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FoxP Genes Subdivide Interneuron Subclasses in the Developing Mouse Spinal Cord

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ABSTRACT OF THE THESIS

FoxP Genes Subdivide Interneuron Subclasses in the Developing Mouse Spinal Cord

by

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I have investigated the role of the FoxP genes in the developing mouse spinal cord using in situ hybridization and immunohistochemistry in conjunction with a variety of genetically modified mice. In E11.5-E12.5 developing spinal cords, my studies show that three members of the FoxP gene subfamily, FoxP1, FoxP2 and FoxP4 are expressed in subsets of V1, V0, di2 and di6 interneurons. FoxP gene expression exhibits a differential expression profile across segmental levels. Finally, mutant analysis suggests interneuronal expression of the FoxP genes is controlled by dorsoventral factors.

INTRODUCTION

NEURAL TUBE PATTERNING – THE MORPHOGENS AND THEIR EFFECTS

The cells of the nervous system are derived from an embryonic structure, the neural plate that in turn forms the neural tube. Formation of the neural tube in vertebrates coincides with the development of other embryonic structures that are integral to the proper specification of neural tube neurons. These important developmental structures include the notochord, located ventral to the neural tube, the somites, present bilaterally, and the ectoderm that overlies the dorsal aspect of the neural tube. Each of these structures is a source of crucial factors known as morphogens that play an instructive role in neuronal patterning. Secreted morphogens are believed to pattern the neural tube in a manner that is dependent upon their concentration. The concentration gradients of morphogens are critical to the proper spatial organization of the neural tube and the establishment of distinct domains of neural progenitors that are ultimately responsible for the production of individual classes of post-mitotic neurons.

Morphogens that contribute to the dorso-ventral patterning of the spinal cord include RA (retinoic acid), BMPs (bone morphogenic proteins), Wnts, and Shh (sonic hedgehog). Dorsally, BMPs and Wnts are secreted by the roof plate (Chizhikov et al., 2004). Ventrally, Shh originates from the notochord and the floorplate (Lupo et al., 2006), while RA is secreted by the laterally positioned somites (Lupo et al., 2006; Wilson

& Maden, 2005). In addition to the dorsoventral patterning of the neural tube by morphogens, FGFs (fibroblast growth factors) act along the anterior-posterior axis. Fgf8 is expressed by the caudally migrating organizer node. FGF-signaling appears to maintain neural cells in a neural progenitor state and strong evidence implicates FGF signaling as a requirement for proper axial organization by controlling the expression of Hoxc genes (Dasen et al., 2003). Finally, in order for neuronal differentiation to proceed in the neural tube RA signaling is required, as it antagonizes Fgf8 (Diez del Corral et al., 2004; Novitch et al., 2003).

Much work has been done in determining the role of Shh in the patterning of the developing spinal cord. The node and subsequently the notochord secrete the distally acting morphogen, Shh, which sets up a receding gradient in the ventral to dorsal direction. The gradient is such that the presence of Shh is virtually non-existent in the intermediate regions of the neural tube. Recent work suggests a possible mechanism for the establishment of the Shh gradient. The main contributor to forming the gradient is the Ptc1 receptor, which has a downstream negative feedback system that when bound suppresses the expression of the Ptch1 receptor. Further the Ptch1 receptor has a strong affinity for the Shh molecule which is believed to contribute to gradient formation (Dessaud, 2008). Moreover, the duration of Shh exposure also affects the patterning of the ventral neural tube. It appears that there is a gradual cessation in a cell's sensitivity to Shh which is explained by a negative feedback mechanism that upregulates Ptch1 which is known to not only be a Shh receptor, but an inhibitor of Shh mediated response. This “temporal adaptation” hypothesis about neuronal precursor sensitivity to Shh suggests

that cells initially respond vigorously to Shh signaling and upregulate Gli transcription strongly, but in the presence of varying concentrations of Shh, only a high concentration of Shh will overcome the desensitization that occurs in response to Shh (Dessaud et al., 2007; Jeong and McMahon, 2005; Stamataki et al., 2005). Therefore this combination of varying concentration of Shh and cell-sensitivity to duration give rise to a varying transcriptional domains in the ventral neural tube.

The homeodomain and bHLH transcription factors that are expressed in discrete dorsoventrally established domains are not the primary transcriptional targets of Shh. Shh mediates its patterning effects through modulation of the 3 vertebrate homologues of the cubitus interruptus TF, which was first isolated and studied in *Drosophila.* These vertebrate homologues are the zinc finger proteins Gli1, Gli2 and Gli3 (Jacob & Briscoe 2003). The results of various knock in and knock out experiments in mice with the Gli genes indicate that Gli2 has a role as a transcriptional activator that is proportional to the level of Shh signalling (Jacob & Briscoe, 2003; Bai & Joyner, 2001).

A second dorsally-derived morphogen gradient also contributes to the dorsoventral patterning of the neural tube. The roof plate is the primary source of these morphogens, which are members of the BMP and Wnt families of secreted proteins that induce proper specification of the dorsal half of the spinal cord (Lee and Jessell, 1999; Liem et al., 1997). It is known that Wnt plays an antagonistic role to BMP, keeping progenitor cells from proliferating and potentially being involved in proper di1-di3 interneuron specification (Ille et al., 2007). Induction by BMP in the dorsal half of the

spinal cord gives rise to six distinct progenitor domains called the pdi1-pdi6 regions which can be classified by their combinatorial expression of bHLH transcription factors. The subsequent patterning of the dorsal neural tube is produced by differential expression of the bHLH transcription factors Math, Mash, Ngn and LIM including the homeodomain transcription factors Lbx and Lmx (Helms and Johnson, 2003).

The instructive role of the roof plate in patterning of the dorsal neural tube is illustrated by genetic ablation of this structure leading to defects in gene expression in the dorsal neural tube. The level of Pax7, which is expressed in the dorsal half of the neural tube is reduced. Furthermore, there is an expansion of Pax6 expression further dorsal compared to wild-type (Lee et al., 2000). The importance of the BMPs that are secreted by the roof plate have been examined by overexpression studies. Ectopic expression of a constitutively active BMP receptor showed that in ventrally located electroporated cells there was expression of the dorsal genes Pax7, Msx1/2, Cath1 (Timmer et al., 2002). Moreover there was also a downregulation of Pax6, Dbx2, Dbx1, Evx1, En1, all genes expressed in the ventral spinal cord. These results seem to indicate that Shh and BMP are both acting as concentration dependent morphogens that seem antagonistic towards each other. In order to test this, notochord grafting experiments in chick were performed near the roof plate where they found overall a suppression of dorsal neuron status in zebrafish, which corroborates the antagonistic Shh and BMP hypothesis (Yamada et al., 1991; Goulding et al., 1993).

CROSS REPRESSIVE FACTORS DEFINE THE PROGENITOR DOMAINS

The inductive effects of these morphogens and the downstream transcriptional programs result in the portioning of the neural tube into two distinct domains. Much work has been done to identify and classify the different progenitor regions by their gene expression profiles.

In the ventral spinal cord graded Shh signaling sets up the five identified progenitor domains: p0, p1, p2, pMN, p3 (Briscoe et al., 2000). In the ventral spinal cord the differential expression of the Shh-responsive class I homeodomain proteins, Pax7, Irx3, Dbx1, Dbx2 and Pax6 and the class II homeodomain proteins, Nkx6.1 and Nkx2.2 are critical in the generation of these distinct domains. Studies using retroviral and electroporation approaches to drive ectopic expression of the various class I and class II proteins provide strong evidence of the mutual repression of the class I and class II patterning factors (Briscoe et al., 2000). For example, the loss of Pax6 results in an expansion of the p3 domain, and conversely, loss of Nkx2.2 results in an expansion of Pax6 and a loss of p3 progenitors and neurons (Ericson et al., 1997; Briscoe et al., 1999). Irx3 and Nkx2.2 provide spatial demarcations for the dorsal and ventral pMN region, Dbx2 and Irx3 mark the dorsal and ventral boundaries for the p2 progenitor domain, Dbx2 and Nkx6.1's cross repressive interactions generate the p2 and p1 boundary, while

Dbx2 and Dbx1 setup the p0 and p1 regions (Briscoe et al., 2000; Pierani et al., 2001). The cross-repressive nature of these class I and class II genes establish sharp boundaries that demarcate each progenitor region.

Finally, there is strong evidence that the combinatorial expression of these homeodomain proteins are crucial in determining the fate of the post-mitotic neurons that come from these domains (Briscoe et al., 1999; Briscoe et al., 2000). Therefore what exists thus far is a three-step process in ventral cord patterning. First, the Shh gradient establishes a rough progression of differential class I and class II transcription factor expression along the dorsoventral axis of the ventral half of the neural tube. Second, Shh induction of class I and class II genes help to cleanly restrict the boundaries of each domain through cross repression. Third, because the class I and class II pattern of expression tends to span multiple progenitor domains, we have a combinatorial transcription factor code that exists in each progenitor domain. This suggests that the unique combination of expression plays a crucial role in the proper specification of post mitotic spinal neurons (Briscoe et al., 2000). In the ventral neural tube, these five progenitor domains generate many of the post-mitotic neurons that form the core motor circuitry (Goulding et al., 2002).

Similar mechanisms appear to function in the patterning of the dorsal spinal cord. After BMP induction, the dorsal half of the spinal cord can be segregated into two distinct domains. Within these domains, the bHLH transcription factors Math1, Ngn1 and Mash1 operate through cross-repressive interactions, much like that of the ventral cord, to

further demarcate the dorsal neural tube (Caspary & Anderson, 2003). More generally, the dorsal neural tube can be divided into two classes of neurons, the Class A neurons and Class B neurons, each of which can be subdivided into three dorsal interneuron regions, better known as the di1-di3 for Class A, and di4-di6 for Class B. (Zhuang & Sockanathan, 2005). Class A precursor neurons are marked by the expression of the bHLH transcription factor Olig3 while Class B neurons are well marked by the post-mitotically expressed Lbx1 homeodomain transcription factor (Muller et al., 2005; Gross et al., 2002; Muller et al., 2002). In Lbx1 mutants, the di4-di6 adopt a more dorsal fate at least partially trans-differentiating into di1-di3s. This is interesting since Lbx1 is a post-mitotic transcription factor it suggests that the di4-di6 post-mitotic neurons are still multi-potent.

The function of the bHLH family of transcription factors in dorsal patterning has been examined in mice by the creation of targeted null alleles. In studies involving the loss of Math1 (expressed in di1s) dorsal expansion of Ngn1 (expressed in di2s) was observed. Conversely, Ngn1/Ngn2 mutants exhibited an expansion of Math1 into the di2 region indicating mutual repressive activities between the genes (Caspary & Anderson, 2003). A loss of Mash1, which is expressed in the di3-di5 domain, results in a dorsal expansion of Ngn1. Furthermore, Mash1 overexpression leads to ectopic expression of di3 and di5 markers with an equivalent loss of di2 and di4 cell subtypes, further highlighting a cross-repressive paradigm similar to that in the ventral cord also operating in the dorsal spinal cord (Zhuang & Sockanathan, 2005).

The cross-repressive activities of homeodomain and bHLH transcription factors subdivide the neural tube into discrete progenitor domains along the dorsoventral axis (Briscoe et al., 2000; Caspary & Anderson, 2003). Within each progenitor domain, a combinatorial transcription factor profile generates the genetic programming that allows for specification of the 11 major classes of spinal neurons (Goulding et al., 2002). Throughout development of the neural tube and as development progresses, the activities of Hox genes in the proper specification of MNs would suggest that dorsoventral patterning works in conjunction with the rostrocaudal factors to properly specify neuronal subtypes that comprise all sorts of functions in the spinal cord (Dasen et al., 2003; Dasen et al., 2005). Important strides towards understanding the differential genetic code involved in programming these post-mitotic neurons have helped to answer many questions about these different neuronal subclasses.

THE GENETIC PROGRAMMING OF VENTRALLY LOCATED NEURONS

Having established the cardinal progenitor domains in the ventral spinal cord via cross repressive interactions between homeodomain Class I and Class II transcription factors, neuronal precursors begin to steadily exit the cell cycle and become post-mitotic neurons on their way to more specific fates. Molecular markers and combinatorial genetic profiles unique to the four ventral interneuron subtypes have been determined and serve as important genetic loci to decipher the functions of each class of the ventral

interneurons. The V0s are marked by Dbx1 expression and the V1s by the post-mitotic markers Foxd3 and En1. V2 neurons comprise of two subclasses: the V2a's that express Chx10 and the V2b's that express Gata3 (and Gata2). Finally the V3 neurons express Sim1 (Goulding et al., 2002; Zhang et al., 2008).

