxc9 Expression Activate SE2 Throughout the Spinal Cord. Embryos were electroporated with SE2-GFP reporter plasmids plus HA-tagged Hoxc9. Images show cryostat microtome spinal cord sections from either brachial (Br) or thoracic (Th) spinal cord regions. Embryos were immunostained for GFP (A & D) and Hoxc9 (B & E).

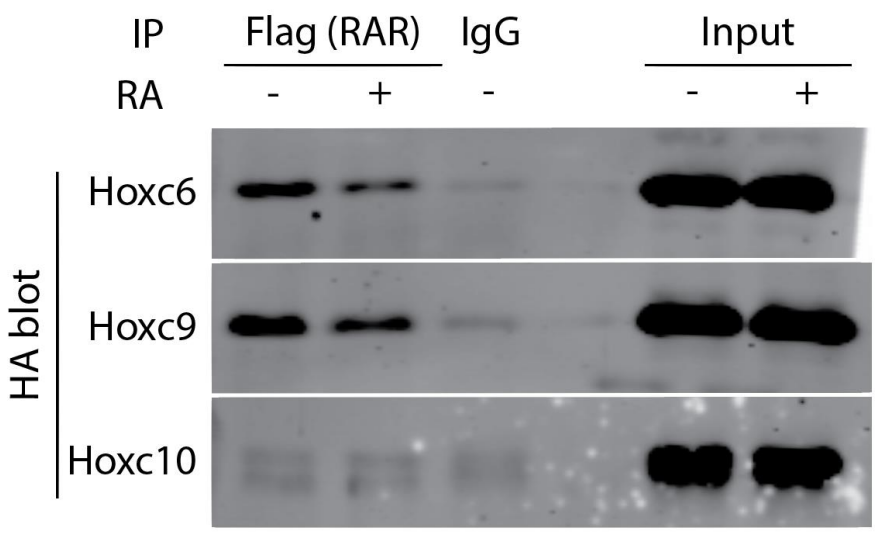

Figure 19: RAR Specifically Interacts with Hoxc6 and Hoxc9

Coimmunoprecipitation Experiments in cultured HEK293 cells. Cells were transfected with Flag-tagged RAR plus HA-tagged Hoxc6, HA-tagged Hoxc9 or HA-tagged Hoxc10. Flag-tagged RAR was precipitated from cell lysates. Results show western blots of the precipitate which was probed for HA-tagged Hox genes.

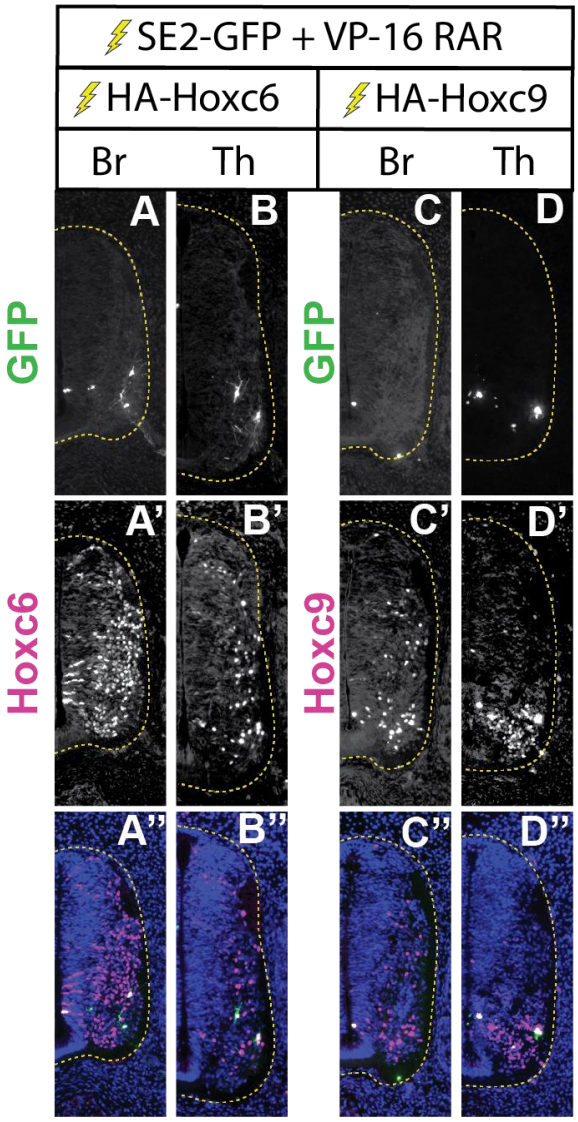

Figure 20: RAR and Hoxc6 or Hoxc9 are Not Sufficient to Activate SE2 Outside of Embryonic Motor Neurons

