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Hox Repertoires for Motor Neuron Diversity and Connectivity Gated by a Single Accessory Factor, FoxP1

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SUMMARY

The precision with which motor neurons innervate target muscles depends on a regulatory network of Hox transcription factors that translates neuronal identity into patterns of connectivity. We show that a single transcription factor, FoxP1, coordinates motor neuron subtype identity and connectivity through its activity as a Hox accessory factor. FoxP1 is expressed in Hox-sensitive motor columns and acts as a dose-dependent determinant of columnar fate. Inactivation of *Foxp1* abolishes the output of the motor neuron Hox network, reverting the spinal motor system to an ancestral state. The loss of FoxP1 also changes the pattern of motor neuron connectivity, and in the limb motor axons appear to select their trajectories and muscle targets at random. Our findings show that FoxP1 is a crucial determinant of motor neuron diversification and connectivity, and clarify how this Hox regulatory network controls the formation of a topographic neural map.

INTRODUCTION

The versatility of motor behaviors relies on the ability to activate, on demand, a select few of the many hundred skeletal muscle groups. Motor neurons (MNs) lie at the core of this action plan. Each muscle is innervated by a dedicated set of MNs, and many of the inputs to the spinal cord are designed to activate MNs in temporal patterns that meet the mechanical requirements of motor performance. How the neurons that implement these motor programs are assembled into functional circuits remains poorly understood.

During development spinal MNs segregate into discrete columns, each innervating a different peripheral domain (Figure 1A). Median motor column (MMC) neurons innervate axial muscles, hypaxial motor column (HMC) neurons innervate body wall muscles, preganglionic motor column (PGC) neurons innervate sympathetic ganglia, and lateral motor columns (LMC) innervate limb musculature (Fetcho, 1992; Gutman et al., 1993; Landmesser, 2001). Within these columns, MNs exhibit finer-grained positional identities that are also matched with the location of their targets (Laskowski and Sanes, 1987; McHanwell and Biscoe, 1981; Gutman et al., 1993). The developmental logic that underlies nerve-muscle connectivity is best understood for neurons of the LMC, which acquire divisional and pool identities that determine their axonal trajectory and muscle target within the limb (Jessell, 2000).

The construction of this topographic motor map is directed by molecular programs that link neuronal subtype identity and target innervation (Figures 1B and 1C). Graded Hedgehog and Wnt4/5 signals control the dorsoventral expression of homeodomain (HD) transcription factors that specify MMC and HMC fates (D. Agalliu and T.M.J., unpublished data; Briscoe and Ericson, 2001). In contrast, PGC and LMC neurons are specified by a rostrocaudal FGF signaling gradient that establishes regional domains of Hox transcription factor activity: Hox6 paralogs specify brachial LMC neurons, Hox9 paralogs specify PGC neurons, and Hox10 proteins specify lumbar LMC neurons (Dasen et al., 2003; Shah et al., 2004; Wu et al., 2008). Within the LMC, a more intricate network, built from over a dozen Hox proteins, imposes discrete motor pool identities (Dasen et al., 2005). Hox proteins appear to be primary determinants of MN connectivity since changing the profile of Hox expression in specific pools results in corresponding changes in the pattern of muscle innervation (Dasen et al., 2005). This core Hox network appears to direct motor innervation patterns by activating a diverse array of downstream transcription factors and cell surface receptors (De Marco Garcia and Jessell, 2008; Livet et al., 2002; Kania and Jessell, 2003).

Evidence for a Hox-dependent program of MN columnar specification is supported by phylogenetic studies which indicate that motor columns induced by the dorsoventral and rostrocaudal signaling pathways emerged at different stages of evolution, in parallel with the elaboration of peripheral target structures. Early aquatic vertebrates with simple locomotor behaviors that are driven by axial and hypaxial muscles appear to possess neurons of MMC and HMC character but lack PGC and LMC neurons (Fetcho, 1992; Kusakabe and Kuratani, 2005). The appearance

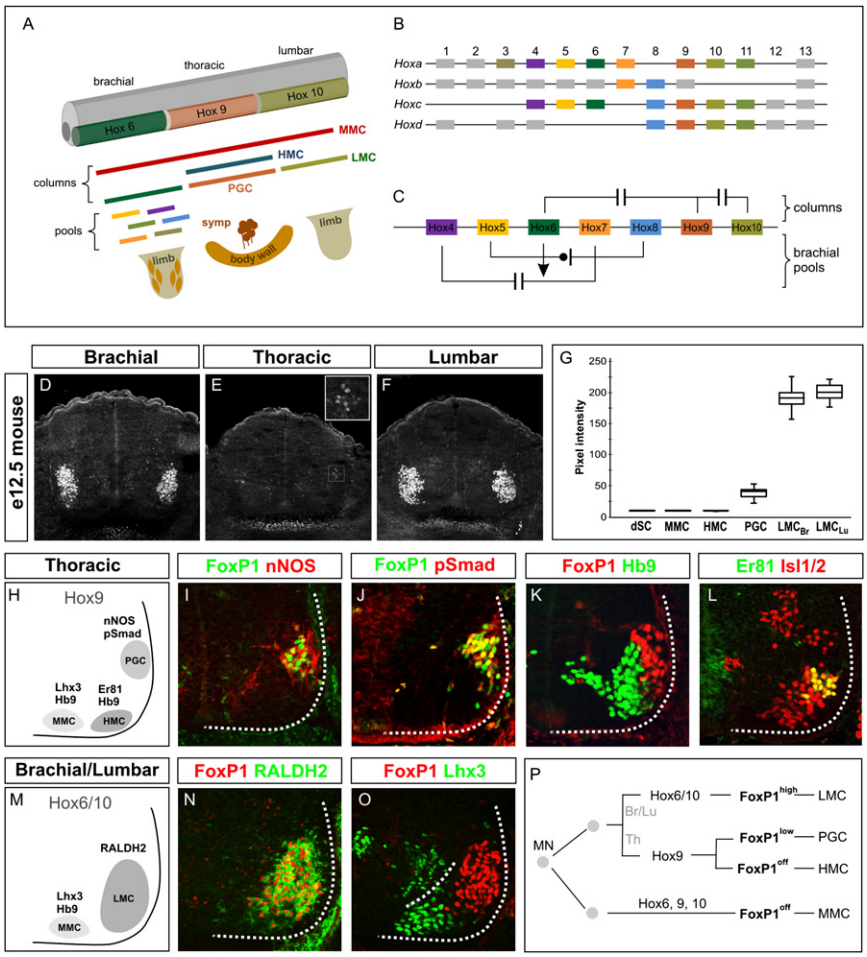

Figure 1. Expression of FoxP1 in Hox-Dependent Motor Columns

(A) Organization of Hox proteins, motor columns and pools. MMC: median motor column; HMC: hypaxial motor column (after Fetcho, 1992, formerly lateral MMC; Gutman et al., 1993); PGC: preganglionic motor column; LMC: lateral motor column. Symp: sympathetic chain ganglion neurons.

(B) 21 Hox proteins assign spinal MN identity.

(C) Hox interactions specifying MN identity (Dasen et al., 2005).

(D–F) FoxP1 in e12.5 spinal cord. Inset: magnified view.

(G) FoxP1 levels in motor columns and dorsal spinal cord (dSC). Similar *Foxp1* mRNA levels are detected (Figures S1K–S1P).

(H) Profile of columnar subtypes in thoracic (Th) spinal cord.

(I–L) FoxP1 and markers of Th motor columns.

(M) Markers of MN columnar subtypes at brachial (Br) and lumbar (Lu) levels.

(N and O) Expression of FoxP1 in RALDH2$^+$ LMC neurons but not Lhx3$^+$ MMC neurons or V2 neurons. Dotted line in L separates MNs and interneurons.

(P) FoxP1 and Hox expression in MN columns.

of LMC and PGC neurons is linked to the formation of paired appendages (lateral fins and limbs) and a sympathetic nervous system - structures that emerged later in vertebrate evolution (Fetcho, 1992; Freitas et al., 2006; Funakoshi and Nakano, 2007). The Hox-dependent program of MN diversification may therefore have evolved to meet the demands of a new and diverse set of peripheral target tissues, and more elaborate motor behaviors.

The workings of the motor neuron Hox regulatory network remain obscure. One puzzle is that Hox proteins are expressed by all spinal MNs, yet PGC and LMC neurons alone depend on Hox function (Dasen et al., 2003), implying a constraint on Hox activity within certain motor columns. It is also unclear whether Hox programs of columnar specification involve common downstream effectors. Consequently, there has not been an effective analysis of the contribution of the Hox network, as a whole, to the formation of topographic motor maps. We reasoned that insight into these issues might emerge from an examination of the spinal motor system under conditions in which the entire Hox-dependent program of MN subtype differentiation has been inactivated, while leaving intact the Hox-independent program. Such a perturbation might consign spinal MN differentiation to an ancestral vertebrate state, yet encase these primitive neurons in a body that is hundreds of millions of years more advanced.

Analysis of patterns of motor innervation in such atavistic chimeras could reveal how Hox regulatory networks enable MNs to innervate their targets with specificity. Achieving this condition through direct genetic perturbation of Hox genes is unrealistic given the involvement of so many. We therefore searched for other ways to disable the Hox-dependent program of MN specification.

