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A Hox Regulatory Network Establishes Motor Neuron Pool Identity and Target-Muscle Connectivity

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Summary

Spinal motor neurons acquire specialized "pool" identities that determine their ability to form selective connections with target muscles in the limb, but the molecular basis of this striking example of neuronal specificity has remained unclear. We show here that a Hox transcriptional regulatory network specifies motor neuron pool identity and connectivity. Two interdependent sets of Hox regulatory interactions operate within motor neurons, one assigning rostrocaudal motor pool position and a second directing motor pool diversity at a single segmental level. This Hox regulatory network directs the downstream transcriptional identity of motor neuron pools and defines the pattern of target-muscle connectivity.

Introduction

The precision of neural-circuit assembly has a crucial role in defining the innate repertoire of animal behaviors. The task of establishing appropriate patterns of connectivity is at its most challenging in the vertebrate central nervous system (CNS), where hundreds of neuronal types are required to form thousands of synaptic contacts, each with a selective subset of targets. The mechanisms that direct connectivity within the vertebrate CNS, indeed within any nervous system, remain elusive. Recent studies, however, have indicated that the acquisition of subtype identities by developing neurons is a critical determinant of their connectivity—through the regulation of neuronal settling position (Marin and Rubenstein, 2003), axonal trajectory (Tessier-Lavigne and Goodman, 1996), and target choice (Yamagata et al., 2002). And in large part, a neuron's identity is encoded by the profile of transcription factors expressed by its ancestral progenitors and by the postmitotic neuron itself (Pearson and Doe, 2004). Defining the transcriptional logic that links the inordinate diversity of neurons found in the vertebrate CNS to patterns of target connectivity may, therefore, reveal general principles of neural-circuit assembly.

Some insights into the mechanisms that drive neuronal diversification have come from the study of one of its major neuronal classes—the spinal motor neuron (MN) (Jessell, 2000). From the perspective of locomotor control, the most critical aspect of MN differentiation is the formation of precise connections with target muscles in the limb (Landmesser, 1978, 2001). Such precision is achieved by conferring MNs with discrete columnar, divisional, and pool identities (Figures 1A–1C). Each of these facets of MN identity appears to govern a distinct step in the projection of motor axons to their limb-muscle targets. The acquisition of a lateral MN columnar (LMC) identity directs motor axons toward the limb, and the emergence of divisional identities within the LMC directs motor axons ventrally or dorsally upon entering the limb mesenchyme. But it is with the specification of their pool identity that MNs within the LMC acquire the ability to form precise axonal trajectories and innervate individual muscle targets (Landmesser, 1978, 2001). The existence of more than 50 muscle groups in a typical amniote limb (Sullivan, 1962) demands a corresponding diversity of motor pool identities (Hollyday and Jacobson, 1990; Romanes, 1942), posing a considerable molecular challenge in connecting MNs to their muscle targets.

Classical embryological studies have provided evidence that LMC neurons have acquired aspects of their pool identity as motor axons invade the limb mesenchyme, well before approaching muscle targets (Landmesser, 2001). Although the molecular logic that links motor pool identity and target-muscle connectivity remains obscure, there is emerging evidence that the selectivity of transcription-factor expression is a determinant of specificity in motor circuits. LIM homeodomain proteins define the medial and lateral subdivisions of the LMC and control motor axon trajectory along the dorsoventral axis of the limb (Kania and Jessell, 2003). In addition, certain MN pools have been defined by ETS transcription factors (Lin et al., 1998; Arber et al., 2000), but these proteins are expressed only after motor axons have begun to form muscle-nerve branches (Lin et al., 1998; Livet et al., 2002). Indeed, genetic studies have shown that ETS proteins are not involved in defining motor axon trajectories to specific muscle targets and instead regulate the clustering of MNs into coherent pools (Livet et al., 2002; Price et al., 2002).