Embryos were electroporated with SE2-GFP reporter plasmids plus VP-16 RAR and either HA-tagged Hoxc6 (A-B) or HA-tagged Hoxc9 (C-D). Images show cryostat microtome spinal cord sections from either brachial (Br) or thoracic (Th) spinal cord regions. Embryos were immunostained for GFP and Hoxc6 or GFP and Hoxc9.

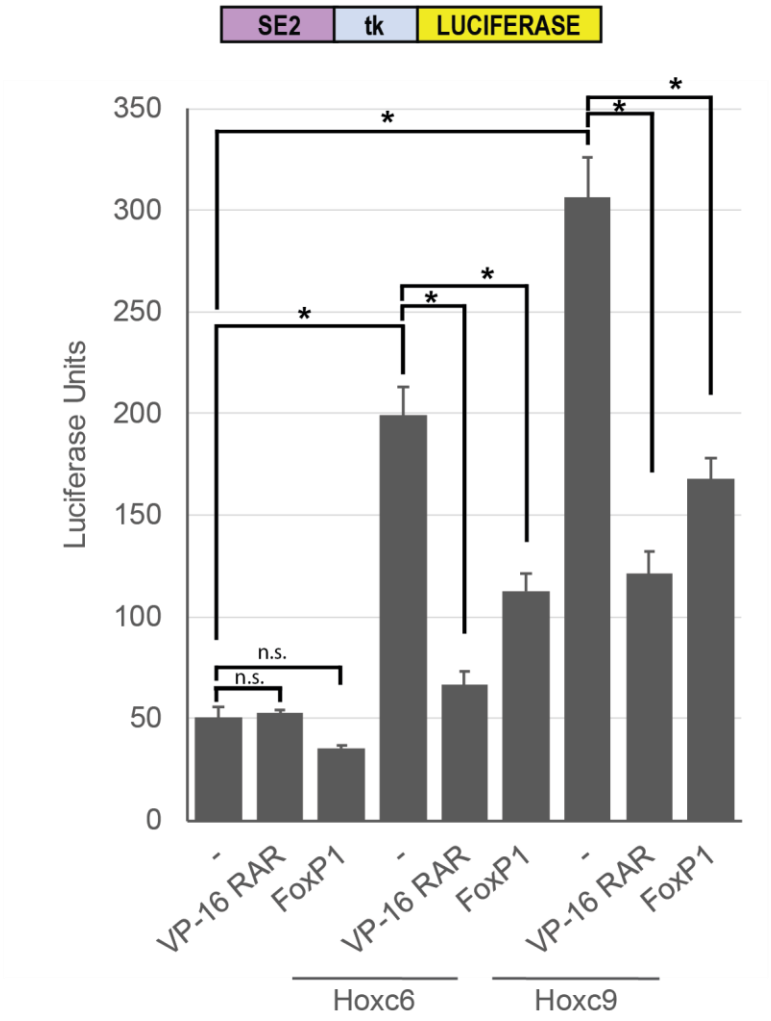

Figure 21: Hoxc6 and Hoxc9 Activate SE2 in Tissue Culture

Luciferase assays in cultured HEK293 cells. Cells were seeded in 48 well plates, and transfected using calcium phosphate. Cells were transfected with SE2-Luciferase reporter plasmid plus empty vector or, transcription factor expression constructs. Results are reported as luciferase units / β-galactosidase units. Results show the average of technical duplicates. Two-tailed t-test assuming equal variance were used to compare transcription levels in response to the

addition of different transcription factors. *p<0.05, n.s: not significant. Error bars represent the standard error of the mean.