Hox proteins typically rely on transcriptional cofactors that refine and constrain their activities (Mann and Affolter, 1998). Two *Drosophila* cofactors, Extradenticle [Exd] and Homothorax [Hth] (Meis and Pbx/Prep proteins in vertebrates), have pervasive roles as regulators of Hox activity (Mann and Affolter, 1998; Moens and Selleri, 2006). In the spinal cord Meis and Pbx/Prep proteins have broad patterns of expression (Dasen et al., 2005), and thus they are unlikely candidates as cell-type specific regulators of MN Hox activity. Recent studies in *Drosophila* have identified a distinct group of Hox 'accessory' factors, notably the HD protein Engrailed and the forkhead proteins Slp1/2 which work together with Exd and Hth to gate Hox activities (Gebelein et al., 2004). These factors have more restricted domains of expression and activity: Engrailed regulates Hox activity in posterior compartment cells whereas Slp1/2 regulate Hox activity in anterior cells (Gebelein et al., 2004). Their vertebrate counterparts, the Engrailed and Fox proteins, are expressed by subsets of spinal neurons (Jessell, 2000; Tamura et al., 2003), but their function and potential roles as regulators of Hox activity have not been defined.

We have explored the role of accessory factors as regulators of Hox-dependent programs of spinal MN differentiation,

focusing on FoxP proteins. Our findings reveal that FoxP1, a transcription factor with functions in cardiac and hematopoetic development (Wang et al., 2004; Hu et al., 2006), controls the output of the entire Hox-dependent program of spinal MN diversity and connectivity.

RESULTS

FoxP1 Is Restricted to Hox-Sensitive Motor Columns

To explore whether Fox and Hox proteins work together in the specification of MN subtype identity we defined *Fox* genes expressed by MNs. Analysis of forty-one *Fox* genes in embryonic mouse spinal cord revealed that three members of the FoxP sub-family, FoxP1, FoxP2, and FoxP4, are expressed by ventral neurons. FoxP2 is expressed by interneurons but not MNs, FoxP4 is expressed transiently by a subset of MNs, whereas FoxP1 is expressed by MNs at brachial, thoracic and lumbar levels of the spinal cord (Figures 1D–1F and Figures S1A–S1G available online). We have focused on the role of FoxP1 as a potential regulator of Hox activity during MN differentiation.

To determine whether FoxP1 is restricted to columnar classes of MNs we compared its expression with that of Hox proteins and other markers of columnar subtype, from e11.5 to e14.5. This analysis revealed that FoxP1 is expressed selectively by LMC and PGC neurons, and that its onset of expression occurs after Hox proteins but before other markers of columnar differentiation (Figures 1H–1O and S2A–S2D). In brachial and lumbar spinal cord, FoxP1 is detected at high levels in LMC neurons, defined by retinaldehyde dehydrogenase-2 (RALDH2) expression (Figure 1N) Brachial FoxP1^{+} LMC neurons coexpress Hoxa6 and Hoxc6, whereas lumbar FoxP1^{+} LMC neurons express Hoxd10 (Figures S3A, E; data not shown). In thoracic spinal cord, the domain of Hoxa9 and Hoxc9, FoxP1 is detected at low levels by PGC neurons, defined by phospho-Smad1/5/8 (pSmad) and neuronal nitric oxide synthase (nNOS) expression (Figures 1I, 1J, and S3C). In contrast, FoxP1 is excluded from MMC neurons, defined by expression of Hb9, Isl1/2 and Lhx3, as well as from HMC neurons, defined by Hb9 and Isl1/2 expression in the absence of Lhx3 (Figures 1K, 1O, and S3H). A similar columnar profile of FoxP1 expression is detected in developing chick spinal cord (Figures S1H–S1J and S4A–S1I). Thus FoxP1 marks Hox-dependent motor columns.

We also examined, more quantitatively, the level of FoxP1 expression in MNs. We found that the level of FoxP1 in the nuclei of LMC neurons is ~6-fold greater than that in PGC neurons (Figure 1G; $p < 0.001$). The difference in FoxP1 expression level is evident at the onset of MN differentiation (~e9.5) and persists until at least e14.5. Thus, LMC neurons constitute a FoxP1^{high}, and PGC neurons a FoxP1^{low}, population (Figure 1P).

Hox and Homeodomain Activities Determine the Columnar Profile of FoxP1

To determine whether FoxP1 expression in MNs depends on Hox activity we used chick electroporation to express engrailed repressor (EnR) derivatives of Hox proteins that permit MN differentiation, yet block the emergence of PGC and LMC identities (Dasen et al., 2003). We examined the impact of EnR-Hoxc6 on FoxP1 expression in brachial MNs, and of EnR-Hoxc9 on FoxP1 expression in thoracic MNs. MNs generated after brachial expression of EnR-Hoxc6 failed to express FoxP1 (Figures 2A and 2C), or RALDH2 (Figures S5A–S5D). The HD profile of this novel set of brachial MNs (Isl1/2^{+}, Hb9^{+}, Lhx3^{off}) matches that of HMC neurons. Similarly, MNs generated after thoracic expression of EnR-Hoxc9 lacked FoxP1 (Figures 2B,C) as well as PGC markers (Dasen et al., 2003), and the number of neurons with an HMC profile was increased (Figures S5E-S5H). Thus Hox activity is required for the expression of FoxP1 in MNs, and the loss of PGC and LMC identity after Hox blockade is accompanied by the appearance of MNs with an HMC profile.

Is the difference in FoxP1 level in PGC and LMC neurons determined by Hox paralog expression? To assess this, we altered the profile of Hox proteins along the rostrocaudal axis of chick spinal cord and examined whether the interconversion of columnar identities is accompanied by changes in FoxP1 expression level. Thoracic misexpression of Hoxc6 or Hoxd10, which represses Hox9 proteins and elicits a switch from PGC and HMC to LMC columnar fates (Dasen et al., 2003; Figures S5I and S5L; data not shown), induced FoxP1 expression at ~5-6 fold greater levels than that in non-electroporated MNs, close to the level normally detected in LMC neurons (Figures 2D-I). Conversely, brachial expression of Hoxc9, which represses Hox6 proteins and generates PGC neurons at the expense of LMC neurons (Dasen et al., 2003), reduced the level of FoxP1 ~5-fold, to values approaching those of PGC neurons (Figures 2J–2L). Ectopic expression of Hox6/9/10 proteins did not induce FoxP1 in Lhx3^{+} MMC MNs (Figures S5J and S5L, and data not shown). These findings provide evidence that the difference in level of FoxP1 in PGC and LMC neurons is set by the Hox paralog profile.

We next examined why FoxP1 expression is excluded from MMC and HMC neurons, despite expression of Hox proteins (see Figure S3). Lhx3 has been implicated in the assignment of MMC fate (Sharma et al., 2000), and Hb9 in the suppression of PGC fate (William et al., 2003). We found that ectopic Lhx3 prevented FoxP1 expression in brachial and thoracic MNs and that ectopic Hb9 repressed FoxP1 expression in prospective PGC neurons (Figures 2M–2R and S5M–S5P). These findings show that the selective expression of FoxP1 by PGC and LMC neurons is achieved through the convergence of rostrocaudal and dorsoventral transcriptional programs: the inductive activity of Hox paralog proteins in concert with a restrictive influence of HD proteins that assign MMC and HMC fates (Figure 2S).

FoxP1 Level Determines Motor Neuron Columnar Identity

Does the level of FoxP1 expression in MNs determine PGC and LMC identities? We first asked whether ectopic expression of FoxP1 in thoracic MNs converts prospective HMC neurons to a PGC fate, and whether an increase in FoxP1 level converts HMC and PGC neurons to a LMC fate. We used *Hb9* and *CAGGs* promoters to generate *Foxp1* transgenic mouse and chick embryos in which the number of thoracic MNs expressing FoxP1 is increased from ~25% to ~70% (Figures S7A and S7R). Founder analysis of e12.5 *Hb9::Foxp1iresGFP* transgenic mice revealed that the level of FoxP1 in individual thoracic MN nuclei was ~3-fold greater than that detected in wild-type PGC