Since motor pool identity emerges within neurons that have acquired a prior columnar identity (Figures 1A–1C), we reasoned that insights into the pathway of motor neuron columnar specification might provide clues about the strategies used to assign motor pool fates. The columnar identity of MNs appears to be determined by the activities of Hox proteins arrayed along the rostrocaudal axis of the spinal cord (Dasen et al., 2003; Shah et al., 2004) in response to graded FGF signaling (Bel-Vialar et al., 2002; Dasen et al., 2003; Liu et al., 2001). Certain Hox proteins are expressed by subsets of LMC neurons (Carpenter, 2002; Lance-Jones et al., 2001), and the analysis of constitutive *Hox* mutants has revealed defects in the axonal projections of spinal-cord and hindbrain MNs (Carpenter, 2002; Gavalas et al., 1998; Studer et al., 1996; Tiret et al., 1998). But the relevance of phenotypic defects found in *Hox* mutants to the establishment of MN pool identity remains unclear.

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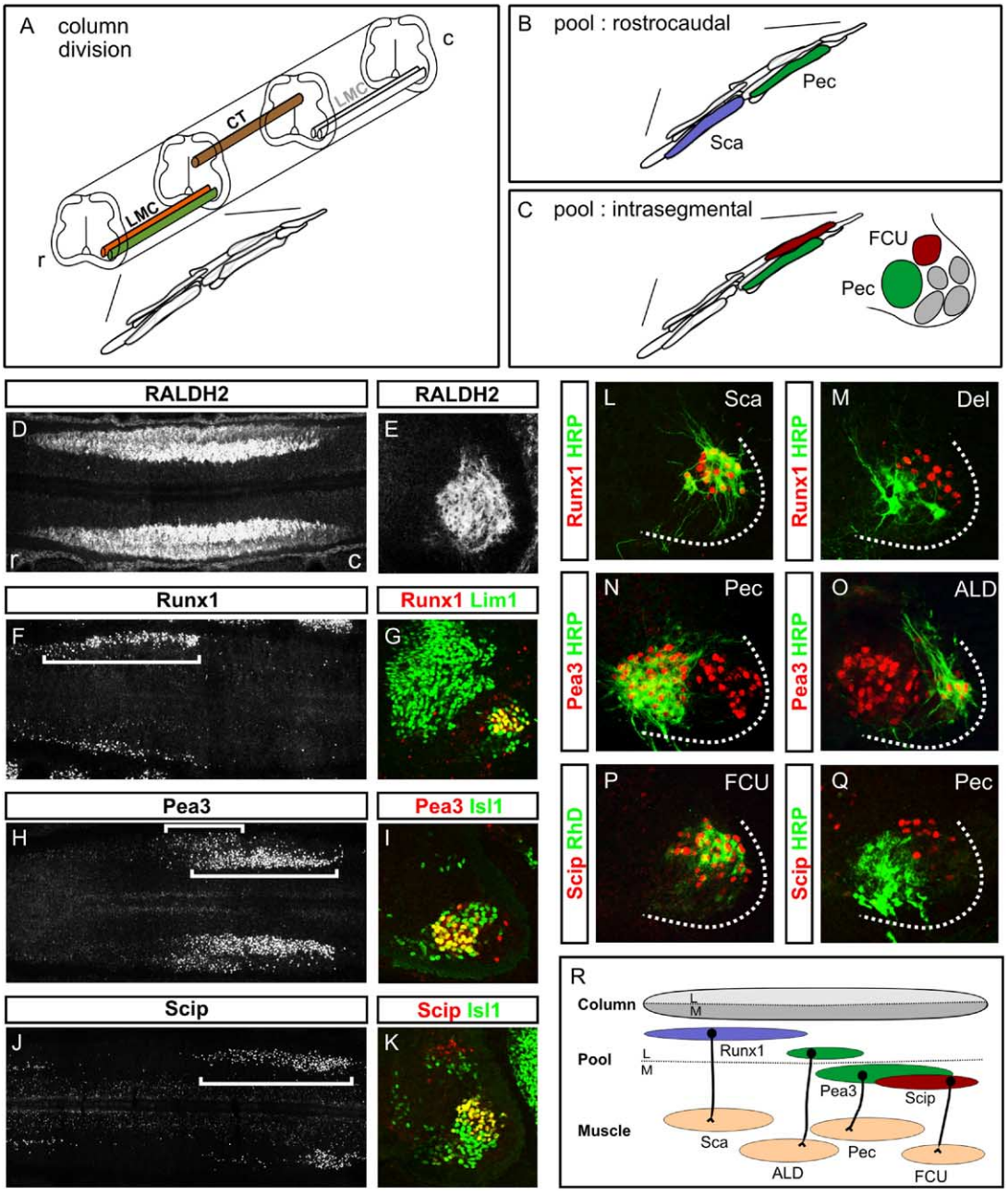