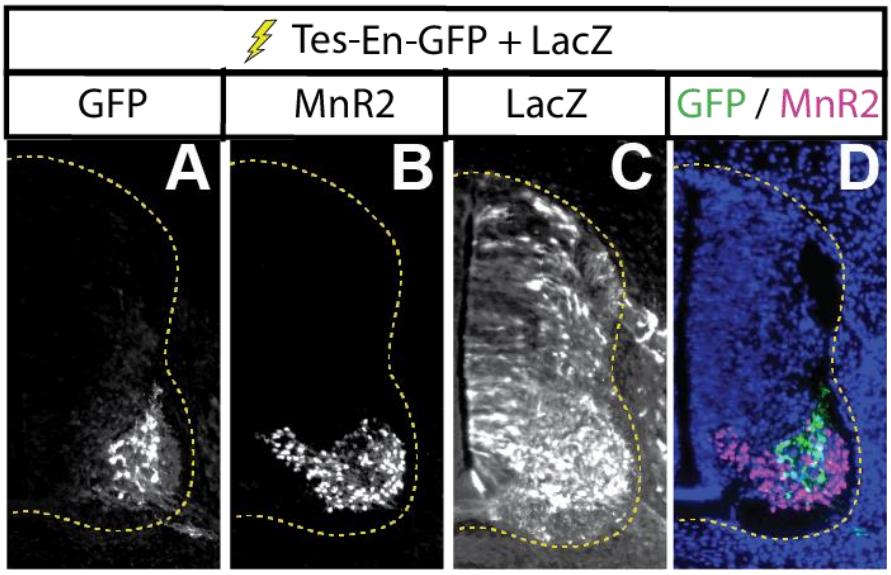

Figure 22: Hoxc9 Genomic Binding Locus Acts as an LMCm-specific Enhancer

Embryos were electroporated with Testin-Enhancer-GFP reporter plasmid (Tes-En-GFP) and LacZ to mark electroporated tissue. Images show cryostat microtome spinal cord sections from either brachial spinal cord regions. Embryos were immunostained for GFP, Mnr2 and LacZ.