Each of the interneuronal progenitor domains in the ventral spinal cord appear to give rise to a generic class of neurons, although it is now becoming apparent that each domain gives rise to multiple cell types. The p0 progenitor domain, which is marked by Dbx1 expression, generates V0 neurons. Interestingly, the dorsal half of the Dbx1 region is Pax7+, while the ventral half is Pax7- and it appears that two classes of early born V0 commissural neurons are generated from these two p0 domains, the Evx1-$V0_d$ neurons and the Evx1+ $V0_v$ neurons. Dbx1 also appears to act in conjunction with Dbx2, which is expressed in both the p0 and p1 domain, to define V0 progenitors. In embryos lacking Dbx1, ectopic expression of En1, a V1 marker, is observed in the p0 domain. This suggests that V0 progenitors are incorrectly specified and instead adopt the fate of ventral interneuron progenitors that express only Dbx2, namely a V1-like fate (Pierani et al., 2001). Furthermore, ectopic expression of Dbx1 suppresses En1 expression and expands Evx1/2 expression into the p1 domain, further corroborating a combinatorial Dbx1/Dbx2 code for differentiating the p0 and p1 domains. Dbx1 is also required for Evx1, which is expressed in$V0_V$s (Pierani et al., 2001). Ectopic Evx1 expression also suppress the V1 fate by downregulating En1, suggesting Evx1 is the downstream post-mitotic switch that guides $V0_v$ fate and represses the V1 fate (Moran-Rivard et al., 2001).

The p1 progenitor domain, which is marked by the expression of Pax6, Dbx2 and Nkx6.2 (Sapir et al., 2004) generates ipsilaterally projecting V1 neurons (Saueressig et al., 1999). The V1 population is a class of inhibitory interneurons that has been conserved across many species. In aquatic vertebrates the V1 neurons are ipsilaterally projecting, glycinergic inhibitory interneurons (Higashijima et al., 2004). In mammals, inhibitory Ia interneurons and Renshaw cells have been shown to be derived from the En1+ V1 population (Sapir et al., 2004; Alvarez et al., 2005). With a well characterized neurotransmitter phenotype, morphology and general projection patterns, they are thought to modulate sensory gating, help in coordinating locomotion and directly modulate MN burst activity. The canonical post-mitotic marker of the V1 interneuron population, En1, is a homeodomain transcription factor that regulates fasciculation and axon pathfinding in the V1s, but does not seem to be required to specify the V1 fate, as En1 mutants do not express markers of neighboring cell types (Saueressig et al., 1999; Sapir et al., 2004). This would seem to indicate that En1 is a downstream branch of the V1 program that controls an aspect of V1 specification. Having multiple roles in spinal cord function clearly indicates that the V1 population can be further subdivided into subclasses, suggesting differential transcriptional activity would be involved in generating these different subclasses. Indeed such is the case for a V1 subtype known as the Renshaw cells.

Lhx3, FoxN4 and Mash1 are all expressed in p2 progenitors (Peng et al., 2007). As these cells differentiate, two classes of postmitotic neurons emerge from this domain: the Chx10+ V2a's and the V2b's which are marked by the expression of Gata2, Gata3 and

Scl. The V2 neurons, like the V1 neurons project their axons ipsilaterally. However, the Chx10-expressing V2a's are primarily excitatory, whereas the Gata3 V2bs are inhibitory (Lundfald et al., 2007). Efforts to understand how the p2 domain gives rise to two classes of neurons have focused on the Notch pathway. Utilizing PS1 KO mice, Peng and colleagues demonstrated that there is a gain of V2a neurons at the expense of V2b neurons. Additional studies have shown Delta4-Notch interactions play a critical role in determining post-mitotic interneuronal fate. The authors were confirmed by misexpressing a mediator of Notch signaling, *mastermind,* that promotes the fate of V2bs (Peng et al., 2007). The involvment of Notch signaling in the generation of these two classes of intenreurons suggests that both cell types arise from a common progenitor. Confirmation of this has come from the observation that a single V2a and a single V2b are generated from a single dividing progenitor cell (Kimura et al., 2008). Questions as to whether or not these two populations can be further subdivided are not yet clear, but due to subtle yet distinct characteristics among neurons of both groups of V2 cells, one would have to imagine there are other genetic cascades involved in specifying unique phenotypes in these cells (Lundfald et al., 2007).

Less is known about the genetic program that specifies V3 interneurons. The p3 progenitor domain is demarcated by its expression of Nkx2.2. P3 progenitors also express the bHLH prproneural factor Ngn3. Both of these factors appear to play a role in the genesis of V3 neurons that are marked by the expression of the PAS-bHLH transcrition factor Sim1 (Briscoe et al., 1999). Somewhat more is known about the properties of the V3 interneurons. They appear to comprise of at least two major populations, a ventral

group of cells that settle in lamina VIII and a group that migrates dorsally to settle in lamina VI and the deep dorsal horn. The V3 interneurons that settle ventro-medially in lamina VIII send excitatory projections to the contralateral side of the cord (Zhang et al., 2008). This was demonstrated with a Sim1-tauLacZ mouse line. Using a Sim1-Cre; Rosa26floxstop-GAP43-GFP genetic background, which labels axonal projections from Sim1 expressing neurons, Zhang et al., (2008) also showed that the V3s make direct connections to V1 interneurons and MNs. Furthermore, transynaptic tracing studies indicate that that at least a portion of the V3 neurons synapse directly onto contralateral MNs (Zhang et al., 2008). Molecular markers that can further delineate and allow for functional molecular studies of the V3 population have not yet been defined.

VENTRAL INTERNEURONS FUNCTION IN LOCOMOTOR CPG

Molecular characterization of these neuronal populations has paved the way for advanced molecular genetic techniques in order to study the roles of the various classes of ventral interneurons. Using a combination of cutting edge genetics and electrophysiology, we have begun to better understand the contributions that these four classes of ventral interneurons have in CPG locomotion.

Lanuza and colleagues (Lanuza et al., 2004) characterized the loss of the contralaterally projecting inhibitory V0s in Dbx1 mutants and the $V0_v$'s in Evx1 mutants. The authors performed root recordings in mice during chemically induced fictive

locomotion in isolated spinal cords to look at whether the loss of$V0_d$'s as well as $V0_v$'s changed the patterns of fictive locomotion following the application of NMDA and 5-HT. In recordings made from L2 and L5, which correspond to hindlimb flexors and extensors, respectively, it was observed that the loss of the V0s caused episodic overlaps in left-right flexor bursting activity, indicating that the CPG lost some of its innate ability to coordinate proper left-right movements during locomotion. Further evidence that the V0s are involved in locomotion was obtained after chemically inducing locomotion and then staining for c-fos which colocalized with Dbx1 expressing neurons (Lanuza et al., 2004).

Similar experiments were performed on the inhibitory and ipsilaterally projecting V1 interneurons. Using various molecular genetic approaches to extinguish the V1 interneurons, Gosgnach and colleagues (Gosgnach et al., 2006) demonstrated that the V1s play a critical role in the pace of locomotor output. One technique involved the Pax6 mutants which are known to lose specification of V1s. Another method was with an En1 KO. The more interesting approaches involved selective ablation of the V1s with an En1-DTA allele and an En1-Cre; AlstR192 genetic background which allowed for a reversible silencing of the V1s while keeping the rest of the spinal cord relatively unperturbed. The AlstR192 allele expressed the *Drosophila* allatostatin receptor in a Cre-dependent manner. The allatostatin receptor is a GPCR that activates inward rectifying K+ channels. Therefore with significant expression of the receptor and in the presence of allatostatin, the AlstR+ neurons have too high a K+ conductance to achieve a supra-threshold potential through excitatory inputs. Using these approaches the authors confirmed that targeted loss of V1 interneurons resulted in an elongated step cycle that maintained left-

right and flexor-extensor coordination and that this errant locomotor phenotype was reproducible in multiple ways (Gosgnach et al., 2006).

The V2a interneurons are marked by their expression of Chx10. After acquiring their fate through Notch signaling, V2a interneurons become ipsilaterally projecting excitatory interneurons that make most of their contacts onto the inhibitory and commissural V0 population as well as MNs (Crone et al., 2008). Utilizing a Chx10-DTA genetic strain, the authors selectively ablated the V2a interneurons and proceeded with ventral root recordings identical to the manner described previously. The results were somewhat surprising in that there was no significant alteration in the mean locomotor cycle period and the normalized burst amplitude and no loss of flexor-extensor coordination, however when individual burst amplitudes were analyzed they noticed erratic behavior as opposed to consistent amplitudes. Furthermore, left-right alternation was disrupted as would be expected based on their anatomical connections to V0s. In all, it suggests that V2a interneurons are involved in maintaining a stable excitation of flexor-extensor muscles during locomotion and aid in providing relevant circuit information to the contralateral side to coordinate a proper left-right alternation (Crone et al., 2008).

Such experiments were also performed on the V3s. The V3s are a predominantly ventromedially settling, contralaterally projecting, glutamatergic interneuron population. Interestingly, the V3s make contacts with MNs, Renshaw Cells, Ia inhibitory interneurons and V2 interneurons; indicating a relatively complex role in spinal circuitry. Taking advantage of a Sim1-Cre driver allele Zhang et al., 2008 crossed it with a ZnG transgenic

reporter that shows strong nuclear GFP staining. Whole-cell recording analysis of these GFP+ V3 interneurons showed a linear relationship between spike frequency and current pulse as well as moderate levels of spike adaptation. Coupled together with anatomical data, this would seem to suggest the V3 interneurons accurately propagate the summation of their presynaptic input across various nodes of the spinal circuit. Locomotor studies using the Sim1-Cre allele with a Rosa26floxstop-TeNT ubiquitous expression system caused neurons that expressed TeNT to no longer mediate synaptic transmission, effectively silencing their output. Ventral root recordings in these mutant neonate spinal cords resulted in a peculiar observation in asymmetrical left/right flexor burst duration. Further, there was no consistency in the step cycle period and overall fictive locomotion was not as easily induced. The authors showed that this phenotype was reproducible with the AlstR192 allele. These data seem to suggest that the V3s are involved in maintaining a balance between the outputs of each side of the spinal cord (Zhang et al., 2008).

The elimination or silencing of such large populations of neurons while recording net outputs can only reveal so much about the underlying circuitry. There is considerable evidence that each of these early generic neuronal classes comprise multiple cell types. Attempts to manipulate smaller, more well-defined populations will be necessary to unravel the underlying locomotor CPG. This will require an identification of further downstream genetic markers to isolate more specific subpopulations of these classes of neurons and detailed anatomical studies to further dissect the circuitry into better understandable discrete units. It is therefore critical to develop experimental approaches that provide better spatio-temporal control of genetically defined classes of neurons. Such

approaches will require the knowledge of downstream molecular codes that program neuronal subsets of these interneuron classes. Based on their diverse and broad roles in biological systems including development and cell differentiation, the Fox genes appear likely to be candidates of interest. The genetic techniques employed in these studies have provided a sturdy platform upon which to move forward in decoding the spinal circuitry. The Fox genes and their loci may well have the potential to be employed as molecular genetic tools described in these studies.

THE FOX GENES

Transcription factors that contain a forkhead domain were first identified in *Drosophila melanogaster*. To this date, genetic screens have identified hundreds of Fox genes across a wide variety of species, from the lowly yeast to us humans. The high divergence of the Fox genes has led to a phylogenetic classification of the gene family into 18 clades, each of which has multiple subfamily members. So far we have identified that humans have genes in each of these subfamilies, while mice have genes in all but one of these subfamilies (Kaestner et al, 2000).

When the crystal structure of the forkhead domain was solved, they found high similarities to that of the linker histone H1. However, further studies revealed that Foxa proteins have the ability to displace linker histones and recruit other transcription factors which may have implications on other Fox genes and their hierarchical importance in

creating transcriptional complexes (Clark et al., 1993; Cirillo et al., 2002). The divergence of this family of genes has been crucial to the evolution of vertebrates and higher vertebrates as evidenced by their involvement in many critical areas of embryological development including node formation, patterning along the left-right and anterior-posterior axes and also regulation of the cell cycle (Tuteja & Kaestner 2007; Ang & Rossant, 1994; Brody et al., 2000).

The Foxa subfamily of genes regulate a broad range of biological processes. The Foxa genes exhibit a similar, yet distinct expression profile during development and they have also been shown to have critical importance in neural tube, liver, lung, gastrointestinal and pancreatic development (Friedman & Kaestner, 2006). Double mutant Foxa mice lack livers, highlighting their importance in hepatogenesis. In the pancreas, Foxa genes are expressed just before the beginning of pancreatic development and pancreatic-conditional Foxa2 mutants exhibit a lack of final stage differentiation of alpha-cells specifically, leading to early post-natal death. Further studies in the lung show a redundancy and necessity of Foxa genes in lung formation and function (Wan et al., 2005). The Foxa genes are also involved in neural development. Ectopic expression of Foxa2 in the brains of transgenic mice causes the downregulation of Pax3 and morphological defects of the cerebellum (Sasaki et al., 1994). More recently, studies in midbrain development show that the dose-dependent expression of Foxa1/2 are critical in the multi-phased specification of dopaminergic neurons (Ferri et al., 2007). Given their numerous roles, it isn’t surprising that the Foxa genes have been shown to be pioneer factors that are crucial in recruiting particular cofactors in an organ-specific manner

(Friedman & Kaestner, 2006).