Figure 1. The Molecular Organization of Motor Columns and Pools

(A) MN columnar organization. The cell bodies of MNs that send axons to the limb are contained within the LMC at brachial and lumbar levels of the spinal cord. Other MNs, notably preganglionic neurons, populate the column of Terni (CT) at thoracic levels. The medial (depicted in orange) and lateral (green) divisions of the LMC are shown. Rostral (r) and caudal (c) are indicated.

(B) Motor pool organization along the rostrocaudal axis of the spinal cord. Motor pools that send axons to individual muscle targets in the limb are generated at different rostrocaudal positions within the LMC. The outlines of different motor pools within the LMC are depicted schematically.

(C) Intrasegmental diversity of MN pools. A single axial level of the spinal cord typically generates 8–10 distinct motor pools.

(D) RALDH2 expression by brachial LMC neurons in a stage 31 embryo, shown in a horizontal longitudinal section.

(E) RALDH2 expression in a transverse section of a stage 31 embryo.

(F and G) Runx1 is expressed by rostral LMC neurons that coexpress Lim1.

(H and I) Pea3 is expressed by two caudal LMC neuron populations.

(J and K) Scip is expressed by caudal LMC neurons that express Isl1.

(L–Q) Identification of motor pools by retrograde HRP or RhD labeling after tracer injection into forelimb muscles.

(L and M) Runx1^{+} MNs are labeled selectively after HRP injection into the scapulohumeralis posterior (Sca) but not the deltoid (Del) muscles.

(N and O) Pea3^{+},Isl1^{+} MNs are labeled after HRP injection into the pectoralis (Pec) muscle, whereas Pea3^{+},Lim1^{+} MNs are labeled after HRP injection into the anterior latissimus dorsi muscles (ALD).

(P and Q) Scip^{+} MNs are labeled after RhD injection into the flexor carpi ulnaris (FCU) but not the Pec muscle.

(R) Molecularly defined LMC motor pools and their muscle targets.

We therefore set out to examine the potential functions of Hox proteins in motor pool specification, addressing three main issues. First, how do different MN pools come to occupy characteristic positions along the rostrocaudal axis of the LMC? Second, how are diverse MN pools generated at a single segmental level of the LMC? Third, do Hox proteins link motor pool identity to target-muscle connectivity?