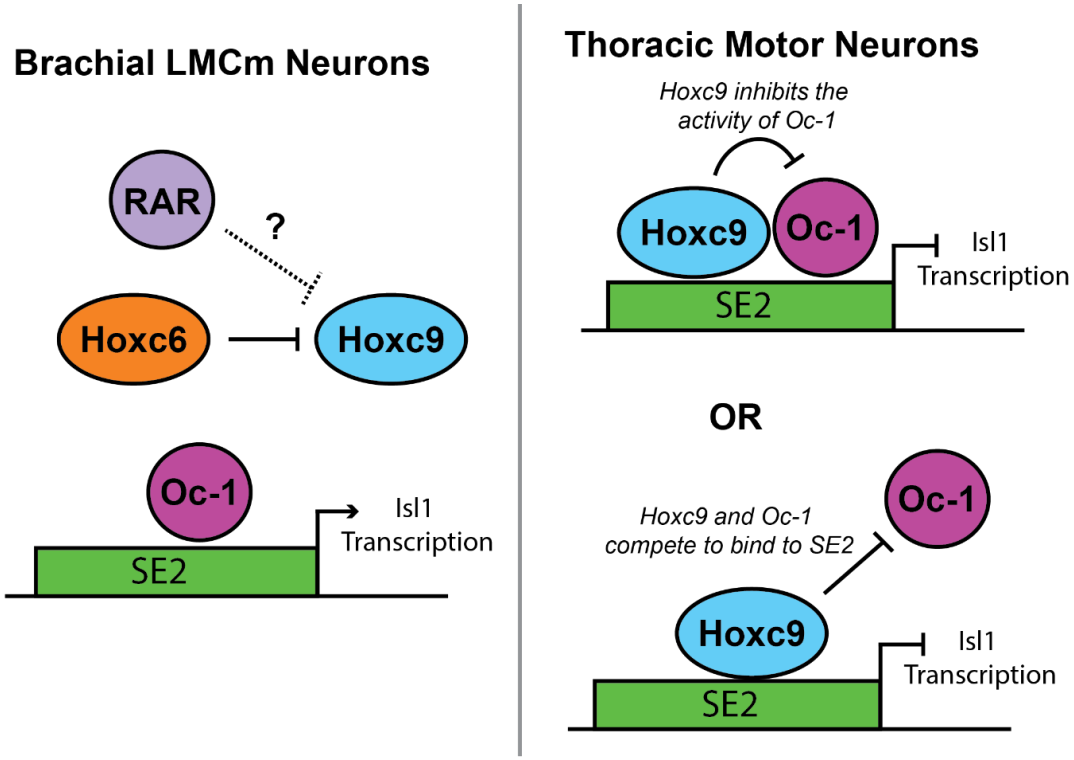

Figure 23: Model for SE2 Regulation by Hoxc6, Hoxc9, RAR and Oc-1. LMCm neurons in the brachial spinal cord express Hoxc6, RAR and Oc-1. Hoxc6 represses the expression of Hoxc9 and directs LMC fate specification. RAR and Hoxc6 activate SE2 expression, but it is unclear whether this activation is mediated through direct interaction with SE2 or if this effect is indirect, perhaps through promoting LMCm fate specification. Thoracic motor neurons express Hoxc9 and Oc-1. Hoxc9 directly interacts with SE2 and represses SE2 activity. We propose that Hoxc9 inhibits the activity of Oc-1 either by recruiting co-repressor complexes that inhibit the activity of Oc-1 or by competing for SE2 binding, thereby preventing Oc-1 from binding to SE2. It is worth noting that although Hoxc9 represses the enhancer activity of SE2, Isl1 is still expressed in thoracic motor neurons, indicating that there are additional transcriptional pathways regulating Isl1 expression in these cells.

SUMMARY AND CONCLUSIONS

To generate functional sensory and motor circuits, the embryonic spinal cord must produce diverse types of neurons, including distinct types of ventral and dorsal interneurons as well as motor neurons. Additionally, these neurons must be generated in the correct location in the spinal cord and at the appropriate developmental time. This myriad of cell types is generated from an initially uniform pool of spinal cord progenitor cells (Jessell, 2000; Price and Briscoe, 2004). Studies investigating ventral spinal cord patterning have shown that a Shh gradient, initiated from the notochord and floorplate, induces differential gene expression along the dorso-ventral axis of the ventral spinal cord to establish discrete progenitor domains (Fig 1) (Jessell, 2000; Price and Briscoe, 2004). Each domain then goes on to generate a specific subset of ventral interneurons or motor neurons (Fig 2) (Jessell, 2000; Price and Briscoe, 2004).

The pMN domain is established through combinatorial expression of Nkx6.1, Nkx6.2, Olig2 and Pax6 (Fig 1) (Ericson et al., 1997; Briscoe et al., 2000; Novitch et al., 2001; Vallstedt et al., 2001). For motor neurons to differentiate, they must downregulate Olig2 expression and upregulate the expression of Mnr2 (Fig 3) (Tanabe et al., 1998; Lee et al., 2005b). Mnr2 then activates its own expression and the expression of the LIM-homeodomain transcription factors Isl1 and Lhx3 (Fig 3) (Tanabe et al., 1998). Isl1 and Lhx3 in turn form the Isl1-Lhx3 transcription complex and activate the expression of Mnr2, as well as genes that are critical for motor neuron differentiation and maturation (Fig 3 & Fig 4) (Thaler et al., 2002; Lee et al., 2008, 2012). The expression of Isl1 and Lhx3 is critical for

motor neuron specification, as embryos that lack either of these genes fail to generate motor neurons (Pfaff et al., 1996; Sharma et al., 1998; Thaler et al., 2002; Song et al., 2009; Liang et al., 2011). Although Mnr2 expression initiates Isl1 and Lhx3 expression, Isl1 and Lhx3 expression are maintained after Mnr2 expression is downregulated (Tanabe et al., 1998).

Likewise, as motor neurons mature into distinct motor neuron subtypes, they differentially regulate the expression of Isl1 and Lhx3. Isl1 expression is maintained in all motor neuron subtypes except for LMCl neurons, and Lhx3 expression is only maintained in MMCm neurons (Table 1) (Tsuchida et al., 1994; Rousso et al., 2008). The temporal and spatial regulation of Isl1 and Lhx3 expression is critical for both motor neuron specification and motor neuron subtype development (Sharma et al., 2000; Roy et al., 2012). However, the mechanisms that regulate the transcription of these two genes throughout motor neuron development remain unclear.

Here I've shown that, during motor neuron specification, Isl1 and Lhx3 generate a positive transcriptional feedback loop to potentiate their own expression (Fig 15). I propose that this feedback loop acts to rapidly induce Isl1 and Lhx3 expression in differentiating motor neurons, and that it maintains high levels of Isl1 and Lhx3 expression in MMCm neurons. To investigate the pathways that regulate Isl1 expression in motor neuron subtypes, I examined the activity of the Isl1-associated enhancer SE2. I found that SE2 is activated specifically in embryonic LMCm neurons and it is directly repressed by Hoxc9. I

also found that SE2 activity is expanded to thoracic motor neurons by ectopic expression of Hoxc6 or VP-16 RAR.