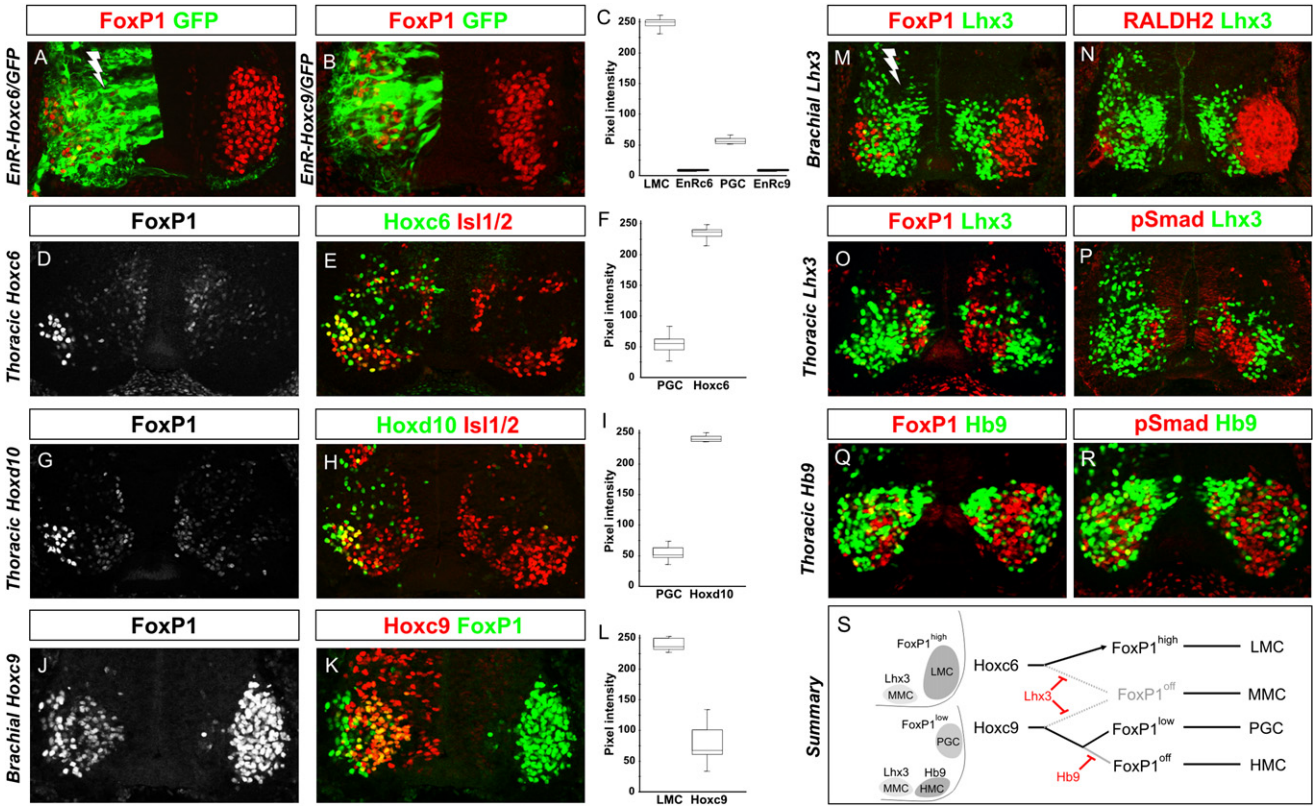

Figure 2. Induction and Restriction of FoxP1 Expression in Motor Neurons

Regulation of FoxP1 expression in chick MNs analyzed by electroporation of genes at stages 14–17 and analysis at stages 26–30.

(A) Expression of EnR-Hoxc6 in brachial (Br) spinal cord prevents FoxP1 expression in MNs. GFP marks neurons that express EnR-Hoxc6. Bolt: electroporated side.

(B) Thoracic (Th) EnR-Hoxc9 expression abolishes FoxP1 in MNs.

(C) Impact of expression of EnR-Hox fusions on FoxP1 levels. FoxP1 levels in the nuclei of GFP$^+$ MNs (labeled EnRc6 and EnRc9) and non-electroporated neurons (LMC and PGC).

(D–F) Expression of Hoxc6 in Th MNs elevates nuclear FoxP1 level.

(G–I) Th Hoxd10 expression elevates nuclear FoxP1 level.

(J–L) Br Hoxc9 expression reduces FoxP1 level. C, F, I, L show nuclear FoxP1 levels from $\geq$ 50 MNs, from electroporated and control sides.

(M and N) Br Lhx3 blocks FoxP1 and RALDH2.

(O and P) Th Lhx3 blocks FoxP1 and pSmad. Lhx3 does not change Br Hoxc6 or Th Hoxc9 (Figures S5M and S5N).

(Q and R) Th Hb9 blocks FoxP1 and pSmad. Expression of Hb9 at limb levels does not block FoxP1 or LMC fate (Figures S5O–S5P).

(S) Interactions of Hox, Hb9, and Lhx3 control MN FoxP1.

neurons (Figures S6A–S6C). In chick, electroporation of a diluted *CAGGs::Foxp1* (*Foxp1$^{[low]}$*) plasmid directed FoxP1 expression at low levels, similar to that of PGC neurons, whereas *Hb9::Foxp1iresGFP* and high concentrations of *CAGGs::Foxp1* (*Foxp1$^{[high]}$*) resulted in expression of FoxP1 at levels ~7-fold greater than that in PGC neurons (Figure S6 and data not shown). Expression of these constructs did not impair general features of MN differentiation, nor did it change the profile of Hox expression (Figures 3N, S7C–S7F, and S7S).

Analysis of columnar fates in the thoracic spinal cord of *Hb9::Foxp1iresGFP* mouse embryos revealed a doubling in the number of pSmad$^+$, and nNOS$^+$ PGC neurons, compared to controls (Figures 3A–3D and 3K). In addition, a few MNs expressed the LMC marker RALDH2 (Figures 3E, 3F, and S7G–S7L). Conversely, the number of neurons with an HMC profile (Isl1/2$^+$, Hb9$^+$, Lhx3^{off}) was markedly reduced (Figures 3K and S7O–S7R). In mouse, ~40% of HMC neurons express the ETS protein Er81 (Figure 1L) (Cohen et al., 2005), and this subset was similarly reduced in *Hb9::Foxp1iresGFP* embryos (Figures 3G, 3H, and 3K). In contrast, the number of Lhx3$^+$ MMC neurons was unchanged (Figures 3I–3K), and the motor nerve projecting to axial muscles was maintained (data not shown). We next compared the impact of FoxP1 dosage on thoracic MN differentiation in chick embryos. Expression of *CAGGs::Foxp1$^{[low]}$* elicited a 50% increase in the number of PGC neurons (Figures 3L and S6F–S6H). In contrast, *Hb9::Foxp1iresGFP* and *CAGGs::Foxp1$^{[high]}$* markedly reduced the number of pSmad$^+$ PGC as well as Hb9$^+$, Isl1/2$^+$, Lhx3^{off} HMC neurons (Figure 3M; data not shown) and induced many more RALDH2$^+$ LMC neurons (Figure 3O). These findings provide evidence that incremental changes in the level of FoxP1 in thoracic MNs result in a step-wise 'HMC to PGC to LMC' interconversion of columnar fate (Figure 3R), under conditions in which Hox paralog profiles are unchanged.

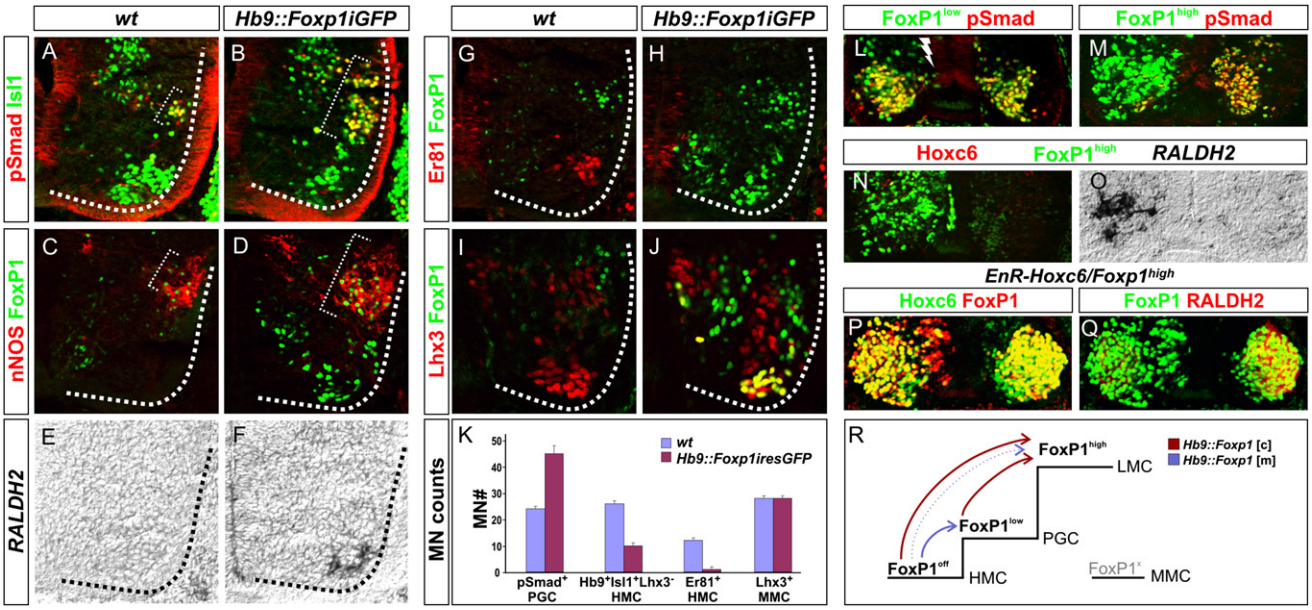

Figure 3. FoxP1 Induces PGC and LMC Identity

(A–K) Thoracic MN differentiation in e12.5-e13.5 *Hb9::Foxp1iresGFP* embryos.

(A–D) Expression of FoxP1 increases the number of nNOS$^+$ and pSmad$^+$ neurons.

(E and F) FoxP1 induces *RALDH2* in a few Th MNs, close to Lhx3$^+$ MNs (see also Figure S7).

(G and H) Ectopic FoxP1 blocks Er81.