Results

Transcription Factors Define MN Pools

To examine the molecular steps that assign MN pool identity, we searched for transcription factors that delineate the rostrocaudal and intrasegmental organization of motor pools within the brachial LMC. LMC neurons, as a whole, were defined by their selective expression of the retinoid synthetic enzyme retinaldehyde dehydrogenase-2 (RALDH2) (Figures 1D and 1E; Sockanathan and Jessell, 1998). Three transcription factors, Runx1, Pea3, and Scip, exhibited informative patterns of expression within the brachial LMC. Expression of all three proteins was detected by stages 24 to 25, soon after motor axons have entered the limb (data not shown). And by stages 29–31, when specific muscle-nerve branches have formed, these transcription factors were restricted to subsets of LMC neurons. Runx1, a Runt-class transcription factor, is expressed by a group of MNs that occupy a rostral (anterior) domain of the LMC (Figure 1F; Theriault et al., 2004). These Runx1$^+$ neurons coexpressed Lim1, a marker for MNs in the lateral division of the LMC that project their axons to dorsal-limb musculature (Figure 1G). Pea3, an ETS-class transcription factor, was confined to two caudal (posterior) groups of LMC neurons: one within the medial, Isl1$^+$ division of the LMC that sends axons to ventral-limb musculature and the other within the lateral, Lim1$^+$ division (Figures 1H and 1I; Lin et al., 1998). Scip, a POU-class transcription factor, was expressed by a distinct group of medial, Isl1$^+$ LMC neurons that occupied a position more caudal than the medial group of Pea3$^+$ MNs (Figures 1J and 1K; Helmbacher et al., 2003). Thus, the differential expression of Runx1, Scip, and Pea3 in the context of Isl1 and Lim1 defines four groups of MNs with distinct rostrocaudal and intrasegmental positions within the brachial LMC.

To determine whether Runx1, Pea3, and Scip define coherent MN pools, we injected horseradish peroxidase (HRP) or rhodamine dextran (RhD) into individual forelimb muscles at stages 35 to 36 and examined the transcriptional status of MNs that accumulated the tracer by retrograde axonal transport. After tracer injection into the scapulohumeralis posterior (Sca) muscle, all retrogradely labeled MNs expressed Runx1 (Figures 1L and 1M). Injections into the pectoralis (Pec) muscle resulted in selective retrograde labeling of Pea3$^+$ MNs located in the medial, Isl1$^+$ division of the LMC (Figures 1N and 1Q), whereas tracer injections into the anterior latissimus dorsi (ALD) selectively labeled Pea3$^+$ MNs located in the lateral, Lim1$^+$ division of the LMC (Figure 1O). Injection into the flexor carpi ulnaris (FCU) muscle selectively labeled Scip$^+$ MNs (Figures 1P and 1Q). Thus, the profiles of Runx1, Pea3, and Scip expression define four brachial motor pools, each with distinct rostrocaudal and intrasegmental coordinates (Figure 1R). We have focused on the differentiation of these four transcriptionally defined sets of MNs in experiments to clarify the logic of motor pool specification and target connectivity.

Patterned Hox Protein Expression by Brachial LMC Neurons

To begin to examine whether Hox proteins are involved in imposing the distinct identities of LMC motor pools, we analyzed the expression of each of the 39 Hox genes, as well as that of *Meis1*, *2*, and *3* and *Pbx1*, *2*, and *3,* genes that encode Hox cofactors (Mann and Affolter, 1998). Expression of 21 *Hox* genes and of *Meis1*, *Meis2*, *Pbx1*, and *Pbx3* were detected in MNs at brachial, thoracic, and lumbar levels of stage 29–31 spinal cord (see Figure S1 in the Supplemental Data available with this article online and data not shown). We generated antibodies against each of these Hox proteins and against selected Meis and Pbx proteins. For each Hox gene, the pattern of protein and transcript in postmitotic MNs was well matched, and paralogous Hox genes were expressed in similar, although not identical, patterns within the spinal cord (Figures S1–S3). Brachial LMC neurons expressed 11 Hox proteins: Hoxa3, a4, a5, a6, and a7; Hoxb7 and b8; and Hoxc4, c5, c6, and c8. Lumbar LMC neurons expressed 9 proteins: Hoxa9, a10, and a11; Hoxc10 and c11; and Hoxd8, d9, d10, and d11 (Figure S2). In this study, we have focused on the link between Hox activity, motor pool identity, and target-muscle connectivity at brachial levels of the spinal cord.