In this dissertation, I've shown that the expression of Isl1 and Lhx3 are differentially regulated during motor neuron specification and motor neuron subtype maturation. First, this dissertation sheds critical insight into the pathways that potentiate and maintain Isl1 and Lhx3 expression in newly differentiating motor neurons. Second, investigating SE2 has illuminated a previously undescribed role for Hoxc9 in directly repressing LMCm genes, and my findings suggest a role for retinoic acid signaling in LMCm specification.

Transcriptional Regulation in Motor Neuron Specification

The first step for generating motor neurons is establishing the pMN domain. Olig2 expression is critical for the establishment of the pMN domain (Fig 3) (Novitch et al., 2001; Lee et al., 2005b). Accordingly, embryos that lack Olig2 fail to establish the pMN domain and cannot generate Hb9-positive motor neurons (Lu et al., 2002). Olig2 has been shown to directly repress the transcription of Hb9 in motor neuron progenitor cells (Fig 3) (Lee et al., 2005b). As motor neuron progenitor cells begin to differentiate and migrate laterally, Olig2 protein levels decrease, and Hb9 is released from Olig2-mediated transcriptional repression. This decrease in Olig2 expression is required for the induction of Hb9 expression and for motor neuron differentiation (Lee et al., 2005b).

During this time, newly differentiating motor neurons also upregulate the expression of Isl1 and Lhx3 (Tanabe et al., 1998). Isl1 and Lhx3 form a

hexameric transcription complex, with NLI that activates the expression of motor neuron genes (Fig 4) (Thaler et al., 2002; Lee et al., 2008, 2012). The Isl1-Lhx3 complex is critical for motor neuron specification, as disrupting the expression of either Isl1 or Lhx3 inhibits motor neuron generation (Pfaff et al., 1996; Sharma et al., 1998; Song et al., 2009). While the induction of Isl1 and Lhx3 expression in newly differentiating motor neurons is critical for motor neuron specification, the transcriptional pathways that regulate their expression in these cells are unresolved. I propose that, during motor neuron differentiation, Isl1 and Lhx3 are also released from transcriptional repression, potentially by Olig2. Our findings show that upon modest expression of Isl1 and Lhx3, these genes generate a positive transcriptional feedback loop to enhance and maintain their own expression (Fig 15).

I examined transcriptional regulation in newly differentiated motor neurons by identifying and characterizing four genomic binding loci of the Isl1-Lhx3 transcription complex. Two of these loci are associated with *Lhx3*, one is associated with *Isl1*, and one is associated with *Lmo4*. I found that each of these genomic loci acts as a motor neuron specific enhancer, activating transcription in newly differentiated motor neurons. I also found that each locus recruits the Isl1-Lhx3 complex in the embryonic spinal cord, and each is transcriptionally activated by the Isl1-Lhx3 complex. These findings show that during motor neuron specification, Isl1 and Lhx3 generate a positive transcriptional feedback loop to potentiate their own expression (Fig 15).

At the same time, the Isl1-Lhx3 complex also activates the transcription of *Lmo4*. LMO4 inhibits the formation of the tetrameric Lhx3 complex by competing with Lhx3 for binding to NLI, which increases the pool of free Lhx3 (Fig 4) (Lee et al., 2008). This increase in free Lhx3 potentiates the formation of the Isl1-Lhx3 complex, which in turn, potentiates the transcription of Isl1, Lhx3 and Lmo4 (Fig 15). Isl1-Lhx3 also competes with LMO4 for binding to NLI, and high levels of LMO4 have been shown to inhibit the activity of the Isl1-Lhx3 complex (Song et al., 2009). Thus, LMO4 likely generates a negative feedback loop, with high levels of LMO4 inhibiting Isl1-Lhx3 complex formation (Fig 15).

The experiments in this dissertation demonstrate the potency of each of these enhancers and strongly support a transcriptional autoregulation model. However, it is less clear if there are compensatory mechanisms facilitating *Isl1*, *Lhx3* and *Lmo4* transcription during motor neuron specification. Effective techniques utilizing Cas-9 guide RNAs have recently been developed to recognize and inhibit endogenous enhancer activity *in vivo* (Gilbert et al., 2012). Future experiments blocking the activity of the endogenous Isl1, Lhx3 or LMO4 enhancers and then examining the expression of each of these genes and motor neuron specification will allow us to better assess the role of each enhancer in motor neuron specification. Likewise, blocking these enhancers could prove to be an effective mechanism for knocking out, or knocking down Isl1, Lhx3 or LMO4 expression relatively late in motor neuron development. This late alteration in gene expression could be a useful model for learning more about the roles of

each of these genes in motor neuron development and motor neuron subtype specification.