Various other Fox genes have been identified as having a role in neuronal development in the spinal cord. The winged-helix transcription factor FoxD3 has been shown in the chick neural tube to be expressed in neural crest cells. Overexpression experiments indicate that FoxD3 promotes neural crest fates even in cells that are in the neural tube. Furthermore FoxD3 is downstream of Pax3, an expansive progenitor domain patterning gene (Dottori et al., 2001). Characterization of FoxD3 by Gross and colleagues (Gross et al., 2002) showed that FoxD3 is expressed in neurons exiting the di2 progenitor domain as well as cells exiting the p1 region, indicating that FoxD3 is a reliable marker for di2 and V1 neurons.

Detailed work with FoxN4 has shown it to be a master regulator of V2 fating. Del Barrio and colleagues (Del Barrio et al., 2007) showed that FoxN4 is expressed early in the p2 progenitor domain and occupies an elevated seat in the transcriptional hierarchy of V2 fating. The authors demonstrate that FoxN4 has a dual role in V2 differentiation. First it sets up the transcriptional program that leads to the expression of Delta-like-4 in all V2 progenitors. Then after the V2s begin to exit the cell-cycle, delta-notch signaling creates the two separate classes of V2s: the V2a's and the V2b's. Subsequently in the V2a's which express Chx10, FoxN4 transcription is turned off, but remains on in only the V2b's where it may have a role in further specifying specific V2b characteristics (Del Barrio et al., 2007). The dual role that FoxN4 appears to play in early generation and later specification is reminiscent of the Foxa function in pancreatic cell, suggesting that FoxN4

may also have a similar pioneering role as a transcription factor in neuronal differentiation.

The forkhead box genes have diverse roles in development and disease due to their complex divergence and many evolutionary generations. What is known about the genes suggests that it is difficult to speculate as to what functions other sub-families have in other biological systems. The focus of my research has been on the FoxP genes and their roles in the developing cord. Knowing that the Fox genes play essential yet differing roles in spinal cord development raises questions about the potential roles that the FoxP transcription factors may play in neuronal fating.

THE FOXP SUBFAMILY OF GENES

The FoxP subfamily of the Fox genes has recently come to the forefront in medical and developmental research. In 2001 when a single point mutation in the DNA binding forkhead domain of FoxP2 was identified to be highly suspect in severe speech and language impediments in humans, the gene was thrust into the popular and scientific limelight. The impact of this discovery was such because it was the first gene to be implicated in the maintenance of a highest of order function that only humans possess (Lai et al., 2001). This discovery prompted many subsequent studies on the 4 FoxP genes, FoxP1, FoxP2, FoxP3 and FoxP4.

A unique aspect that the FoxP genes have compared to previously characterized Fox genes is their ability to form hetero and homodimeric complexes (Wang et al., 2003). The FoxP genes are identified by their well-conserved forkhead domains, a zinc finger domain as well as a coiled-coils structure in between those two regions and a leucine zipper motif, all of which are occupying roughly the same sequential positions in their respective transcripts (Teufel et al., 2003; Li et al., 2004a). For a rough comparison, the authors aligned the FoxP4 sequence with each of the other three and found that FoxP4 shares 54%, 60% and 47% identity with FoxP1, FoxP2 and FoxP3, respectively. This indicates high homology between the four sister genes, but FoxP4 has been shown to be missing a “subdomain” when compared to FoxP1 and FoxP2 which confers it slightly modified transcriptional regulation (Li et al., 2004a). These data raise questions as to whether or not the FoxP genes perform primarily redundant roles or have significantly evolved their abilities to recruit and form different complexes with other proteins, thereby leading to divergent transcriptional behavior.

Of substantial interest are FoxP1, FoxP2 and FoxP4 due to their broad array of expression in the CNS. To begin, FoxP1 and FoxP2 are known transcriptional repressors of lung-specific promoters (Shu et al., 2001). FoxP1 has also been implicated in cancer cells as a tumor suppressor gene. FoxP2 has been linked to critical neural development involving crucial speech and language centers (Hannenhalli & Kaestner, 2009). Less is known about FoxP4, but in an attempt to compare transcriptional activity to FoxP1 and FoxP2, Li and colleagues used GAL4 DNA binding domains and yeast two-hybrid screens with varying sequences of the three FoxP genes and demonstrated in some cases

a differing role in transcriptional regulation with FoxP4 (Li et al., 2004a).

FoxP1, FoxP2 and FoxP4 have been shown to be expressed at elevated levels in lung, gut and the CNS. Although similar, the expression patterns are distinct and overlap. Expression of these FoxP genes are present in many areas of the CNS. In situ hybridization analysis in rat forebrain slices show differential expression of the FoxP genes. More specifically, in the striatum, the authors noticed expression of both FoxP2 and FoxP4 in the LGE and SV, while FoxP1 only in the SV. Likewise in the cerebral cortex, FoxP1, FoxP2 and FoxP4 exhibited spatially similar yet distinct patterns in the various cortical layers all while displaying differing temporal regulation throughout development. Expression in the basolateral amygdala was limited to FoxP2 and FoxP4 while FoxP1 was undetected (Takahashi et al., 2008). The expression patterns of the three FoxP genes are unique yet overlap in many ways suggesting a combinatorial FoxP code in regulating differentiation in different neuronal subtypes in the forebrain. However neuronal fating studies have not yet been undertaken and the specific downstream pathways being regulated by the FoxP genes in the CNS are as of yet unknown.

Of the four FoxP genes, FoxP3 has the most distinct role in embryonic development. In the immune system, a class of T cells that are required for the suppression of self-antigenic effectors called the natural regulatory T cells are marked by FoxP3 expression. In experiments involving FoxP3 KOs, experimenters find the mice die of fatal autoimmune lymphoproliferative disease which is characterized by a complete loss of these natural regulatory T cells and subsequent death. In studies involving dual

reporter plasmid constructs cotransfected into cells, the transcriptional regulatory behavior of FoxP3 indicated that it also had a primary role as a transcriptional repressor (Buckner & Ziegler, 2008).

Because they form hetero and homodimeric complexes to regulate transcription it is likely that dosage of these FoxP genes is crucial to proper regulation of gene expression. Furthermore, differential and coexpression of the FoxP genes and their potential ability to form heterodimers with other as of yet unidentified proteins points to a diverse range of transcriptional targeting. FoxP1 is known to be expressed at differing levels of concentration and is known to be crucial in its interactions with different downstream Hox genes in the spinal cord, leading to specific MN column fates (Dasen et al., 2008). However, the critical roles of other FoxP genes in the developing nervous system have yet to be elucidated.

FOXP1 – A MASTER SWITCH IN SPINAL CORD DEVELOPMENT

Very recently groundbreaking research has defined a critical role that FoxP1 plays in higher vertebrate spinal cord development. The Hox genes were known to be integral in the generation of the various MN columns, but other transcription factors that intersect with the Hox pathway to determine MN differentiation had not been identified. Analyzing the ectopically expressed genes in an Olig2 mutant, Rousso et al, 2008 found the gene FoxP1, analyzed its expression pattern in the neural tube.

FoxP1 is expressed at varying levels along the rostrocaudal axis. At the brachial and lumbar levels, high levels are found in the LMC MNs while in thoracic segments, it is expressed at medium levels in the PGC MNs (Rousso et al., 2008; Dasen et al., 2008). Interestingly, FoxP1 is not coexpressed with Lhx3 in MMCm MNs, which suggests a possible repressive role of FoxP1 by Lhx3 (Dasen et al., 2008). Indeed this was the case, as electroporation of constructs driving Lhx3 under the CMV promoter showed a significant reduction of FoxP1 expression as well as LMC MN markers RALDH2 and Lhx1 (Rousso et al., 2008). Further, generation of a transgenic Hb9::FoxP1 mouse revealed a marked decrease in Lhx3+ MMCm MNs and an increase in LMC MNs, suggesting cross repressive interactions between the Lhx3 and FoxP1 in determining MMCm and LMC fates respectively. Analysis at thoracic levels revealed similar results, where an increase in PGC MN markers was observed.

However it wasn't until FoxP1 mutant mice were analyzed that its critical role in defining MN columns was understood. FoxP1 mutants exhibit striking phenotypes with respect to their motor column layouts. At E12.5 in the brachial and lumbar segments, there is almost a complete loss of the LMC which appears to be respecified to a HMC fate, while at thoracic levels, the PGC is apparently respecified to a HMC fate as well (Dasen et al., 2008). Deeper analysis shows that FoxP1 mutants have increased numbers of MMC MNs as well, further demonstrating FoxP1's critical role proper specification of MN columns (Rousso et al., 2008). Finally the question as to where FoxP1's role is in the interplay with Hox genes was answered using HoxC10; HoxD10 double mutants where

FoxP1 expression was reduced significantly reduced, suggesting that Hox genes are upstream of FoxP1 and that a FoxP1 expression is combinatorially regulated via various Hox genes.

In higher vertebrates, the HMC does not exist at the brachial and lumbar levels. This implicates that the recruitment of FoxP1 may have been a critical step in the evolution from lower vertebrates to higher vertebrates as FoxP1 is necessary for the generation of the LMC MNs which are involved in more complex limb movements that are present in mammals such as mice. This possibility is further corroborated by the loss of the PGC in FoxP1 mutants which is responsible for many critical autonomic functions that are found in higher vertebrates. The observation that FoxP1 has a significant role in proper spinal cord development naturally leads to many questions about what roles if any do other members of the FoxP subfamily of genes play in neuronal development in the spinal cord. The results presented in this thesis are the first crucial steps towards being able to answer such questions.

CHAPTER 1 – STUDYING THE FOXP GENES IN THE SPINAL CORD

FOXP2 AND FOXP4 ARE EXPRESSED IN VENTRALLY SETTLING INTERNEURONS

Previous studies have established that FoxP1 is expressed in a subset of motor neurons (Dasen et al., 2008). In contrast, less is known about the expression patterns of FoxP2 and FoxP4 during spinal cord development. In order to explore the profile of FoxP2 and FoxP4 gene expression in the developing cord, I performed in situ hybridization and immunohistochemistry on embryonic day 11 and 12 cryosectioned tissue. FoxP2 protein was detected as early as E11.5 in the developing spinal cord, where it was expressed in dorsal cells that were migrating ventrally, as well as in more ventrally located cells (Fig 1a). FoxP2 expression was not detected prior to E11.5, indicating it is primarily expressed in postmitotic neurons. I also observed strong expression of FoxP4 protein in a subset of brachial MNs, as has previously been reported (Dasen et al., 2008). Additional neurons expressing FoxP4 in a manner similar to FoxP2 were also observed at all levels of the spinal cord. These cells were not motor neurons (Fig 1d,j). I also observed that FoxP4 protein was expressed either slightly later, or at lower levels compared to FoxP2 (Fig 1g,h). For both FoxP2 and FoxP4, the expression of protein or mRNA was in cells exiting the progenitor domain, suggesting that they are part of a post-mitotic neuronal program.

Analysis of FoxP2 and FoxP4 expression at E12 revealed continued expression in cells exiting the p1 and pd2 domains of the ventricular zone (Fig 1b,e,k). However by E12.5 cells exiting the ventricular zone no longer expressed either FoxP2 and FoxP4,. The earlier born neurons that had all migrated to the ventral half of the spinal cord appeared to maintain expression of these genes (Fig 1c,f). Interstingly both the FoxP2 and FoxP4 neurons were seen to settle in two distinct ventral domains and thus appear to comprise of ventro-medial and ventro-lateral subpopulations. (Fig 1c,f,l). The medially settling interneurons are known to be primarily contralaterally projecting neurons while laterally settling interneurons are predominantly ipsilaterally projecting. I therefore decided to classify the ventro-medial populations as $FoxPX_m$ and the ventro-lateral populations as $FoxPX_l$.

QUANTIFICATION OF THE FOXPS

FoxP1 is known to show high levels of expression in the LMCs at brachial and lumbar segments, and at lower levels in the thoracic PGC. Furthermore, this difference in the level of FoxP1 expression leads to specific MN columnar fates at limb versus thoracic levels. In light of this finding, I set out to ask whether similar differential levels of expression might be exhibited by FoxP2 and FoxP4. To do this, I characterized the expression FoxP1, FoxP2 and FoxP4 at the three segmental levels in the developing

spinal cord at E12.5.