(I and J) Expression of FoxP1 has no effect on Lhx3$^+$ neurons.

(K) MN columnar identity after Th FoxP1 expression. Similar results obtained in 3 founder embryos. Mean ± SEM/ventral quadrant/15 μm section. Experimental and control embryos differ at $p < 0.01$.

(L–O) FoxP1 expression in chick Th spinal cord. (L) More pSmad$^+$ neurons after expression of FoxP1 at low levels. (M) Loss of pSmad$^+$ neurons after expression of FoxP1 at high levels. (N) FoxP1 does not induce Hoxc6. (O) FoxP1^{high} expression induces *RALDH2*.

(P and Q) Lack of RALDH2 after expression of FoxP1 under *CAGGs* control in the presence of EnR-Hoxc6.

(R) MN columnar fate after Th elevation of FoxP1 expression in chick [c] and mouse [m].

Is FoxP1 simply a Hox intermediary, or an accessory factor that functions together with ongoing Hox activity? To resolve this issue we sought to eliminate Hox activity while maintaining FoxP1 expression. We expressed a EnR-Hoxc6 construct which blocks Hox6 activator functions at brachial levels (Dasen et al., 2003), and in addition expressed *CAGGs::Foxp1$^{[high]}$*, to compensate for the loss of endogenous FoxP1 caused by repression of Hox6 activity. If FoxP1 functions as a Hox intermediary, its target, *RALDH2*, should be expressed robustly despite the loss of Hox activity. Conversely if FoxP1 requires ongoing Hox activity, the expression of *RALDH2* should be abrogated despite high level FoxP1 expression. *RALDH2* expression was drastically reduced in brachial MNs, despite evident high level FoxP1 expression (Figure 3P,Q). This finding reveals an ongoing requirement for Hox activity during the FoxP1-dependent assignment of LMC identity, and indicates that FoxP1 and Hox proteins act in a convergent manner to specify MN columnar fates.

Switches in Columnar Fate and Connectivity in *Foxp1* Mutants

To test the requirement for FoxP1 activity in the assignment of MN columnar fates we analyzed Hox expression profiles, MN subtype identity and connectivity in *Foxp1* mutant mice.

In e11.5 *Foxp1* mutants the expression of Hb9, Isl1/2, and the cholinergic marker *VAChT,* was similar in *Foxp1* mutant and wild-type embryos (Figures S8A-H). Thus, FoxP1 is not required for the emergence of generic MN characteristics. Moreover, the profile of Hox4 to Hox10 paralog expression was unchanged in *Foxp1* mutants (Figures S8I-V). Thus Hox protein expression regulates, but is not itself regulated by, FoxP1.

We next analyzed how the loss of *Foxp1* activity influences MN columnar differentiation. At thoracic levels we detected a > 90% reduction in PGC neurons, assessed by the absence of dorsal Isl1$^+$ MNs, as well as by the loss of pSmad and nNOS expression (Figures 4A–4F and 4K; data not shown). At brachial and lumbar levels we detected a > 90% reduction in *RALDH2* expression (Figures 4L–4O). The persistence of a few neurons of PGC and LMC character is likely to result from a compensatory activity of FoxP4, which is expressed transiently by MNs and shares FoxP1's inductive activity (Figures S1Q–S1S). RALDH2-dependent retinoid synthesis by LMC neurons provides a feed-back signal that promotes the proliferation of MN progenitors (Jessell, 2000) and the loss of this signal may account for the ~30% decrease in the number of MNs detected at limb levels of e13.5 *Foxp1* mutants (data not shown).

What becomes of prospective PGC and LMC neurons in *Foxp1* mutants? At thoracic and limb levels of *Foxp1* mutants

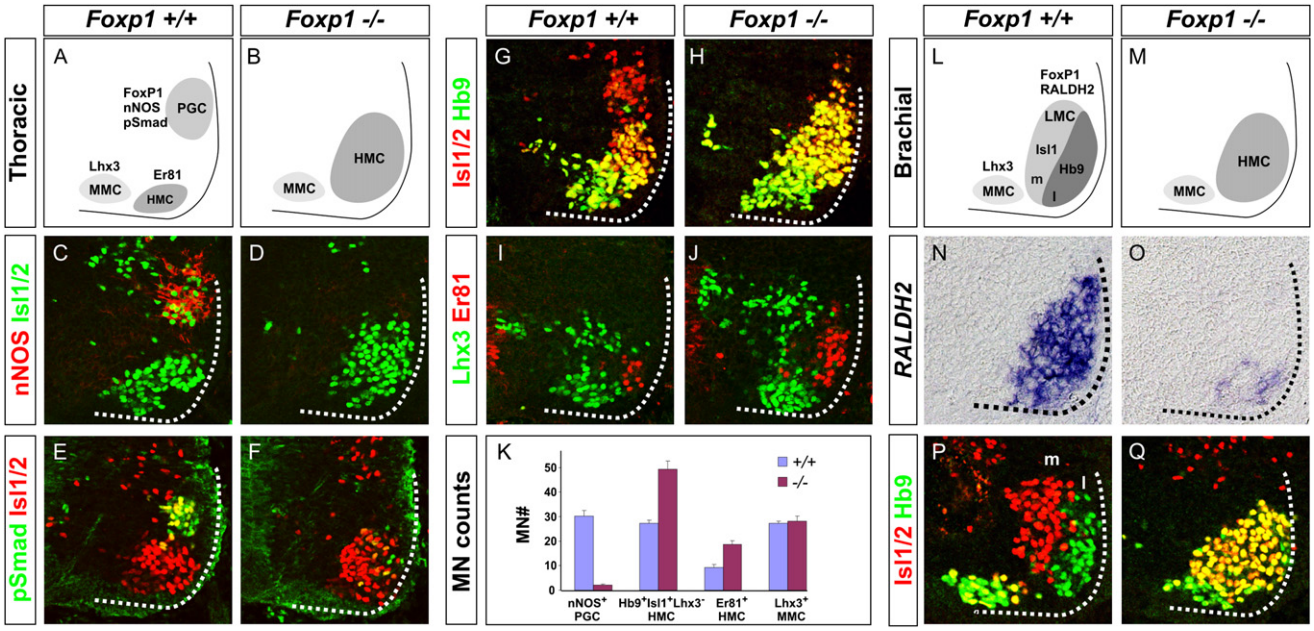

Figure 4. FoxP1 Is Required for the Differentiation of PGC and LMC Neurons

(A and B) Motor columns at Th levels in wild-type and *Foxp1* mutants.

(C–F) In *Foxp1* mutants, Isl1^{+} MNs are not detected in a dorsal position, and little nNOS or pSmad is detected.

(G and H) Increase in the number of Hb9^{+}/Isl1/2^{+} MNs.

(I and J) Increase in Er81^{+} MNs in *Foxp1* mutants. The number of Lhx3^{+} neurons is unchanged.

(K) Quantification of MN columnar subtype markers in Th spinal cord of wild-type and *Foxp1* mutant embryos/15 μm section, mean ± SEM. Values for PGC and HMC differ in experimental and control embryos ($p < 0.01$).

(L and M) Motor columns at Br levels in e12.5 wild-type and *Foxp1* mutant embryos.

(N and O) Loss of *RALDH2* in *Foxp1* mutants. Residual expression likely reflects a redundant role of FoxP4 (Figures S1Q-S1S).

(P and Q) At e13.5, medial (m) and lateral (l) divisions of the lumbar LMC are defined by segregated expression of Isl1 and Hb9. In *Foxp1* mutants, Lhx3^{off} MNs coexpress Hb9 and Isl1.

most Lhx3^{off} MNs coexpressed Isl1/2 and Hb9 - the profile of HMC neurons (Figures 4G, 4H, 4K, 4P, and 4Q). We also detected a 2-fold increase in Er81^{+} HMC neurons at thoracic levels of *Foxp1* mutants (Figure 4I- K). In contrast, the number of MMC (Hb9^{+}, Isl1/2^{+}, Lhx3^{+}) neurons was unchanged (Figure 4I–4K, data not shown). Thus in *Foxp1* mutants the spinal cord loses PGC and LMC neurons and gains HMC neurons, and as a consequence is transformed along its length into a twinned columnar system that comprises MMC and HMC neurons (Figure 6S). These findings provide genetic evidence that FoxP1 activity normally diverts MNs from an HMC-like ground state toward PGC and LMC fates.

We next examined how the appearance of a continuous HMC column influences MN axonal projections. We introduced an *Hb9::GFP* transgene (Arber et al., 1999) into *Foxp1* mutant and heterozygote backgrounds, and analyzed the trajectory of GFP-labeled motor axons from e11.5 to e14.5. At thoracic levels of *Foxp1* mutants we observed a severe reduction in the projection of the axons of PGC neurons to sympathetic chain ganglia (Figures 5A–5C). We also injected rhodamine-dextran (RhD) into the intercostal nerves that supply body wall muscles in *Foxp1* $^{-/-}$ and wild-type embryos and monitored the transcriptional profile of retrogradely-labeled MNs at e12.5. Virtually all RhD-labeled MNs in *Foxp1* mutants exhibited an HMC-like profile (Isl1/2^{+}, Hb9^{+} , Lhx3^{off} ; Er81^{+}; Figures 5G–5L). Thus thoracic MNs deprived of FoxP1 fail to pursue a PGC-like trajectory and instead project their axons distally toward body wall muscles, the normal target of HMC neurons. Lhx3^{+} MMC neurons were not labeled after intercostal tracer injections in *Foxp1* mutants (Figures 5G and 5H), indicating that their axons continue to project to axial muscles.