At brachial levels, each of the 11 Hox proteins was detected in newly generated LMC neurons by stage 20, and their expression persisted until at least stage 35 (Figure S2 and data not shown). Within the brachial LMC, defined by Hox6 and RALDH2 expression (Dasen et al., 2003), the rostrocaudal domains of Hox5 (Hoxa5 and Hoxc5) and Hoxc8 proteins were segregated, with a transition midway along the LMC (Figures 2A and 2B): rostral LMC neurons expressed Hox5 proteins, whereas caudal LMC neurons expressed Hoxc8. In the transitional zone, Hox5 and Hoxc8 proteins were never coexpressed at high levels by individual LMC neurons (Figure 2B; Dasen et al., 2003). In contrast, Hox3, Hox4, and Hox7 proteins were initially expressed in domains that overlapped each other and overlapped with Hox5 and Hoxc8 proteins (Figure 2I and Figure S4). Members of each paralog group exhibited distinct rostral and caudal limits of expression within the LMC (Figure S4). Thus, the initial profiles of Hox3, Hox4, Hox5, Hox7, and Hox8 proteins divide the LMC into rostrocaudal subdomains (Figure 2I).

We examined how the rostrocaudal profile of Hox proteins within the LMC is established. The initial position of the brachial LMC, defined by Hox6 and RALDH2, appears to be determined by the exposure of neural-tube cells to graded FGF signaling (Liu et al., 2001; Dasen et al., 2003). We examined whether the rostrocaudal positioning of Hox proteins within the LMC is also regulated by FGF signaling. In ovo electroporation was used to elevate the level of expression of *FGF8* in the