Numerical analysis of two wild-type E12.5 embryos of FoxP2 and FoxP4 expression at brachial, thoracic and lumbar segments revealed increased numbers of FoxP2 and FoxP4 cells in the lateral ventral population at brachial and lumbar levels of the cord. On average there were 68.3 and 93.4 $FoxP2_l$ cells at brachial and lumbar segments, respectively and 40.9 and 41.9 $FoxP4_l$ cells at similar levels. By way of comparison at thoracic levels, there were on average 62.3 and 25.1 $FoxP2_l$, $FoxP4_l$ cells, respectively (Table 1). These lower numbers of lateral FoxP2/4-expressing cells may reflect a general decrease in overall interneuron numbers at thoracic levels of the thoracic spinal cord compared to brachial and lumbar cord. Differences may also reflect other factors such as rostrocaudal patterning, with brachial and lumbar spinal segments. I must note that there were differences in brachial-level lateral cell counts with FoxP2 between embryo B and embryo A were noticeable. Embryo A exhibited higher thoracic $FoxP2_l$ counts relative to brachial; however embryo B had opposite results, where brachial $FoxP2_l$ numbers were substantially higher compared to thoracic. Throughout numerous experiments that I've performed I have generally observed higher numbers in brachial spinal cord relative to thoracic. Given that, I feel the embryo B cell counts best reflect reality, but further counts must be performed to obtain a statistically significant data set.

Analysis of the medial populations also revealed a difference in FoxP2 expression along the rostrocaudal axis, and comparatively stable numbers of $FoxP4_m$ cells. $FoxP2_m$

cells on average numbered 45.4, 54.4, 65.3 at brachial, thoracic and caudal levels, respectively. $FoxP4_m$ cells averaged 21.4, 22.8, 28.4 at brachial, thoracic and caudal levels, respectively (Table 1). Overall, a progressive increase as we descend caudally along the spinal cord is also indicative of a rostrocaudal influence on the medial FoxP populations. Another alternative is that FoxP2 andFoxP4 are expressed as these cells migrate ventrally and expression is extinguished once these cells settle in the ventral horn.

In general, the expression patterns of FoxP2 and FoxP4 are very similar in terms of the relative ratios of medial cells to lateral cells as well as their overall expression patterns along the rostrocaudal axis. FoxP2 and FoxP4 are share 60% overall homology and have highly conserved DNA binding domains. This together with the observation that this class of Fox proteins have leucine zippers that confer the ability to form hetero and homodimers, highlighted the necessity to quantify the number of cells that coexpress FoxP2 and FoxP4. In order to quantify in a logical manner, I tallied the number of FoxP+ cells and calculated the percentage that were FoxP2+/FoxP4+ or singly positive for either FoxP gene (Fig 7). On average, 28% of the medial at brachial levels expressed both proteins, with 62% of the FoxP2 cells being single positive and 10% of the FoxP4 cells being single positive. Brachial lateral populations exhibited 42% coexpression with 39% of the cells being single positive $FoxP2_l$ and 19% being $FoxP4_l$ single positive cells. The vast majority, if not all, single-positive $FoxP4_l$ cells comprise brachial MNs. At the thoracic levels, medial populations had 38% coexpression of FoxP2 and FoxP4, 53%

were FoxP2 single positive and 9% were FoxP4 single positive. Brachial-lateral populations had 41% coexpression, 55% FoxP2 single positive and 4% FoxP4 single positive cells. Although the number of sections counted is not statistically significant, based on what I observed throughout other FoxP2/FoxP4 costaining, these numbers appear to accurately reflect reality.

Overall I found a high level of coexpression between FoxP2 and FoxP4, but there were noticeable differences between brachial and thoracic levels. Brachial levels exhibited higher numbers of single positive FoxP4 cells overall, a majority of which are likely to be FoxP4+ MNs. Thoracic levels, where FoxP4 is only expressed in interneurons we see very high levels of coexpression between the two genes. This suggests that in these neurons both of these factors may function redundantly. Alternatively, there may be a combinatorial FoxP genetic code that operates in these interneurons. The role of such a code is not known.

CHARACTERIZING THE LATERAL FOXP2/FOXP4 POPULATION

In order to better understand of role of the FoxP genes in neuronal differentiation, I set out to characterize these neurons in greater detail. It is known that lateral neurons consist primarily of MNs and inhibitory interneurons. While FoxP2 appears to be expressed in only interneurons and not MNs, FoxP4 is expressed in a small subset of

brachial MNs as well as other ventral interneurons (this study; Dasen et al., 2008).

To determine whether or not the FoxP2$_l$ and FoxP4$_l$ interneurons are inhibitory or excitatory, I asked if FoxP2$_l$ and FoxP4$_l$ cells expressed Gad67, an isoform of glutamic acid decarboxylase which is expressed only in inhibitory cells. In mice heterozygous for a Gad67-GFP knock-in allele, I observed that virtually all FoxP2$_l$ and FoxP4$_l$ cells expressed Gad67, suggesting that these neurons are indeed inhibitory interneurons (Fig 2a,d).

The V1 and V2b classes of interneurons are both known to be laterally settling inhibitory interneurons.V2a interneurons are located in the lateral ventral horn, but are excitatory interneurons. The V1s can be identified by their post-mitotic expression of En1 while the V2a's and V2b's are marked by their expression of Chx10 and Gata3, respectively (Sapir et al., 2004; Del Barrio et al., 2007). I asked which group of ventral interneurons expressed the FoxP genes. Using En1-Cre; ZnG mice to indelibly mark the V1 interneurons, significant coexpression of both FoxP2$_l$ and FoxP4$_l$ with GFP (Fig 2b,e). This suggests that many, FoxP2+ and FoxP4+ lateral cells are V1s. In heterozygous Gata3-LacZ mice I did not observe FoxP2+/LacZ+ cells or FoxP4+/LacZ+ cells (Fig. 2c,f). Furthermore FoxP4 did not colocalize with Chx10, which is consistent with the FoxP4$_l$ cells being predominantly inhibitory (Fig 2g). Furthermore, the FoxP4 and FoxP2 lateral cells coexpress Lim1/2 a marker of V1,V0, and several others dI class neurons (data not shown), but not V2 interneurons Taken together these data suggest that the

majority of the FoxP2 and FoxP4 lateral neurons are V1 interneurons. Latent and inefficient recombination in the En1-derived neurons could account for the incomplete coexpression of FoxP2, FoxP4 with GFP.

My data here suggests that the FoxP genes marks a subset of V1s, however, there are currently few other markers that can be used to subdivide the V1 populations into molecularly and functionally defined subtypes. I quantified the percentage of FoxP2+/GFP+, FoxP4+/GFP+ and FoxP2+/FoxP4+/GFP+ cells. On average the FoxP's collectively marked ~27% of all En1-derived interneurons, where FoxP2 marked ~24% and FoxP4 marked ~20% and they are coexpressed in ~17% (Fig 8a). So far, two classes of V1 neurons have been identified. These are: 1) Ia inhibitory interneurons and 2) Renshaw cells (Sapir et al., 2004, Alvarez et al., 2005). We know that FoxP2 and FoxP4 do not mark Renshaw cells that express Mafb and Calbindin (Sapir et al., 2004; Stam et al., in preparation). While we do not know the identity of the FoxP2 and FoxP4 neurons, they may be Ia inhibitory INs as these cells are located in lamina VII where Ia inhibitory interneurons are found. Nonetheless, the expression of FoxP2/FoxP4 in the V1s allows us to genetically identify a subset of the V1s.

CHARACTERISTICS OF THE MEDIAL POPULATIONS

To examine the genetic characteristics of the medial FoxP populations, I identified

what other interneuron markers coexpress FoxP2 and FoxP4. Analysis in a heterozygous Gad67-GFP KI neural tube showed that $FoxP2_m$ cells are a heterogeneous population of excitatory and inhibitory interneurons while $FoxP4_m$ cells are predominantly excitatory (Fig 3a,f). I found that a subset of $FoxP2_m$ cells are derived from Dbx1 expressing progenitors, in a heterozygous Dbx1-LacZ KI, while $FoxP4_m$ cells don't express Dbx1 (Fig 3b,g). Furthermore, $FoxP2_m$ cells did not coexpress Evx1, a known marker for the ventral subpopulation of V0 interneurons ($V0_v$), indicating that FoxP2 marks a subset of the dorsal $V0_d$ interneurons (Fig. 3c). In order to quantify the number of V0s marked by FoxP2 at E12.5, I counted the number of FoxP2+/LacZ+ cells as well as the total number of LacZ+ cells that have exited the ventricular zone. On average over the brachial and thoracic cord I found that 19% of Dbx1-derived neurons are FoxP2+, indicating that FoxP2 marks a significant subset the $V0_d$'s (Fig 8b)

The di2 interneurons are also known to be ventrally migrating and are marked by FoxD3. It is known that the dorsally-derived, ventrally-migrating FoxP2 neurons coexpress FoxD3, a marker of di2 cells that settle ventro-medially (Stam et al., in preparation). The FoxP4/FoxD3 experiment could not be done due to both antibodies being generated from the same species.

Finally, another known class of ventro-medially migrating are the di6 interneurons. I found no colabeling of Dmrt3 or Wt1, two known di6 markers, with FoxP2 (Fig 3d,e). Furthermore, Lbx1 which is transiently expressed in di6s does not

coexpress FoxP2 **(**data not shown)**.** FoxP4 also did not show coexpression with Dmrt3 and few brachial $FoxP4_m$ cells coexpressed Lbx1. These data suggest $FoxP2_m$ cells are not di6s, and that $FoxP4_m$ interneurons comprise a small subset of di6 interneurons. Last, I checked for the possibility that $FoxP4_m$ cells could be V3s. In Sim1-Cre; ZnG double heterozygous E12.5 spinal cords, I found no FoxP4/GFP coexpression indicating that $FoxP4_m$ cells are not V3s (Data not shown)

In an attempt to better delineate the medial population in the context of both $FoxP2_m$ and $FoxP4_m$ cells, I analyzed FoxP2 and FoxP4 coexpression at E12 which demonstrates a dorsally-derived, ventrally migrating population that are FoxP2/FoxP4 positive. Colabeling FoxP2/FoxD3 shows these ventrally migrating cells to be di2 interneurons. By extrapolation via adjacent section analysis of FoxP2/FoxP4 staining, I concluded that the majority, if not all $FoxP4_m$ cells are di2s, most of which coexpress FoxP2 (Fig 1i). Furthermore, triple staining in a Dbx1-LacZ KI background with FoxP2/FoxP4 demonstrates that the majority of FoxP2+/Dbx1- medial cells are FoxP4 positive (Fig 3j). All of this together suggests $FoxP2_m$ cells comprise mainly a subset of $V0_d$'s and di2's and $FoxP4_m$ cells comprise predominantly di2s with few di6s. To summarize FoxP2 is upregulated from E11.5-E12 in a subset of post-mitotic $V0_d$’s, V1s, and di2s where expression of FoxP2 appears to persist until at least E12.5. Meanwhile, FoxP4 has already been independently upregulated in a small subset of brachial and thoracic MNs as early as E11.5 (Dasen et al., 2008) and immediately afterwards has a similar expression profile to FoxP2 in V1s, di2s and some di6s from E11.5-E12 (Fig 4).

COMBINATORIAL EXPRESSION OF FOXP1/FOXP2/FOXP4

A major issue of interest in the field of developmental spinal cord research is to further identify genetic markers that can subdivide the 5 cardinal classes of ventral interneurons into more diverse populations. I have shown that FoxP2/FoxP4 do indeed segregate several ventrally settling interneuron populations. However the ability of the FoxP genes to make heterotypic and homotypic dimers raises the possibility that they may be expressed differentially in these neuron populations and that different combinations could direct distinct transcriptional pathways, which could contribute to the development of phenotypically diverse subgroups. In order to analyze their combinatorial expression, I performed triple IHC stainings on wild-type E12.5 embryos with antibodies to FoxP1, FoxP2 and FoxP4. Consistent with previous findings, I found FoxP1 to have high expression in MNs at brachial and lumbar levels and significantly lower expression at thoracic levels. However, I also observed a low level of expression in ventral interneurons in all spinal cord segments. It is likely that this aspect of FoxP1 expression was previously overlooked due to the low level of expression. Furthermore, the interneuron populations marked by FoxP1, similar to FoxP2 and FoxP4, could be separated into a medial and lateral class.

Because FoxP1 is known to exhibit different expression patterns at different

spinal segments, this prompted me to analyze the expression patterns of FoxP2 and FoxP4 in the context of FoxP1 at brachial, thoracic and lumbar sections at E12.5. The results of the triple stains revealed high level of coexpression between all three genes, but a closer look reveals a highly combinatoric genetic code involving the three proteins (Fig 5 d,h,l)

To simplify the analysis, the coexpression of the three genes is described pairwise, two at a time. FoxP1/FoxP2 double staining at brachial and lumbar levels shows no coexpression between FoxP1 marked LMC MNs and FoxP2. Examination of the FoxP2 expressing cells on the whole shows a much higher relative count in both lateral and medial populations compared to FoxP1 and a significant subset of $FoxP2_l$ and $FoxP2_m$ neurons are coexpressed with a low-med level of FoxP1. However there are still single positive FoxP1 cells scattered both laterally and medially along the spinal cord (Fig 5a,e,i). Interestingly, thoracic segments show substantial and strong FoxP1/FoxP2 medial cell coexpression while brachial and lumbar sections show significantly less (Fig 5a,e,i). While laterally, the vast majority of $FoxP1_l$ cells coexpress FoxP2, and there are a substantial number of single-positive $FoxP2_l$ cells.