At brachial and lumbar levels of *Foxp1* mutants we detected a GFP-labeled motor nerve branch that reached axial muscles, suggesting that a MMC-like axonal trajectory is also preserved at limb levels of the spinal cord (Figures 5D–5F). We also detected a distally-directed motor nerve branch that reached the base of the limbs (Figures 5D–5F), and MNs retrogradely labeled after tracer injection into the limbs exhibited an HMC transcriptional profile (data not shown). Thus the HMC-like neurons generated at limb levels of the spinal cord of *Foxp1* mutants embark on a distal trajectory, but by virtue of their aberrant position of origin encounter limbs rather than body wall musculature.

The Fate of Motor Axons in the Limbs of *Foxp1* Mutants

How do the axons of these misplaced HMC neurons behave on entering the limb? The acquisition of LMC divisional and pool identities is thought to impose topographic order in the trajectory

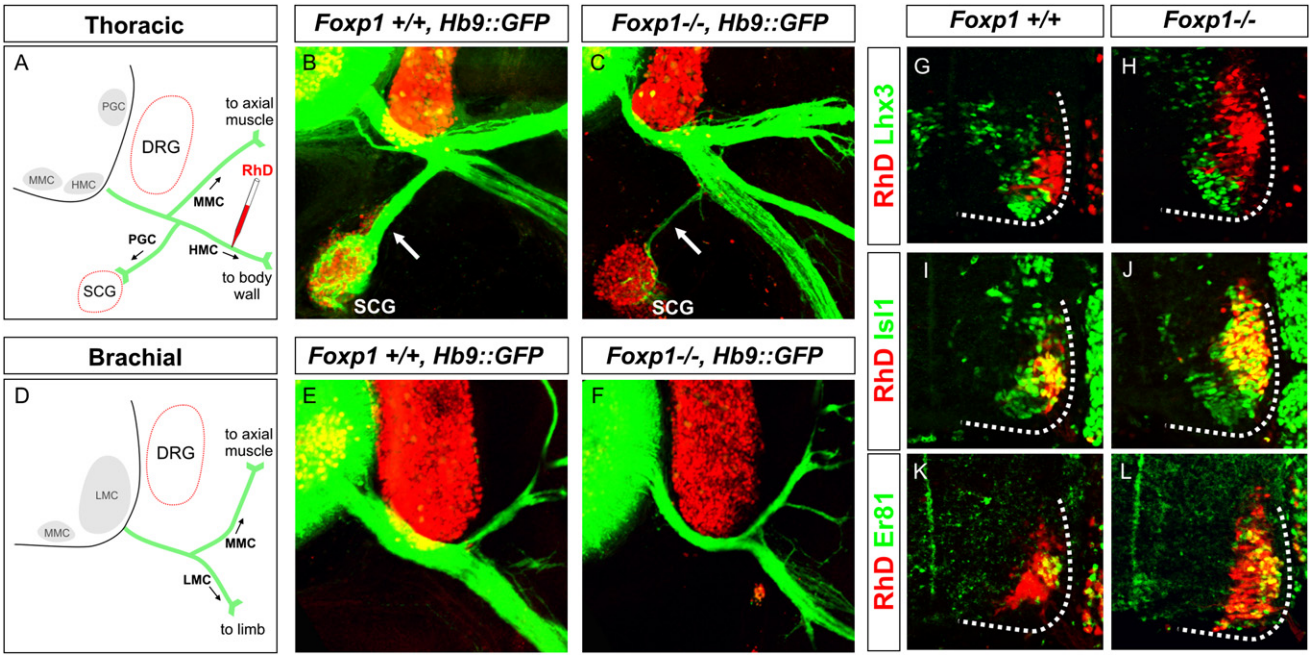

Figure 5. Altered Motor Axon Projections in *Foxp1* Mutants

(A–F) Motor axon projections in wild-type and *Foxp1* mutant embryos.

(A) Projections of Th MNs. Location of RhD injection site for panels (G)–(L) shown.

(B and C) In *Foxp1* mutants, axonal projections to sympathetic chain ganglia (SCG) are dramatically reduced at e13.5 (arrows). GFP-labeled motor axons (green), Isl1/2^{+} DRG and SCG neurons (red). Axonal projections to axial muscles are preserved.

(D–F) Axonal projections at brachial levels in wild-type and *Foxp1* mutants. In *Foxp1* mutants the decrease in thickness of the motor nerve branch projecting to the limb may reflect the depletion of Lhx3^{off} Br MNs. Projections to axial muscles are preserved.

(G–L) Labeled MNs after RhD injection into intercostal nerves. Er81^{+}, Isl1/2^{+}, Lhx3^{off} MNs are labeled in wild-type and *Foxp1* mutants.

of motor axons within the limbs (Figure 7A). Medial and lateral divisional identity dictates the selection of ventral and dorsal axonal trajectories (Kania and Jessell, 2003). And diverse MN pool identities direct the formation of muscle nerve branches as well as the pattern of axonal arborization within target muscles (Livet et al., 2002; De Marco Garcia and Jessell, 2008). To determine the contribution of the FoxP1/Hox program to these patterns of connectivity we compared the extent of LMC divisional and pool differentiation with the trajectory of motor axons within the limbs of *Foxp1* mutants.

We found that FoxP1 is needed for the expression of divisional transcription factors and surface receptors that normally determine the dorsoventral trajectory of LMC axons in the limb. In wild-type embryos, medial LMC neurons express Isl1 and lateral LMC neurons Hb9 and Lhx1 (Figures 4P and 6A) (Kania and Jessell, 2003). The HMC-like MNs generated at limb levels of *Foxp1* mutant embryos did not segregate Isl1 from Hb9, lacked Lhx1 expression (Figures 4Q and 6B) and failed to express *EphA4*, a guidance receptor that directs the axons of lateral LMC neurons into the dorsal limb (Figures 6C and 6D) (Kania and Jessell, 2003). FoxP1 also controls the expression of transcription factors and receptors implicated in MN pool positioning and connectivity. Three Hox-regulated transcription factors normally expressed in selected LMC motor pools, Nk6.1, Pea3 and Er81, were absent from HMC-like MNs in *Foxp1* mutants, (Figures 6G–6L) (De Marco Garcia and Jessell, 2008). These MNs also lacked expression of *Cad-8* and *Cad-20*, type II cadherins involved in motor pool sorting and of *Sema3E*, a neuropilin/plexin ligand implicated in motor axon growth and guidance (Figures 6M–6R) (Livet et al., 2002; Price et al., 2002). In contrast, expression of *Npn-1*, a Sema receptor expressed by many classes of spinal MNs (Huber et al., 2005) was maintained by HMC-like neurons at limb levels of *Foxp1* mutants (Figures 6E and 6F). Thus, FoxP1 activity controls LMC divisional and pool character (Figure 6S).

Analysis of the projections of motor axons in the limb of e11.5 *Foxp1* $^{-/-}$*; Hb9::GFP* mice revealed an apparently normal bifurcation of the main motor nerve trunk into dorsal and ventral divisional branches (Figures 7B and 7C). And between e11.5 and e14.5, the overt pattern of muscle nerves was similar to that observed in control *Hb9::GFP* embryos (Figures 7D–7G and S9A–S9F). Muscle nerves branches tended to be thinner in *Foxp1* mutants, probably a consequence of the reduction in MN number at limb levels. In addition, a few nerve branches to specific muscles were missing (Figure S9). Nevertheless, most limb muscles were innervated by motor axons in *Foxp1* mutants (Figures 7H and 7I; data not shown).

To assess the impact of FoxP1 on limb muscle connectivity we focused on the pattern of innervation of muscles supplied by motor pools that express Hox-dependent transcription factors. Nerve branches supplying the tibialis anterior (Ta) and gracilis posterior (Gp) muscles derive from Nkx6.1^{+} motor pools, and