Transcriptional Regulation In Motor Neuron Subtypes

After exiting the cell cycle and acquiring a generic motor neuron identity, motor neurons continue to differentiate into distinct motor neuron subtypes. Motor neuron subtypes have unique gene expression profiles, occupy discrete regions of the spinal cord, and project axons to specific muscle targets (Table 1, Fig 5 & Fig 6). While most motor neuron subtypes maintain Isl1 expression, with the exception of LMCl neurons, Lhx3 expression is only maintained in MMCm neurons (Tsuchida et al., 1994; Rousso et al., 2008). As the differential expression of Isl1 and Lhx3 is critical for motor neuron subtype specification (Sharma et al., 2000; Roy et al., 2012), understanding the transcriptional mechanisms that regulate their expression in different motor neuron subtypes is important for understanding this process.

I propose that MMCm neurons utilize the positive transcriptional feedback loop generated by the Isl1-Lhx3 complex to maintain high levels of Isl1 and Lhx3 expression. However, all other motor neuron subtypes must down-regulate Lhx3 expression to differentiate (Sharma et al., 2000). To downregulate Lhx3, motor neuron subtypes must disrupt the positive transcriptional feedback loop generated by the Isl1-Lhx3 complex through pathways that specifically repress Lhx3 expression or activity. These pathways are currently uncharacterized, and could include transcriptional repressive factors, microRNAs, or pathways that

decrease the stability of Lhx3 protein. Identifying and characterizing the pathways that contribute to the decrease in Lhx3 expression will be important for understanding the mechanisms that direct motor neuron subtype specification.

As motor neuron subtypes differentiate, the Isl1-En is specifically activated in MMCm neurons in both mouse and chick (Uemura et al., 2005; Kim et al., 2015). These observations show that, in addition to promoting Lhx3 expression in MMCm neurons, the positive transcriptional feedback loop generated by the Isl1-Lhx3 complex also plays an important role in maintaining Isl1 expression in these cells. As MMCm neurons are the only motor neuron subtype that expresses the Isl1-Lhx3 complex (Tsuchida et al., 1994), other motor neuron subtypes must utilize different transcriptional pathways to maintain Isl1 expression.

To study the transcriptional pathways that regulate Isl1 expression in other motor neuron subtypes, I examined the activity of the Isl1-associated enhancer SE2. SE2 is specifically activated in LMCm neurons. As Isl1 is expressed in multiple motor neuron subtypes, including thoracic motor neurons, it is interesting that SE2 is exclusively activated in LMCm neurons. This observation suggests that LMCm neurons utilize transcriptional pathways to activate and maintain Isl1 expression that are distinct from the transcriptional pathways that MMCm, MMCl and PGC neurons utilize to activate and maintain Isl1 expression. Because Hox genes direct motor neuron subtype specification (Dasen et al., 2003; Jung et al., 2010; Lacombe et al., 2013), I hypothesized that Hox genes could regulate SE2 in motor neurons.

I found that Hoxc9 is recruited to SE2 in mouse embryonic stem cells (Mazzoni et al., 2011), and Hoxc9 expression represses SE2 activity. Hoxc9 repression of SE2 is a surprising finding, because Hoxc9 directs the specification of thoracic motor neurons and thoracic motor neurons express Isl1. Oc-1 and Oc-2 activate Isl1 expression in LMCm and thoracic motor neurons, and Oc-1 is recruited to SE2 in the chick embryonic spinal cord (Roy et al., 2012). It appears that Oc-1 and Hoxc9 have opposing effects on SE2 activity in thoracic motor neurons (Fig 23). Since Isl1 is expressed in thoracic motor neurons, but SE2 is not activated in these cells, Oc-1 likely activates the expression of Isl1 through interactions with different, currently unidentified, Isl1 regulatory elements.