Analysis of FoxP1/FoxP4 reveals a similar expression pattern in the interneuron population. At the brachial level, we see FoxP4 expressed in a small subset of the FoxP1+ MNs. The MN population can be differentiated between the interneurons by their relative size. At lumbar levels FoxP4 also appears to be expressed in a subset of the FoxP1 LMC

MNs **(**Data not shown**)**. The majority of the lateral interneuron populations marked by either gene coexpresses the other with few single positive $FoxP1_l$ and $FoxP4_l$ cells at all levels (Fig 5b,f,j). The medial neuron populations also demonstrate substantial coexpression at thoracic and lumbar levels but significant numbers of single positive neurons for both genes exist (Fig 5b,f,j). Interestingly, at brachial levels, the double positive FoxP1/FoxP4 medial cells are present in relatively lower numbers and have comparatively more single positive cells (Fig 5b).

FoxP2/FoxP4 double staining again demonstrates a much higher relative count of total FoxP2 positive cells compared to FoxP4 and high coexpression between the two proteins. At brachial levels, the most noticeable difference is the subset of LMC MNs that are marked by FoxP4, and single positive $FoxP4_m$ cells; however most or all other FoxP4+ interneurons appear to costain with FoxP2 (Fig 5c). The trend is the same when observing the thoracic sections, high coexpression between the two genes at both lateral and medial locations with significant FoxP2 single positive cells and few FoxP4 single positive cells (Fig 5g). This trend is slightly different at lumbar levels, where there is an increased number of $FoxP4_m$ single positive cells, but by and large FoxP4+ cells coexpress FoxP2 (Fig 5k).

Evaluation of FoxP1/FoxP2/FoxP4 triple staining is more complex, but reveals similar results to double staining analysis. At brachial levels, the non-MN lateral cells predominantly express all three FoxP genes, with several single positive FoxP2 and

FoxP1 cells. Of particular interest is the apparent difference in protein levels as evidenced by the different signal intensities. Furthermore, the brachial medial populations show few to no triple labeled cells, few to no double labeled cells and significantly higher numbers of single positive neurons marked by each individual FoxP gene (Fig 5d). Descending caudally to thoracic levels, the same trend among lateral cells continues with the majority of FoxP labeled neurons expressing all three genes with significant FoxP2 single positive and some FoxP1 single positive cells. Medially, there are strongly labeled triple positive cells, some double labeled FoxP2+/FoxP4+ cells and a few FoxP1+/FoxP2+ cells with a large number of FoxP2+ single labeled cells, few to no FoxP1 and FoxP4 single positive cells (Fig 5h). At lumbar levels, the majority of the lateral FoxP+ presumptive interneuron population (differentiated by cell size) was again FoxP1+/FoxP2+/FoxP4+, with some single FoxP2+ interneurons as well as some single FoxP1+ interneurons. Medially, there are many single FoxP2+ cells, several FoxP1+ cells, significant FoxP2+/FoxP4+ cells, and some triple positive cells (Fig 5l).

The FoxP genes are known to form heterotypic and homotypic dimers (Li et al., 2004), with each combination potentially having the ability to form different protein complexes. Furthermore, FoxP1 dosage levels are known to determine the MN column phenotype. All of this expression analysis suggests a complex, combinatorial, dose-dependent code that could potentially be driving phenotypic differences in these neuronal populations. Finally, it has given us a method by which we can further define neuronal subtypes within the medial and lateral neuron populations.

UPSTREAM REGULATORS OF FOXP2/FOXP4

In order to investigate the transcriptional hierarchy the FoxP genes play in the spinal cord, I focused on several known transcription factors that are expressed in the various interneurons that are positive for FoxP2 and FoxP4. bHLHB5 is known to be expressed in the di6, v1 and v2 progenitor domains (Liu et al., 2007) suggesting it could be a determinant of FoxP2/FoxP4 expression. However, in bHLHB5 KOs, I observed no appreciable difference in expression pattern or cell counts, indicating that bHLHB5 expression isn't crucial for driving FoxP4 transcription (Fig. 6e,f, data not shown)

As a post-mitotic determinant of di4-di6 identity at the expense of di1-di3 fates, Lbx1 was a potential regulator of FoxP2 and FoxP4 expression in the dorsal interneurons. In Lbx1 mutants, I observed an ectopic expression of Foxp2 in the dorsal half of the spinal cord consistent with the finding that ectopic di2 interneurons are generated in the Lbx1 mutant (Gross et al 2002) (Fig 6c,d). This indicates expression of FoxP2 and FoxP4 in the di2 population is repressed by Lbx1.

Dbx1 plays a critical role in establishing the p0 domain as well as being a key determinant of the V0 fate; in Dbx1 mutants a significant portion of V0s become fated to V1s (Pierani et al., 2001). To address the possibility that Dbx1 is an upstream regulator of

FoxPs, I examined Dbx1 mutants. Upon initial inspection, the expression pattern in the Dbx1 KO did not differ much from Dbx1 heterozygous embryos, but a closer look revealed ectopic expression of FoxP4 and FoxP2 (Fig 6b,h). To better assess the effects of the loss of Dbx1 function, I quantified the numbers of $FoxP2_m$, $FoxP2_l$, $FoxP4_l$, $FoxP4_m$, cells at brachial and thoracic levels relative to a heterozygous control. In the lateral populations I noticed a significant increase in both $FoxP2_l$ and $FoxP4_l$ cells at both brachial and thoracic segments. Brachial $FoxP2_l$ cells averaged 76.4 in the KO versus 58 in the control while brachial $FoxP4_l$ cells averaged 57.9 in the KO versus 45 in the control. Similarly, thoracic $FoxP2_l$ cells averaged 65.3 in the KO versus 48.7 in the control, thoracic $FoxP4_l$ cells in the KO averaged 33.5 versus 25.3 in the control. This increased expression in the lateral population is consistent with Dbx1 mutants having increased numbers of V1s due to the absence of Dbx1's guidance towards a V0 fate. This finding also coincides with my observation that FoxP2 and FoxP4 lateral cells are primarily V1s. (Fig 9)

The medial FoxP2 and FoxP4 populations have mixed results. On average for FoxP4 medial cells, in the KO I counted an increase of 8.6 cells relative to control in brachial sections, and a decrease of 3.33 in thoracic sections. The increase in brachial $FoxP4_m$ cells could potentially be an increased expression of FoxP4 in the di6 neurons. For FoxP2, there was little to no difference in the brachially located $FoxP2_m$ cells; 30.9 versus 31.3 in the KO and control respectively. However, thoracic $FoxP2_m$ cells were noticeably lower in the KO compared to control: 28.33 to 36.5. Furthermore, I found

virtually no medially settling FoxP2+/Dbx1+ V0s in the Dbx1 KO, suggesting that the FoxP2+/Dbx1 cells became fated to a V1 phenotype. It is interesting to note that the number of $FoxP2_m$ cells at brachial levels remains the same in both the KO and control while few to none of the $FoxP2_m$ KO cells are Dbx1+, which suggests ectopically expressed FoxP2 in an undetermined population of $FoxP2_m$ cells (Figure 9).

The increase in the numbers of $FoxP2_l$ and $FoxP4_l$ in the Dbx1 KO is consistent with a subpopulation of the V0s being fated to V1s when lacking Dbx1. Furthermore, it suggests that Dbx1 is responsible for a reduction of FoxP2 and FoxP4 expression. However the presence of FoxP2+ V0s in a Dbx1-LacZ heterozygous spinal cord suggests a more complicated underlying transcriptional control than a simple Dbx1 repression of the FoxP2 and FoxP4 program. The slight increase of $FoxP4_m$ cells at brachial levels could represent ectopically generated di6s and would be consistent with my finding that FoxP4 is expressed in some di6 neurons at brachial levels. The maintained numbers of $FoxP2_m$ cells in a Dbx1 KO spinal cord is more difficult to interpret as one would expect a decrease in $FoxP2_m$ cells due to FoxP2+ V0s acquiring a V1 fate. It could perhaps reflect ectopic expression of FoxP2 in di6 interneurons when Dbx1 is absent.

FIGURES AND TABLES

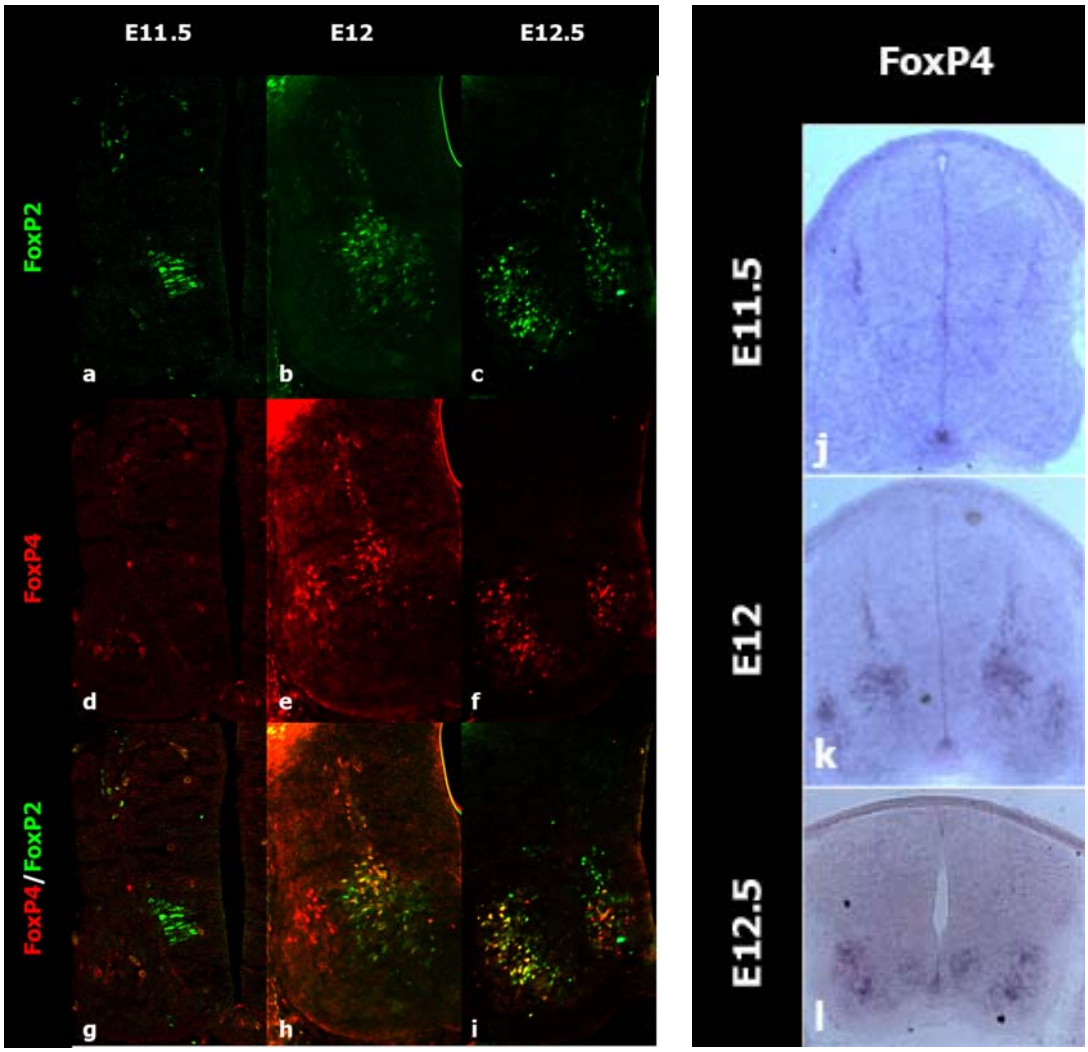

Figure 1. Time Course of FoxP2 and FoxP4 Expression

(a) FoxP2 expression pattern at E11.5.

(b) FoxP2 expression pattern at E12.

(c) FoxP2 expression pattern at E12.5.

(d) FoxP4 expression pattern at E11.5.

(e) FoxP4 expression pattern at E12 (note strong expression in MNs already present).

(f) FoxP4 expression pattern at E12.5.

(g,h,i) FoxP2/FoxP4 expression pattern at E11.5, E12, E12.5.

(j,k,l) FoxP4 mRNA expression at E11.5, E12, E14.5.