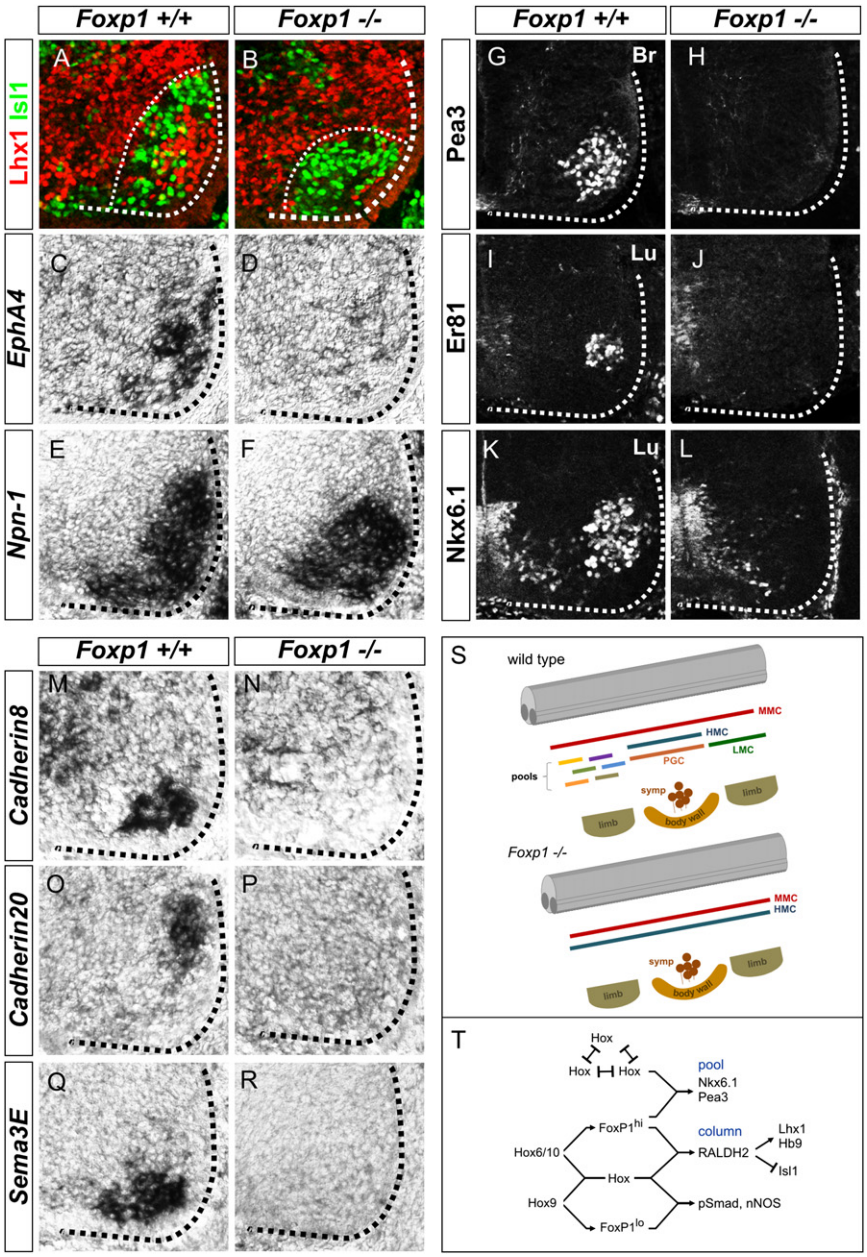

Figure 6. FoxP1 Specifies the Divisional and Pool Identity of LMC Neurons

(A and B) Loss of expression of Lhx1 by Br MNs in e13.5 *Foxp1* mutants. LMC confines marked with dotted line. Similar results obtained at Lu levels.

(C and D) Loss of *EphA4* expression from Br MNs in *Foxp1* mutants.

(E and F) Persistence of *Npn-1* in Br MNs in *Foxp1* mutants.

(G–R) Loss of pool markers in Lhx3^{off} MNs in *Foxp1* mutants at Br and Lu levels.

(S) Summary indicating how FoxP1 controls the formation of PGC and LMC columns and LMC pools.

(T) FoxP1 gates the output of Hox networks that assign MN columnar and pool identities.

in *Nkx6.1* mutants the axons that normally project to these muscles are re-routed to different targets (De Marco Garcia and Jessell, 2008). In *Foxp1* mutants we found that the Ta and Gp muscles were innervated by motor axons despite the loss of Nkx6.1 (Figures 7F,G, S9A,B). This paradoxical finding appears to have its basis in the erosion of Ta and Gp pool identities in *Foxp1* mutants, whereas these pools retain aspects of their molecular character in *Nkx6.1* mutants (De Marco Garcia and Jessell, 2008, see Discussion). In contrast, motor pool programs that specify the pattern of axon terminal arborization within target muscles were severely affected by the loss of FoxP1. MNs that project to the cutaneus maximus (CM) forelimb muscle normally establish an expansive intramuscular arbor through a developmental program that depends on the pool-restricted ETS protein Pea3 (Livet et al., 2002). Analysis of *Foxp1* $^{-/-}$ *; Hb9::GFP* embryos revealed a failure of motor axon arborization within the CM muscle (Figures 7J-M), a phenotype similar to that observed in *Pea3* mutants (Livet et al., 2002). Thus this later, muscle-type specific, connectivity program is drastically impaired in *Foxp1* mutants.

Erosion of Motor Neuron-Muscle Topography in *Foxp1* Mutants

Does the overtly normal pattern of motor nerves observed in the limbs of *Foxp1* mutants conceal a disruption in the topographic link between MN position and muscle nerve trajectory? To address this possibility, we injected HRP and RhD into dorsal and ventral divisional branches of e13.5 wild-type and *Foxp1* mutant embryos, and assessed the position of labeled neurons within the cohort of HMC-like neurons present at limb levels of the spinal cord. In wild-type embryos, axons that enter the ventral divisional branch derive from medially-positioned LMC neurons whereas axons that enter the dorsal divisional branch derive from laterally-positioned LMC neurons (Figure 7N). In *Foxp1* mutants there was no discernable relationship between the mediolateral position of RhD- or HRP-labeled MNs within the HMC-like cohort and the selection of ventral or dorsal divisional branches (Figures 7N, S9G-I; data not shown). This finding, together with the erosion of LMC divisional identity revealed by loss of Lhx1 and *EphA4* expression, provides strong evidence that the inactivation of FoxP1 scrambles the selection of dorsal and ventral axonal trajectories in the developing limb.

We also examined the topographic relationship between MN position and axonal projections into individual muscle nerves. We labeled the forelimb ulnar and hindlimb obturator muscle nerves by focal RhD injection, and assessed the position of

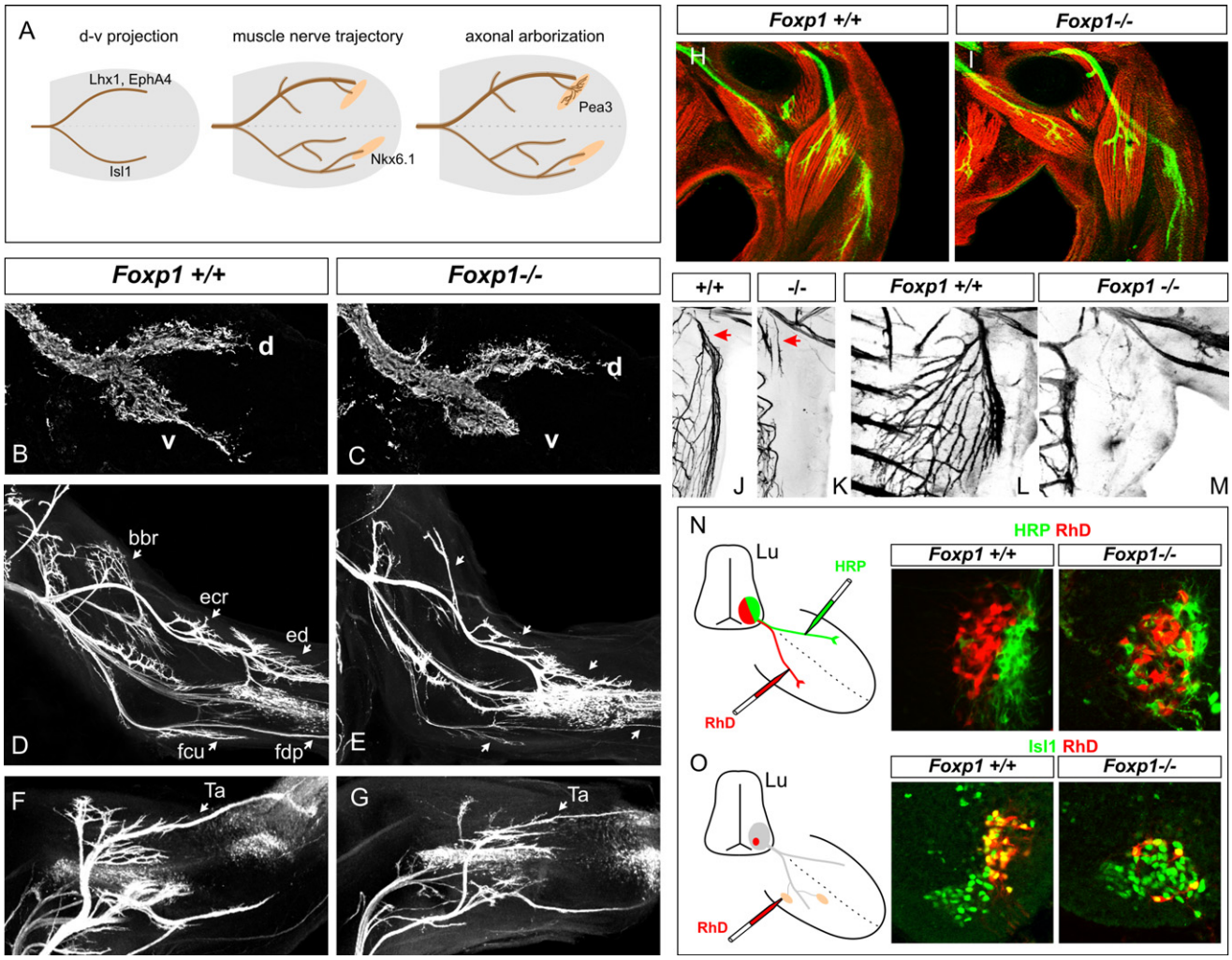

Figure 7. Limb Innervation in *Foxp1* Mutants

(A) Development of limb innervation.