I also found that ectopic expression of Hoxc6 or VP-16 RAR activates SE2 expression in thoracic motor neurons. However, this expansion of SE2 activation did not extend to LMCl neurons. These results suggest that, rather than directly activating SE2, the ectopic expression of Hoxc6 or VP-16 RAR converts thoracic motor neuron subtypes to brachial-like motor neurons. This conversion would alter gene expression in thoracic motor neurons and activate transcriptional pathways to facilitate the expression of brachial motor neuron genes. Hoxc6 has been shown to repress the expression of Hoxc9 and convert thoracic motor neurons to neurons with LMC gene expression profiles (Lacombe et al., 2013). Activation of SE2 via ectopic expression of Hoxc6 could be mediated partially or entirely through repression of Hoxc9. Since Hoxc9 represses SE2, diminishing Hoxc9 expression in the thoracic spinal cord would release SE2 from transcriptional repression and allow it to be activated.

Little is known regarding retinoic acid signaling and motor neuron subtype specification. Secretion of retinoic acid from LMCm neurons is critical to enhance motor neuron generation in the brachial spinal cord, and to specify LMCl neurons (Sockanathan and Jessell, 1998). However, LMCm neurons are also exposed to retinoic acid during motor neuron maturation, and whether or not this signaling pathway contributes to LMCm specification or maturation remains unclear. VP-16 RAR activates SE2 expression, and this activation is restricted to thoracic motor neurons. These results suggest that, like Hoxc6, VP-16 RAR expression in the thoracic spinal cord affects motor neuron subtype specification and alters the gene expression profiles of thoracic motor neurons, thereby indirectly activating SE2.

LMCl neurons do not activate SE2, even when Hoxc6 or VP-16 RAR is ectopically expressed. LMCl neurons likely express transcriptional repressor proteins that inhibit the expression of non-LMCl genes, including Isl1. As Lim1 is specifically expressed in LMCl neurons (Tsuchida et al., 1994), and ectopic expression of Lim1 has been shown to repress Isl1 expression (Kania and Jessell, 2003), it is a good candidate for an SE2 transcriptional repressor. Investigating whether Lim1 is recruited to SE2 and whether Lim1 represses SE2 activity will be informative in delineating the transcriptional pathways that regulate the expression of Isl1 in motor neuron subtypes.

Because Hoxc9 directs thoracic motor neuron specification (Jung et al., 2010), I hypothesized that it represses additional LMCm specific enhancers in parallel with SE2 repression. I found that a novel Hoxc9 binding locus, the Tes-

Enhancer, is specifically activated in LMCm neurons. This finding supports a model in which Hoxc9 broadly represses LMCm genes by repressing enhancers that resemble SE2. Mutational analysis has identified 3 sequences within SE2 that mediate Oc-1 activation of this enhancer (Kim et al., 2015). However, the sequences that mediate Hoxc9 repression of SE2 remain unknown.

I predict that conserved Hox binding sites mediate Hoxc9 repression of SE2 and the Tes-Enhancer in thoracic motor neurons. Experiments mutating putative Hox binding sites and examining the activity of both of these enhancers will be important for understanding the genetic mechanisms that regulate the interactions between LMCm enhancers and Hoxc9. Likewise, it will be informative to test whether Oc-1 also activates the Tes-Enhancer. Comparing the genetic elements and the behavior of SE2 and the Tes-Enhancer will shed critical insight into the transcriptional mechanisms that regulate LMCm gene expression.

Conclusions

Temporal and spatial regulation of gene expression during embryonic spinal cord development is critical for generating diverse cell types in the appropriate location at the appropriate developmental time. This process is essential for the generation of spinal cord sensory and motor circuits. The impairment of motor neuron circuit development leads to severe pediatric motor neuron disorders. Understanding the genetic mechanisms that regulate motor neuron specification and motor neuron subtype specification will provide us with crucial insights into the cellular and molecular processes that dictate motor neuron circuit formation.

Here I've shown that the Isl1-Lhx3 complex activates a potent autoregulatory transcriptional feedback loop to potentiate and maintain its own expression in newly differentiating motor neurons, while simultaneously activating the transcription of Lmo4. Later in motor neuron subtype development, Hoxc9 recognizes and represses the Isl1-associated enhancer SE2 and the previously unidentified Tes-Enhancer. My findings have revealed novel transcriptional pathways that regulate the expression of Isl1, Lhx3 and LMO4 in newly differentiating motor neurons, and Isl1 expression in mature motor neuron subtypes. Understanding these pathways will be a valuable contribution towards developing effective treatments for pediatric motor neuron disorders and neurodegenerative disorders that affect spinal cord motor circuits, such as Amyotrophic Lateral Sclerosis.

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