Table 1. FoxP Cell Counts at E12.5 at Different Segmental Levels

For embryo B: Brachial counts n = 8 hemisections, thoracic counts n = 12 hemisections, lumbar counts n = 6 hemisectionsFor embryo A: Brachial counts n = 8 hemisections, thoracic counts n = 14 hemisections, lumbar counts n = 8 hemisections

B embryo avg	$FoxP2_m$	$FoxP2_l$	$FoxP4_m$	$FoxP4_l$
Brachial	46.4	76.9	21.5	47.8
Thoracic	52.9	60.4	19.2	21.1
Lumbar	59.3	89.5	24.5	37.2
A embryo avg				
Brachial	44.4	59.6	21.4	34
Thoracic	55.7	63.9	25.8	28.4
Lumbar	69.8	96.3	31.4	45.5
A + B avg				
Brachial	45.4	68.3	21.4	40.9
Thoracic	54.4	62.3	22.8	25.1
Lumbar	65.3	93.4	28.4	41.9

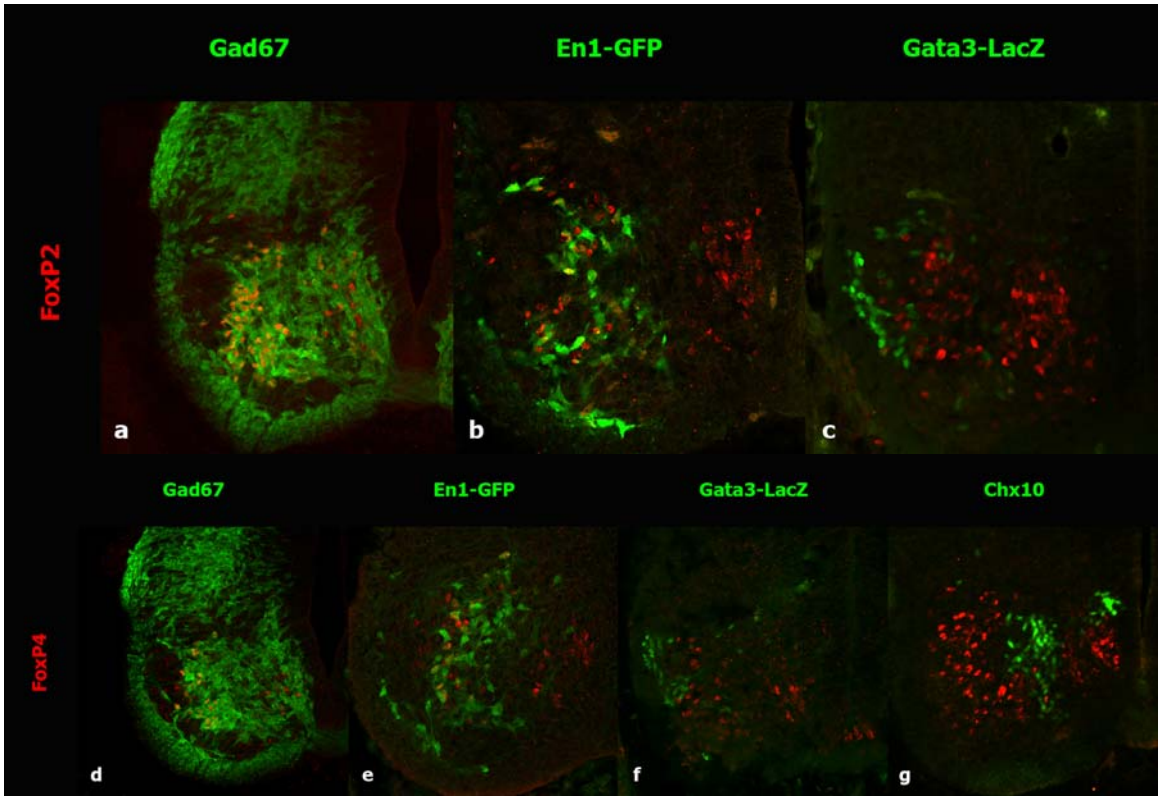

Figure 2. Lateral Population Characterization

(a,d) E12.5 Gad67-GFP heterozygous tissue with FoxP2 and FoxP4 staining. Note the lateral cells are predominantly inhibitory.

(b,e) E12.5 En1-Cre; ZnG double heterozygous background with FoxP2 and FoxP4 staining. Note that significant portions of both $FoxP2_l$ and $FoxP4_l$ populations are GFP+.

(c,f) E12.5 Gata3-LacZ heterozygous tissue with FoxP2 and FoxP4 staining. FoxP2 and FoxP4 do not coexpress Gata3.

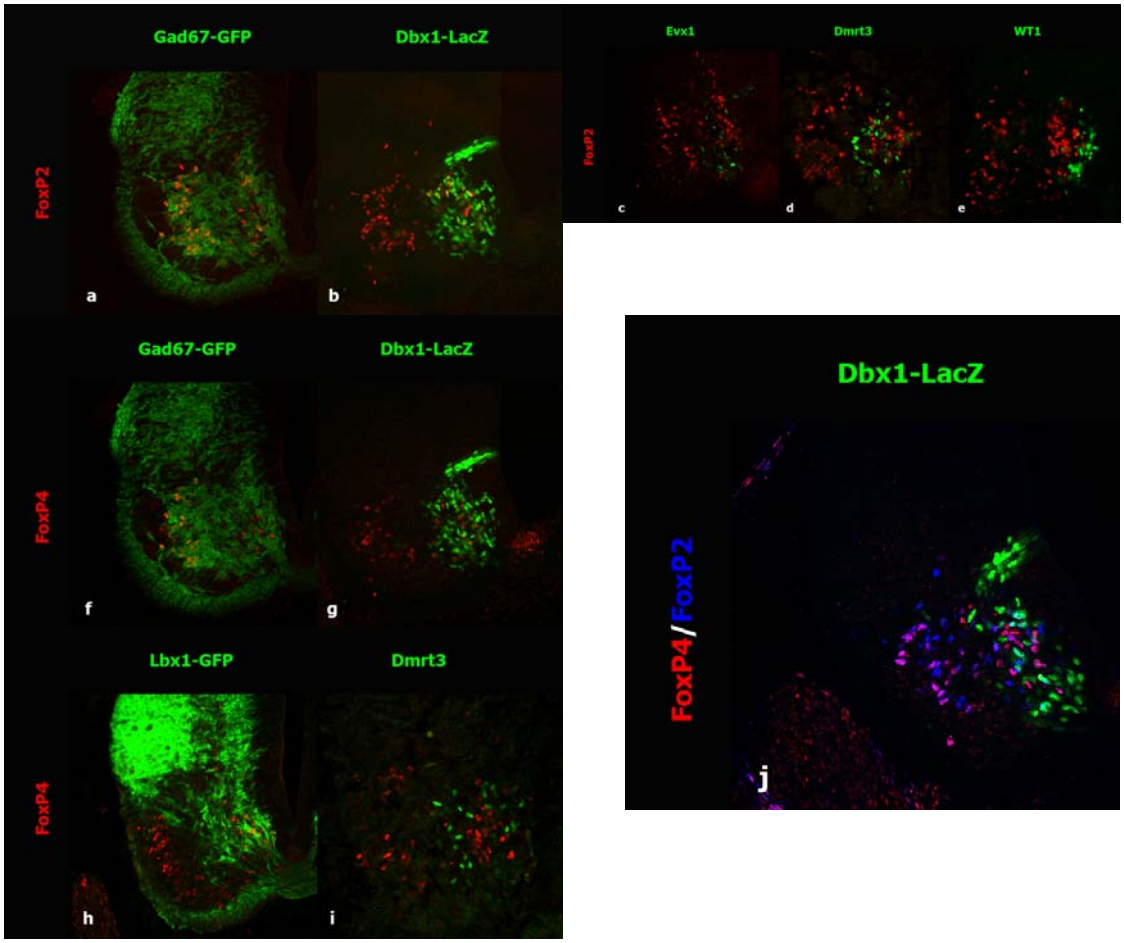

Figure 3. Medial Population Characterization

(a,f) E12.5 Gad67-GFP heterozygous tissue with FoxP2 and FoxP4 staining. Note that $FoxP2_m$ cells are a mixed inhibitory and excitatory population. $FoxP4_m$ cells are predominantly excitatory.

(b,e) E12.5 En1-Cre; ZnG double heterozygous background with FoxP2 and FoxP4 staining. Note that significant portions of both $FoxP2_l$ and $FoxP4_l$ populations are GFP+.

(c,f) E12.5 Gata3-LacZ heterozygous tissue with FoxP2 and FoxP4 staining. FoxP2 and FoxP4 do not coexpress Gata3.

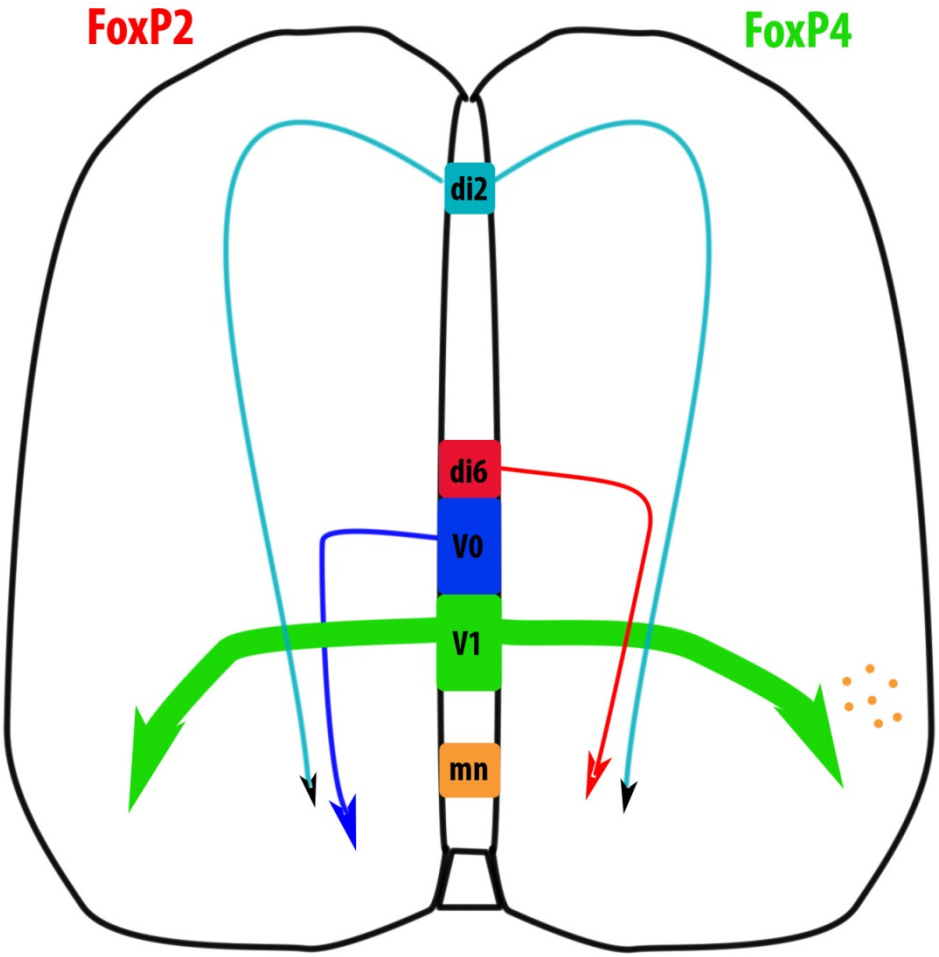

Figure 4. Schematic of FoxP2/FoxP4 Migration Pattern

FoxP2 and FoxP4 interneuron expression in the ventricular/subventricular zone begin at ~E11.5 until ~E12. Then they proceed along ventral migration routes and settle ventro-medially and ventro-laterally.