(B and C) Projection of motor axons along dorsal (d) and ventral (v) divisional branches in e11.5 wild-type and *Foxp1* mutants.

(D and E) Forelimb innervation (dorsal view) in wild-type and *Foxp1* mutants at e14.5. Motor nerve branches are similar in wild-type and *Foxp1* mutants. bbr, biceps brachialis; ecr, extensor carpi radials longus and brevis; ed, extensor digitorum; fcu, flexor carpi ulnaris; fdp, flexor digitorum profundis. Motor axons visualized by GFP in *Hb9::GFP* and *Foxp1*$^{-/-}$ *; Hb9::GFP* embryos.

(F and G) Motor axons in the tibialis (Ta) nerve in wild-type and *Foxp1* mutant embryos at e14.5.

(H and I) Near-normal pattern of motor innervation of distal forelimb muscles (My32: myosin light chain, red) in e13.5 *Foxp1* mutant embryos.

(J–M) A vestigial motor nerve is oriented toward the CM muscle (arrow in K), but the arborization of motor axons is drastically reduced in e13.5 *Foxp1* mutants.

(N) Labeled MNs after HRP and RhD injection into the dorsal and ventral branches of the hindlimb in wild-type and *Foxp1* mutant embryos. Similar results obtained at forelimb levels (Figure S9H).

(O) RhD-labeled, Isl1/2^{+} MNs after tracer injection into the obturator nerve branch of wild-type and *Foxp1* mutant embryos. Similar results obtained after ulnar nerve injection (Figure S9J).

labeled MNs (Figures 7O and S9J). In wild-type embryos, RhD-labeled MNs that send axons into the ulnar and obturator nerve branches were clustered into coherent pools within the medial LMC (Figures 7O and S9J). But in *Foxp1* mutants RhD-labeled neurons were scattered throughout the HMC-like column (Figures 7O and S9J). Thus the loss of FoxP1 degrades the topography of motor axonal projections into individual muscle nerves, suggesting that MNs deprived of FoxP1 project axons into divisional and muscle nerve paths in a haphazard manner.

DISCUSSION

A network of twenty-one Hox genes endows spinal MNs with identities that define the specificity of target innervation. FoxP1, at one fell swoop, controls the output of the Hox repertoire that directs MN identity and connectivity. Short-circuiting this network by inactivation of FoxP1 has revealed insight into the origins of MN diversity and key aspects of the logic through which Hox genes impose a topographic motor map.

FoxP1 and the Origins of Motor Neuron Diversity

Spinal motor systems exhibit remarkable variation in organization and complexity (Fetcho, 1992). Our findings point to an essential role for the FoxP1/Hox program in expanding the range of MN columnar subtypes so as to ensure effective innervation of peripheral motor targets. In particular they suggest that the formation of HMC neurons from an ancestral MMC-like group was a crucial step in MN diversification; providing a malleable population of MNs that serve as the substrate for the FoxP1/Hox program of columnar and pool specification (Figure 6S).

The ancestral state of spinal MNs is marked by expression of Lhx3, in the context of Isl1/2 and Hb9 (Landgraf and Thor, 2006). This transcriptional profile defines early-born 'primary' MNs in zebrafish and amphibian embryos (Appel et al., 1995; Borodinsky et al., 2004), presumed counterparts of the MNs of jawless vertebrates (Fetcho, 1992), as well as the MMC neurons of birds and mammals (Jessell, 2000). The conserved transcriptional profile of this ancestral set of MNs may reflect common patterns of connectivity—the innervation of segmentally arrayed muscles involved in undulatory locomotor behaviors. The diversification of columnar subtypes from this ancestral group requires relief from the confining influence of Lhx3 (Sharma et al., 2000; William et al., 2003). This evasive step may have involved a decrease in the strength of the Wnt signaling component of the dorsoventral inductive pathway, since reducing Wnt4/5 activity in mice promotes the generation of HMC neurons at the expense of MMC neurons (D. Agalliu and T.M.J., unpublished data). Thus the spinal motor system induced by the dorsoventral signaling pathway comprises MMC and HMC neurons, arrayed in coextensive columns (Figure 6S).

How did this twinned columnar plan undergo further diversification to generate segmentally restricted PGC and LMC neurons? Our findings suggest that prospective HMC neurons, freed of the influence of Lhx3, became sensitive to the rostrocaudal patterning activities of Hox proteins, which diverted them away from their constitutive HMC ground state and toward PGC or LMC fates. FoxP1, through its dose-dependent inductive activity, is a key participant in this Hox program of columnar specification. Under the influence of Hox9 activity, newly-generated $Lhx3^{off}$ thoracic MNs acquire the capacity for low-level FoxP1 expression, and appear to resolve their bi-potential, HMC or PGC, fate through mutual repressive interactions between FoxP1 and Hb9 (Figure S7). In the limb-level realms of Hox6/10 activity, FoxP1 can be induced to high levels without repression by Hb9, which is likely to account for the progression of all $Lhx3^{off}$ neurons to a LMC fate.

How the FoxP1/Hox network was recruited to the task of MN columnar diversification remains unclear. The absence of PGC and LMC neurons from early vertebrates could have its basis in evolutionary changes in *cis*-regulatory elements that control *Foxp1* expression (Shubin et al., 1997; Prud'homme et al., 2007), such that Hox-sensitive elements responsible for expression in spinal MNs were configured only at the time of formation of the sympathetic nervous system and paired appendages. Alternatively, the rostrocaudal pattern of expression of Hox genes in the spinal cord of jawless vertebrates may differ from that in birds and mammals (Force et al., 2002; Takio et al., 2007), and thus may fail to produce a productive Hox code capable of activating FoxP1 expression. An evolutionary change in the rostrocaudal profile of Hox genes in the lateral plate mesoderm has been proposed to regulate the formation and positioning of fins and limbs (Cohn et al., 1997; Cohn and Tickle, 1999). Thus, the coordinate reshaping of mesodermal and neural Hox expression patterns during vertebrate evolution provides a plausible basis for linking the formation and diversification of LMC neurons to the appearance and elaboration of paired appendages (Tabin, 1995; Shubin et al., 1997).

FoxP1 as a Hox Accessory Factor

The pervasive activities of insect and vertebrate Hox proteins are constrained by other transcription factors that enhance the specificity of Hox-DNA interactions and thus determine the selection of Hox target genes (Mann and Affolter, 1998; Moens and Selleri, 2006). Our findings show that FoxP1 is an indispensible Hox accessory factor during the programming of spinal MN diversity and connectivity. FoxP1 appears to act jointly with Hox proteins rather than as a linear intermediary in the pathway of columnar specification, since forced expression of FoxP1 is unable to induce LMC character under conditions in which Hox activity is repressed. The idea that FoxP1 and Hox proteins interact to specify MN columnar identity is also supported by analysis of mice lacking *Hox10* gene function, where the erosion of lumbar LMC character is accompanied by the appearance of neurons with an HMC-like transcriptional profile (Wu et al., 2008), a phenotype similar to that of *Foxp1* mutants.

Two distinct sets of Hox regulatory interactions specify the columnar and pool character of LMC neurons. Our data suggest that FoxP1 gates the output of both Hox networks (Figure 6T). But the burden of specificity during the transcriptional programming of MN columnar and pool identity alternates between Hox proteins and FoxP1. Initially, distinctions in Hox6, 9, and 10 paralog identity set the level of MN FoxP1 expression. Once induced, however, FoxP1 is the primary determinant of columnar specificity, since its dose-dependent assignment of PGC and LMC fate is independent of Hox6/9/10 paralog status. But for the program of motor pool allocation, the onus of specificity in target gene activation reverts to Hox proteins, since the Hox code that determines pool identity (Dasen et al., 2005) acts in the context of uniformly high-level FoxP1 expression.

The contributions of FoxP1 to the Hox programming of spinal MN differentiation have parallels with those of the *Drosophila* Slp1/2 forkhead proteins, which serve as accessory factors for Ubx and Abd-A during embryonic patterning (Gebelein et al., 2004). The *Drosophila* Hox protein scr directly activates expression of the *forkhead* gene (Joshi et al., 2007), raising the possibility that cofactor induction is a common strategy in Hox transcriptional networks. This FoxP1/Hox network constitutes a 'coherent feed-forward loop' that can introduce delays in target gene activation (Mangan et al., 2003), and may represent an intrinsic mechanism for matching the time of expression of guidance receptors to the arrival of axons at relevant cues, on the path to their targets.

FoxP1 is critical for the output of the entire Hox program of spinal MN subtype specification, but not all MN Hox functions depend on FoxP1. Expression of FoxP1 in PGC and LMC neurons is induced by Hox proteins, and thus this early phase of

Hox activity does not depend on FoxP1. Moreover, mutual repressive interactions between Hox6/10 and Hox9 paralogs are evident in MMC and HMC neurons (Dasen et al., 2003), even though they lack FoxP1 expression. As a general rule, regulatory interactions between Hox genes occur independently of FoxP1, whereas Hox activation of downstream effectors relies on FoxP1. The well-documented activities of Hox genes in hindbrain MN specification (Trainor and Krumlauf, 2000) must also be independent of FoxP1, since FoxP proteins are not expressed by hindbrain MNs (Figure S1). But Meis and Pbx/Prep cofactors are expressed in the hindbrain, and Pbx proteins regulate cranial MN identity (Moens and Selleri, 2006). Thus, the activities of FoxP1 as an arbiter of Hox output during spinal MN differentiation may be exerted in a broader context of Meis and Pbx/Prep activities. Meis and Pbx/Prep cofactors may impart a first level of Hox specificity (Joshi et al., 2007), with FoxP1 providing additional filters on target gene activation.