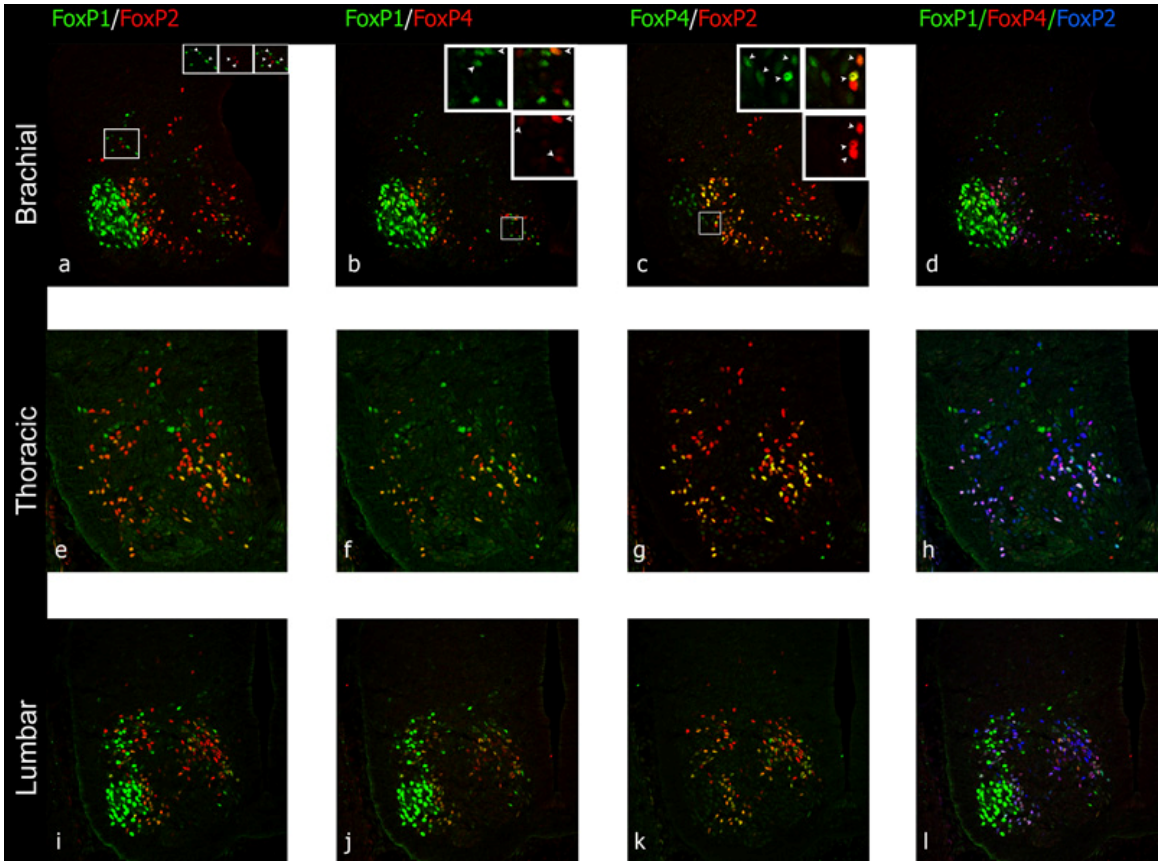

Figure 5. Differential and Coexpression of FoxP1/FoxP2/FoxP4 at E12.5

(a-d) Brachial section of E12.5 mouse neural tube with FoxP1, FoxP2 and FoxP4 staining at 25x. The three-channel image was deconstructed and recombined as three red/green images and the original triple staining. The insets suggest no cross-reactivity between specific FoxP antibodies with “sister” FoxP proteins.

(e-h) Thoracic section of E12.5 neural tube with FoxP1, FoxP2 and FoxP4 staining at 25x. Image was deconstructed in the same manner as a-d. A reduction in the number of FoxP+ neurons was observed throughout this section of the spinal cord. Substantial decrease in FoxP1 positive motor neurons, but high coexpression of the lateral populations between the three proteins was observed.

(i-j) Lumbar section of E12.5 neural tube with FoxP1, FoxP2, and FoxP4 staining at 25x. Note the reemergence of FoxP1+ motor neurons and an on overall increase in FoxP+ interneurons.

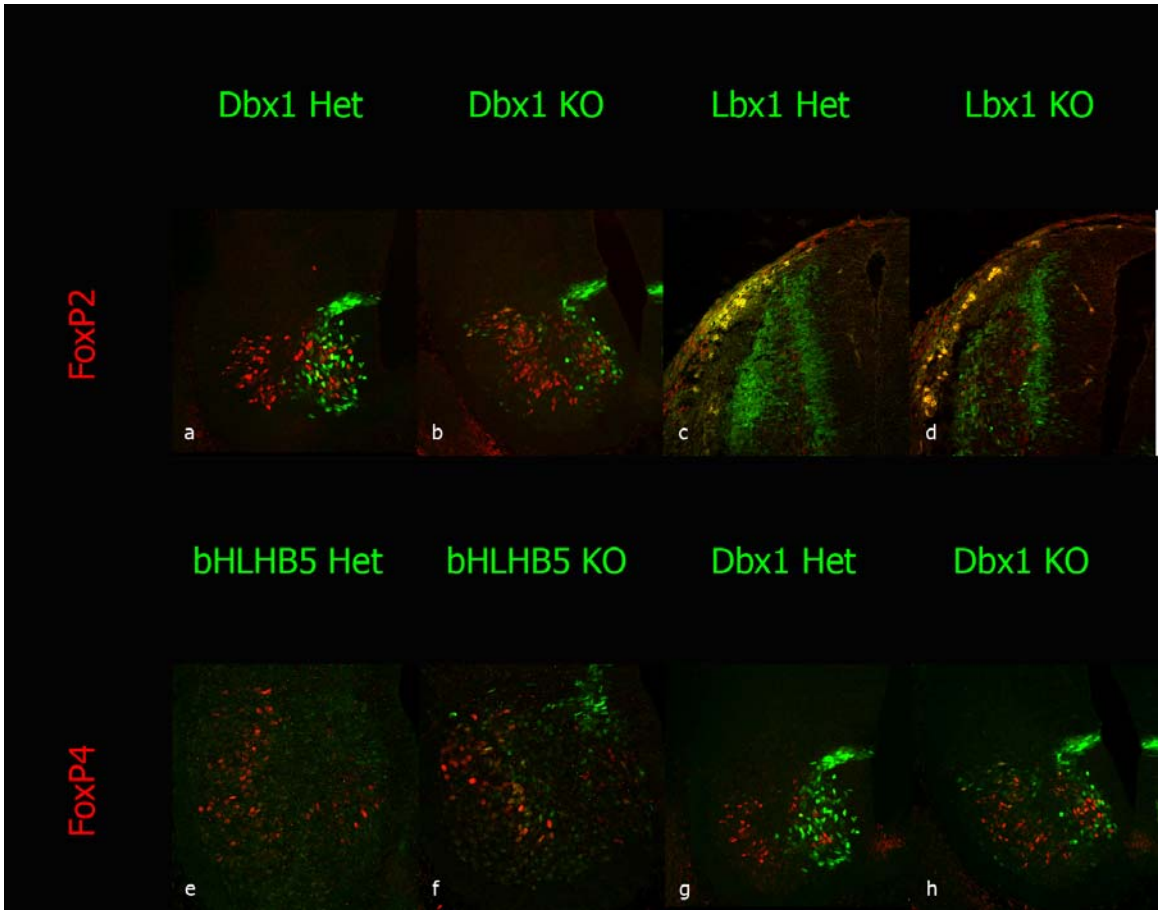

Figure 6. Upstream Regulators of FoxP2/FoxP4

(a,b) FoxP2 expression in E12.5 Dbx1$^{LacZ/+}$ and Dbx1$^{LacZ/LacZ}$ sections.

(c,d) FoxP2 expression in E12 Lbx1$^{GFP/+}$ and Lbx1$^{GFP/GFP}$ sections.

(e,f) FoxP4 expression in E12.5 bHLHB5$^{LacZ/+}$ and bHLHB5$^{LacZ/LacZ}$ sections.

(g,h) FoxP4 expression in E12.5 Dbx1$^{LacZ/+}$ and Dbx1$^{LacZ/LacZ}$ sections.

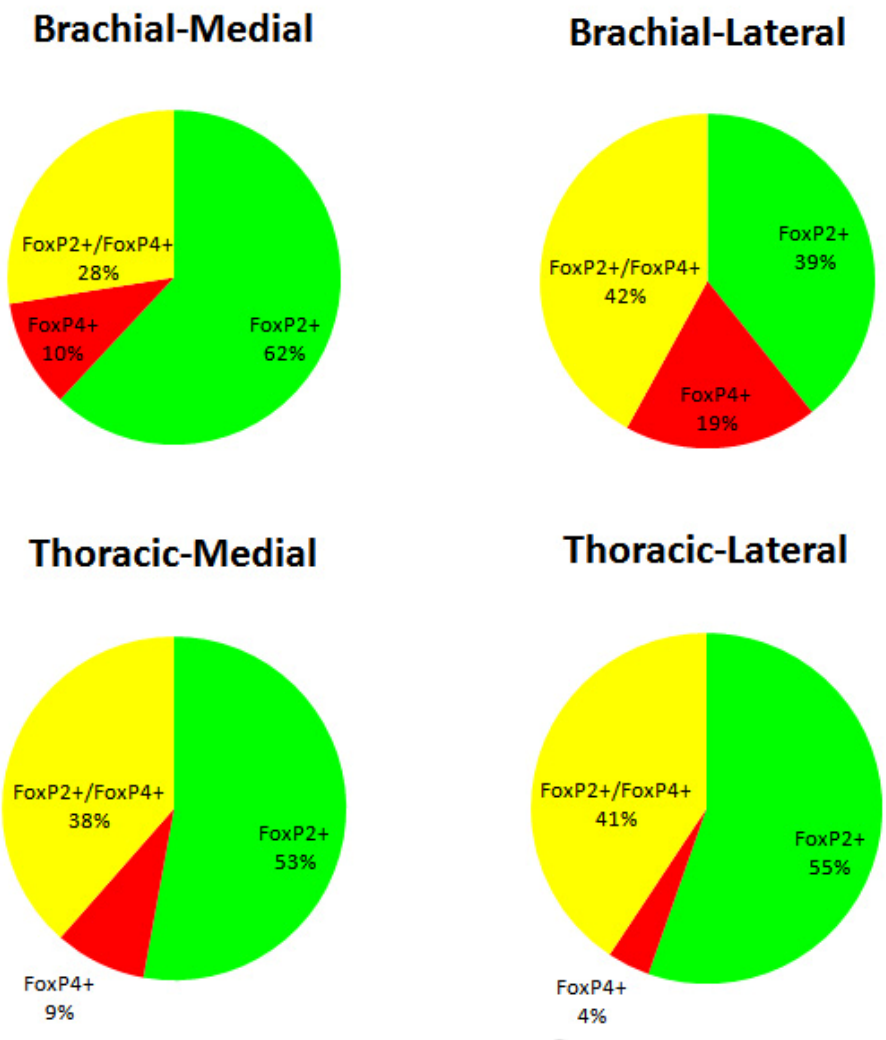

Figure 7. FoxP2/FoxP4 Coexpression at Brachial and Thoracic Levels

For brachial, n = 6 hemisections.
For thoracic, n = 11 hemisections.

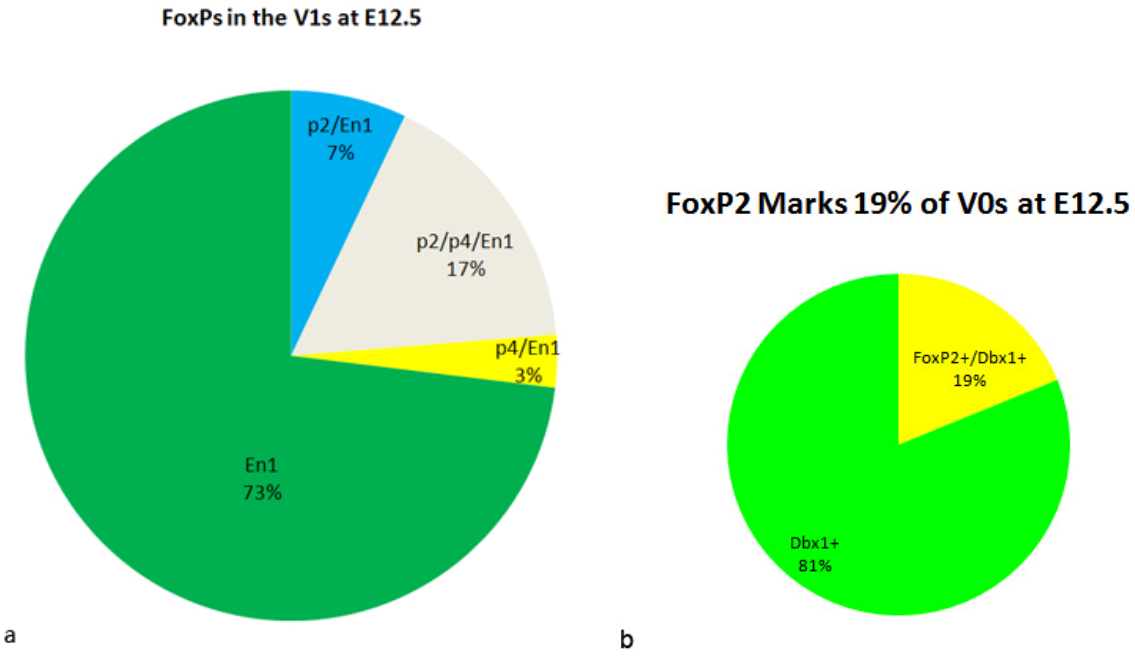

Figure 8. FoxPs Subdivide the V1s and V0s

(a) FoxP2 and FoxP4 are expressed in 24% and 20% of the V1 population at E12.5. FoxP2 and FoxP4 are coexpressed in 17% of the V1 population at E12.5.

n = 20 hemisections

(b) FoxP2 is expressed in 19% of the V0 population at E12.5.

n = 23 hemisections

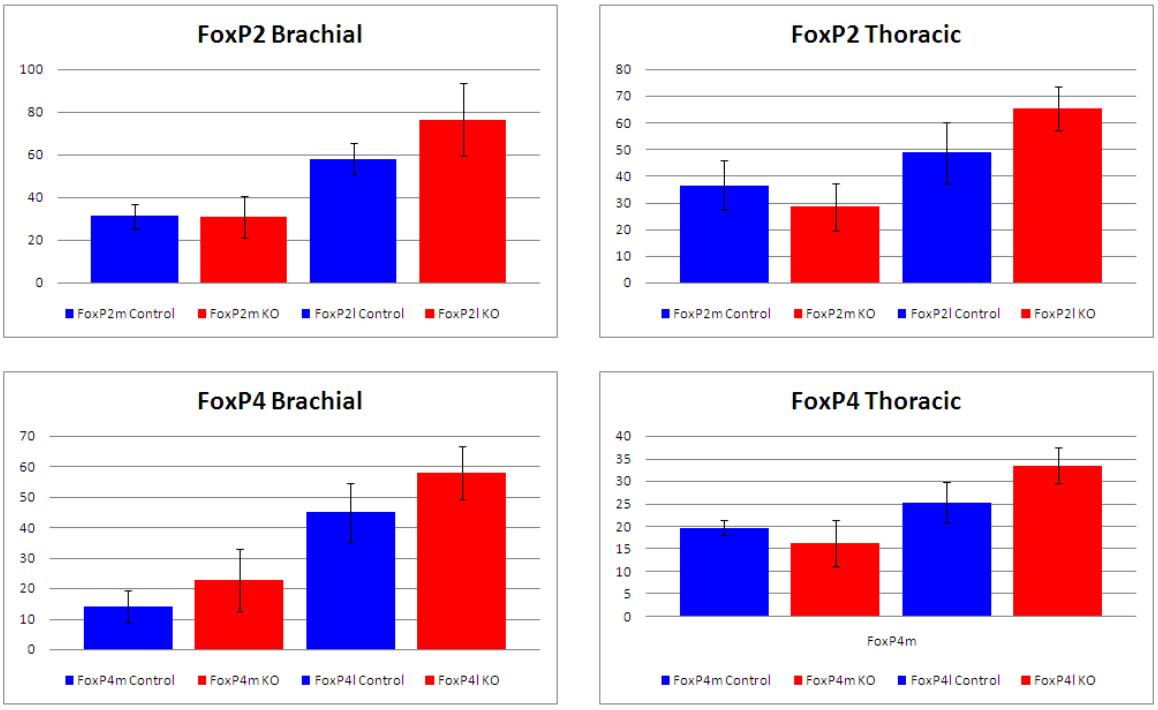

Figure 9. Dbx1 Mutants Ectopically Express FoxP2 and FoxP4

Note the lateral populations for both FoxP2 and FoxP4 all have significant increases compared to control.

Control counts: Brachial n = 4 hemisections, thoracic n = 6 hemisections

KO counts: Brachial n = 8 hemisections, thoracic n = 6 hemisections

Error bars indicate standard deviation

CHAPTER 2 - DISCUSSION

Research in the field of spinal cord development has progressed to a point where we've genetically defined 11 classes of neurons and have begun to make strides in understanding some of the complex anatomical connections these neurons make in general and how these classes contribute to locomotor behavior. My work with the FoxP genes helps put a piece of the complex genetic puzzle into perspective. So far most of the well-characterized transcription factors involved in specifying neuronal fates have been persistently expressed in the progenitor domains or in the post-mitotic neurons derived from a given progenitor domain. Here I present a family of genes that exhibit a tight temporal regulation in spinal cord development and have varied expression profiles in different spinal segments. Additionally they are expressed in distinct but partially overlapping patterns across multiple populations of neurons that have been derived from multiple progenitor domains. This result raises the possibility that the genetic control of neuronal diversity utilizes a highly combinatorial code of transcription factors that are differentially expressed in these populations.

A TIGHT WINDOW OF POSTMITOTIC EXPRESSION

I have demonstrated that the FoxP genes are likely markers of ventrally settling

post-mitotic interneurons. The expression of FoxP genes begins in the subventricular zone, specifically, adjacent to the p0,p1 domains in the developing spinal cord. FoxP expression in cells exiting the ventricular domain persists from ~E11.5 until ~E12 suggesting that there are factors temporally controlling the expression of the FoxP2/FoxP4 in the ventricular/subventricular zone. Systems that could explain such a tight temporal control of gene expression in the developing nervous system are as of yet unknown. However, it is known that differentiated neurons have the potential to send feedback signals to neuronal precursors that induce differential gene expression via the Notch signaling pathway (Namihira, 2009). Such a mechanism could explain the delayed upregulation of FoxP2/FoxP4 in differentiation neurons. In MN specification, temporally regulated phosphorylation of Ngn2 modulates differential transcriptional activity and works to drive different MN fates. Post-translational modification of upstream homeodomain or bHLH type proteins could also be crucial to controlling the temporal expression of the Fox genes (Ma et al., 2008). However the question as to how this entire process is temporally controlled still remains to be elucidated. The combinatorial specification of the progenitor domains in the spinal cord suggest that the interplay of many different factors on top of the classical dorsoventral and rostrocaudal patterning genes including growth factors, neurotrophic factors, and notch-like signaling could all be involved in the time-dependent regulation of FoxP gene expression in the spinal cord.

EXPRESSION PROFILE ALONG THE ROSTROCAUDAL AXIS

The fully developed spinal cord exhibits anatomical differences in size and in neuronal mapping along the rostrocaudal axis. Such an organization suggests that varied patterns of gene expression at specific segmental levels are needed to confer the many types of distinct neurons that are required to serve the myriad functional requirements of the spinal cord. Recent insights into the Hox factors that are expressed differentially along the anterioposterior axis, have been critical in pushing forward our understanding of spinal cord development. Very recent studies have shown FoxP1 activity is controlled by the combinatorial activities of different Hox genes throughout the different spinal segments (Dasen et al, 2008). A direct result of this is that FoxP1 has a varied rostrocaudal expression pattern, driving distinct MN column fates at different levels. Here I've shown that the FoxP genes, FoxP2 and FoxP4 also exhibit a varied expression pattern along the anterioposterior axis, perhaps suggesting that their upregulation could also be dependent on Hox activity. On top of a differential AP-axis expression profile, the Hox genes are also expressed in spatially distinct manners along the dorsoventral axis further meshing with the possibility that the Hox genes are responsible for FoxP2/FoxP4 activity given the different rostrocaudal profile of both medial and lateral FoxP populations (Dasen et al., 2003). Furthermore, increased expression at limb levels, suggests an important role of FoxP2 and FoxP4 expressing neurons in regulating complex movements such as locomotion.

POSSIBLE NEURONAL SUBTYPE SPECIFICATION

Given the discrete expression of FoxP genes in different classes of neurons it is possible they are playing a neuronal fating role. I have shown that the FoxP genes are expressed MNs, V1s, V0s and di2s. Moreover, they display a combinatorial profile of expression across the lateral and medial populations. The FoxP proteins share highly conserved forkhead DNA binding domains, zinc fingers and leucine zippers with an overall homology between them ranging from 40-60% (Teufel et al., 2003). The highly conserved forkhead domains and zinc fingers are known to be DNA binding domains. Unique to the FoxP subfamily is the well-conserved leucine zipper domain known to be the critical sequence that confers the FoxP genes their ability to homo and heterodimerize and without the leucine zipper domain, the FoxP proteins lose their ability to regulate transcription (Li et al., 2004a). The known characteristics of these regions suggest that the highly homologous regions of the FoxP proteins reside on the interior of the dimeric complexes. Such a setup could result in significantly different exterior tertiary structure, allowing for a recruitment of various distinct cofactors and consequently regulate transcription in a specific dimer depedent-manner.

Indeed, analysis of FoxP1/FoxP2/FoxP4 coexpression indicates a highly combinatorial expression profile in neurons expressing FoxP genes. There exist single positive cells for each of the genes, as well as combinations only two and all three. The combinatorial FoxP expression profile of the medial and lateral cells could very well be defining different genetic programs depending on which FoxP genes they express. To make matters more interesting, FoxP1 is known to regulate fates in a dose-dependent

manner in the spinal cord (Dasen et al., 2008), suggesting that on top of the combinatorial profile, the concentration of FoxP dosage in these cells could very well have a defining role in guiding different neuronal fates.

FoxP1 mutant embryos exhibit varied genetic and morphological phenotypes. The switched fating of LMC to HMC suggests a vital role of the FoxP genes in directing proper interneuronal fates (Rousso et al., 2008; Dasen et al., 2008). FoxP1 is also in some cases critical for the proper innervation and arborization at target muscles. A similar role for the FoxP genes in proper innervation and spinal circuit generation is a likely possibility. Moreover, the varied axial expression of Hox genes and the different genetic phenotypes they drive in conjunction with FoxP1 expression points to the differential FoxP interneuronal expression to be Hox-dependent as well. Segmental specific interneuron subtypes have yet to be characterized, varied FoxP expression in particular Hox contexts could very well be directing distinct neuronal characteristics along the anterior-posterior axis.

REGULATION OF THE FOXP GENES

The regulation of FoxP1 is highly context and Hox-dependent, where loss of function mutants of specific Hox genes leads to a downregulation of FoxP1 expression. However, the expression FoxP1 in MN columns is switched on in MNs that have already

become post-mitotic and have begun expressing MN specific factors like Isl1/2, Hb9 and the Hox-genes. In this case I demonstrate that FoxP2 and FoxP4 are temporally switched on in cells becoming post-mitotic, which suggests a different pathway of regulation in these particular neurons. I've shown that Dbx1, a dorsoventral patterning factor, does indeed play a role in the regulation of FoxP2 and FoxP4. Dbx1 is known to play a restrictive role in specifying V0s in conjunction with Dbx2 expression. The loss of Dbx1 allows the p1 and di6 domains to expand (Lanuza et al., 2004) where there is an increase in V1 and di6 interneuron generation. The ectopic expression of FoxP2 and FoxP4 in laterally migrating cells is consistent with the $V0_v$'s being fated to V1s and suggests that the transcriptional control of FoxP2 and FoxP4 in the V1 population could well depend on Dbx1 activity. Experiments with a Dbx2 KO would aid in understanding questions about how FoxP2 and FoxP4 are regulated. At first glance it would seem logical to suggest that Dbx1 represses the upstream factors of FoxP2 and FoxP4, but I have shown that FoxP2 is expressed in Dbx1+ cells. Therefore the mechanism is likely to be more complex. One potential explanation would be that other dorsoventral patterning factors that slightly encroach into the p0 domain and promote FoxP2 expression or cofactors could modify the effect of Dbx1 on FoxP2 expression between activation and repression. It is interesting to note that the numbers of $FoxP2_m$ cells does not change in the Dbx1 mutant, while $FoxP2_l$ cells exhibit a significant increase in numbers

Further evidence that dorsoventral patterning factors are critical in regulating FoxP2/FoxP4 expression was apparent in the Lbx1 mutant. Known to be a post-mitotic

repressor of di1-di3 fates and to promote di4-di6 fates, Lbx1 mutants exhibit ectopic expression of FoxP2 providing further evidence that ectopic di2s are generated in this mutant. This suggests that FoxP2 is downstream of factors that specify di2 fates specifically and that FoxP2 expression in the dorsal spinal cord is spatially restricted by Lbx1. Put together, the Lbx1 and Dbx1 loss of function data show that the temporal control of FoxP2 and FoxP4 expression is still intact and functioning in the absence of these critical patterning genes. Furthermore it indicates that the temporal control isn't defined in spatially segregated regions by unknown factors.

My research suggests that the FoxP genes could well be an example of important proteins that are a genetic intersection/link between differential dorsoventral factors and differential rostrocaudal factors, resulting in distinct axial subtypes. Higher expression profiles at limb levels suggest an important role in these FoxP interneurons in locomotion, and their combinatorial expression could be specifying distinct neuronal phenotypes. However many questions as to what specific roles the FoxP genes play in the intereurons remain to be asked. Genetic mutants in FoxP1 and FoxP4 are embryonic lethal due to developmental defects of vital organs, making such analysis difficult (Rousso et al., 2008; Li et al., 2004b). A way around this would be to generate floxed FoxP alleles, and generate context dependent FoxP KO's, for example with a Nestin-Cre or En1-Cre driver. There is very likely some level of compensatory activity between the similar genes further complicating these loss-of-function studies, which would require multiple FoxP dependent KOs. The FoxP genes are also expressed in a significant

population of V1s, which are known to be involved in the regulation of the speed of locomotor output. Characterization of these FoxP genes will allow for further genetic dissection of the V1s via Knock-ins of various molecular tools at these FoxP loci. There are many avenues to be explored regarding the FoxP+ interneurons, my work has paved the way for many future experiments.

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