FoxP1 and Strategies of Motor Axon Targeting

How does FoxP1/Hox network activity control the decisions taken by motor axons as they project to their targets? The finding that loss of FoxP1 blocks the output of the entire MN Hox repertoire provided an opportunity to assess, wholesale, the contribution of this network to target connectivity.

The switch from PGC to HMC fate in *Foxp1* mutants is accompanied by a redirection of motor axons from sympathetic ganglia to body wall targets. In contrast, HMC-like MNs generated at brachial and lumbar levels project to limb rather than body wall muscles, following a proximal path that resembles that of LMC axons. Is this the expected behavior of HMC neurons, or an indication that cryptic elements of LMC character have been preserved? Evidence that this is the predicted trajectory of HMC neurons unlucky enough to find themselves aligned with limbs comes from the finding that ectopic limbs induced adjacent to thoracic spinal cord are innervated selectively by axons of HMC neurons (Turney et al., 2003 ; J. Brown, J.D. and T.M.J., unpublished data). Thus, HMC and LMC neurons are alike in their initial pursuit of a distal trajectory that takes them to body wall or limb targets.

In the limb, LMC axons establish stereotypic projections to individual muscles (Landmesser, 2001). Transcription factors, recognition molecules and guidance receptors contribute to the establishment of this topographic motor map. The loss of FoxP1 abolishes the expression of all molecular features of LMC divisional and pool identity yet motor nerve branching and limb muscle innervation is remarkably well preserved. One possible explanation for this dichotomy is that elimination of FoxP1 simply scrambles MN cell body position, leaving intact the programs that direct axonal trajectory. The loss of expression of genes that determine the trajectory of LMC axons (*Lhx1, EphA4,* and *Nkx6.1*) as well as those that control motor pool sorting (*Pea3*, *Cad8, Cad20*) argues against this. Our findings fit best with the view that the overall pattern of motor nerve branching within the limb is determined by pre-established permissive or inhibitory domains within the limb mesenchyme, with the FoxP1/Hox program providing LMC neurons with identities that enable axons to respond to local cues that promote the selection of just one of many available axonal conduits (Figure S9K). In this view, the trajectory of MNs deprived of FoxP1 activity will still be constrained by the existence of preordained paths, but without intrinsic molecular programming, individual axons will be reduced to choosing randomly among their permitted options.

The loss of all determinants of motor pool identity in *Foxp1* mutants may underlie the preservation of muscle nerve branches that are missing in mice deprived of downstream pool transcription factors. The persistence of expression of instructive pool determinants in *Nkx6.1* mutants accounts for the re-direction of motor axons away from their intended muscles, to alternate targets (De Marco Garcia and Jessell, 2008). But in *Foxp1* mutants, where every aspect of pool identity is erased, all axonal paths will be available. This view also helps to explain why motor axon arborization within the CM muscle is similarly stunted in *Foxp1* and *Pea3* mutants. We surmise that the random selection of axonal paths ensures, by chance, that some motor axons will project toward the CM muscle, but on arrival they will be unable to activate the Pea3-dependent program that promotes the expansive arborization of motor axons (Livet et al., 2002).

Motor axon arborization is but one of several late steps in motor pool differentiation that drive sensory-motor circuit assembly. Others include dendrite patterning and synaptic input specificity - programs that are also controlled by motor pool transcription factors (Livet et al., 2002; Vrieseling and Arber, 2006). The dependence of these pool determinants on the FoxP1/Hox network therefore predicts that the loss of FoxP1 will perturb the specificity of inputs to spinal MNs that innervate an individual muscle target. And since Hox and FoxP proteins are coexpressed by certain interneuron classes, their convergent activities in interneurons may also contribute to the assembly of local microcircuits that coordinate motor output.

EXPERIMENTAL PROCEDURES

Screening for *Fox* Gene Expression in the Spinal Cord

PCR and in situ hybridization-based screens were performed to identify *Fox* genes expressed in motor neurons. We initially screened against forty one *Fox* genes by RT-PCR (One-Step, Invitrogen), using four or more unique combinations of oligonucleotides directed against each gene. RT-PCR analysis was performed on e12.5-e14.5 mRNA isolated from tissue containing the spinal cord and surrounding mesoderm. Twenty-one *Fox* genes were amplified by RT-PCR from this initial screen and were subsequently cloned into a pcrII-TOPO vector (Invitrogen). Template plasmid DNAs were used to generate cRNA probes for in situ hybridization on e12.5-13.5 spinal cord. Of the twenty-one probes tested, seven (*Foxa2*, *Foxb1*, *Foxd3*, *Foxn4*, *Foxp1*, *Foxp2*, and *Foxp4*) were found to be expressed by neuronal populations within the spinal cord.

Quantification of FoxP1 Levels

Nuclear FoxP1 levels were determined on cryostat sections at sub-saturating antibody concentrations. Images were obtained by confocal microscopy using identical laser and gain configurations. All analyses were performed on sections on the same slide. Nuclear pixel intensity was determined using Photoshop. Mean pixel intensities for > 50 MN nuclei are shown.

Plasmid Constructs for Transgenic Animals

Expression constructs for Lhx3, Hb9, Hox, and Engrailed-repressor (EnR) Hox derivatives were subcloned into a *CMV/β−actin* promoter driver vector (*CAGGs*) as described previously (Dasen et al., 2003, 2005; William et al., 2003). The murine *Foxp1* gene was cloned into an expression vector containing the regulatory elements of the *Hb9* gene (Arber et al., 1999; Wichterle et al., 2002). The *Foxp1* cDNA was followed by an internal ribosomal entry site (ires)

to allow expression of enhanced green fluorescent protein (GFP). For generation of *Hb9::FoxP1iresGFP* transgenic mice the expression cassette was excised from the plasmid prior to pronuclear injection.

In Ovo Electroporation

Electroporation was performed on stage 12–16 chick embryos (Dasen et al., 2003). Levels of FoxP1 approximating those in PGC neurons were achieved by diluting the *CAGGs::Foxp1* plasmid to 10–15ng/μl using pBKS plasmid DNA. Levels of FoxP1 approximating those in LMC neurons were achieved using *Hb9::Foxp1iresGFP* at 3 μg/μl or *CAGGs::Foxp1* at 100 ng/μl. Results for each experiment are representative of > 8 embryos with MN electroporation efficiency > 60%.

Mouse Genetics

The *Foxp1* mutant strain is as described in Wang et al., (2004), the *Hb9::GFP* line in Arber et al., (1999). The *Hb9::Foxp1iresGFP* line was generated as described (Arber et al., 1999).

In Situ Hybridization Histochemistry and Immunohistochemistry

In situ hybridization and immunohistochemistry were performed on 15–20 μm cryostat sections as described (Dasen et al., 2005). Whole-mount antibody staining was performed as described (De Marco Garcia and Jessell, 2008) and GFP-labeled motor axons were visualized in projections of confocal Z-stacks (400–600 μm). Antibodies against Hox proteins, LIM HD proteins, and other proteins were generated as described (Dasen et al., 2005). Antibodies against additional proteins were generated in guinea pigs and rabbits using the following peptide sequences FoxP1: ENSIPLYTTASMGNPTC, RALDH2: CERAKRRIVGSPFDPTTE. Additional antibodies were obtained and used as follows: rabbit anti-nNOS 1:5000 (Immunostar), goat anti-Hoxc6 1:2000 (Santa Cruz), goat anti-Hoxd10 1:500 (Santa Cruz), goat anti-FoxP2 1:8000 (AbCam), rabbit anti-FoxP4 1:8000 (kindly provided by Edward Morrisey), goat anti-HRP 1:2000 (Jackson Immunoresearch).

Retrograde Labeling of Motor Neurons

Retrograde labeling of motor neurons was performed as described (Dasen et al., 2005). 3000 MW lysine-fixable dextran-tetramethylrhodamine (RhD, Molecular Probes) or horseradish peroxidase (HRP, Roche) was injected into severed nerves or individual muscles of e12.5-e13.5 embryos. Prior to injections, embryos were decapitated, eviscerated, and dissected to remove the epidermis dorsal to the spinal cord. To aid in the identification of nerves, we used GFP fluorescence from *Hb9::GFP* transgenic mouse embryos, visualized using a MVX10 wide-field fluorescent macroscope (Olympus). Nerves were severed using Oban Bioscissors and HRP or RhD was injected onto the cut terminal. Embryos were incubated for 3–5 hr in oxygenated F12/DMEM (50:50) solution at 32–34°C and subsequently fixed in 4% paraformaldehyde.

SUPPLEMENTAL DATA

Supplemental Data include nine figures and can be found with this article online at http://www.cell.com/cgi/content/full/134/2/304/DC1/.

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