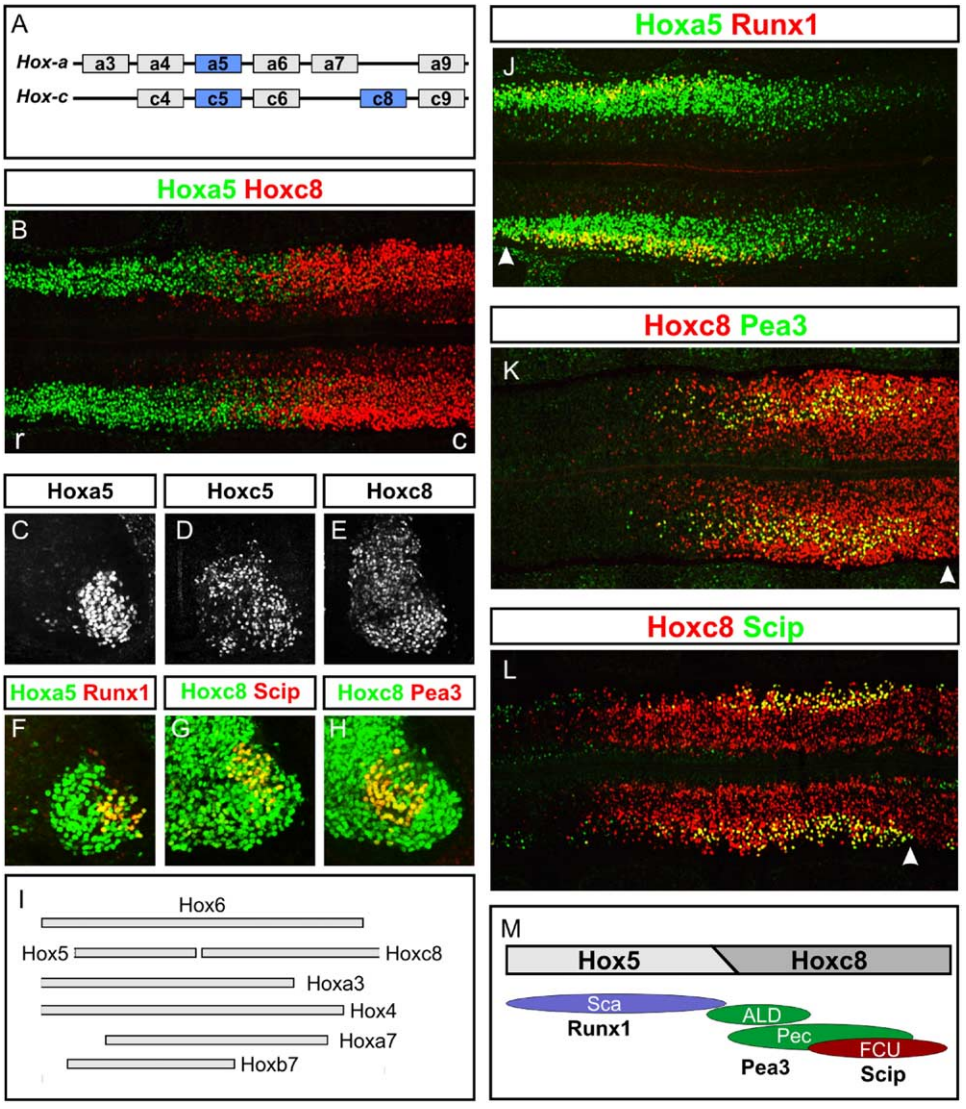

Figure 2. Hox5 and Hoxc8 Expression Subdivides the Brachial LMC and Its Resident Motor Pools

(A) Organization of *Hox3* to *Hox9* genes within the *Hox-a* and *Hox-c* clusters.

(B) Mutual exclusion of Hoxa5 and Hoxc8 by brachial LMC neurons shown in a top-down view of a longitudinal section through a stage 27 embryo.

(C–E) Expression of Hoxa5, Hoxc5, and Hoxc8 by MNs and other ventral spinal neurons (transverse sections).

(F–H) Runx1$^+$ Sca MNs form a subgroup of Hoxa5$^+$ MNs, whereas Pea3$^+$ Pec and Scip$^+$ FCU MNs are subgroups of Hoxc8$^+$ MNs.

(I) Rostrocaudal domains of Hox proteins within the brachial LMC at stage 22. (See Figure S4.)

(J–L) Runx1$^+$ Sca MNs are contained within the Hoxa5 domain; Pea3$^+$ Pec and ALD neurons and Scip$^+$ FCU MNs are contained within the Hoxc8 domain. Arrowheads indicate the rostral limit of the LMC in (J) and the caudal limit of the LMC in (K) and (L).

(M) Domains of Hox5 and Hoxc8 expression in relation to motor pools.

brachial neural tube at stage 12 (Figure S5A; Dasen et al., 2003), resulting in a coordinate rostral shift in the domains of expression of Hox3, Hox4, Hox5, Hox7, and Hox8 proteins, in register with the shift in LMC position, defined by Hox6 and RALDH2 (Figures S5A–S5D and data not shown). The position of differentiation of Runx1$^+$, Pea3$^+$, and Scip$^+$ MNs also shifted in register with Hox pattern (Figures S5E–S5H). These findings provide evidence that an early phase of FGF signaling sets the rostrocaudal limits of expression of Hox proteins and motor pool transcription factors within the LMC.

Hox5 and Hoxc8 Proteins Define the Rostrocaudal Identity and Position of Motor Pools

The reciprocity in Hox5 and Hoxc8 expression along the rostrocaudal axis of the LMC led us to examine whether the position of generation of the Sca, Pec, ALD, and FCU motor pools, as defined by transcription-factor expression and target-muscle connectivity, is established by these Hox proteins. We found that the Runx1$^+$ Sca motor pool is confined to the Hox5 domain, whereas the Pea3$^+$ Pec and ALD pools, as well as the Scip$^+$ FCU pool, are confined to the Hoxc8 domain (Figures 2C–2H and Figures 2J–2L). The interface of exclu-

sion of Hox5 and Hoxc8 therefore distinguishes the domain of generation of the Sca motor pool from that of the Pec, ALD, and FCU pools (Figure 2M). These findings raise the question of whether altering the profiles of Hox5 and Hoxc8 within the LMC changes the rostrocaudal position at which specific MN pools are generated.

We examined whether a caudal-to-rostral switch in Hox5 and Hoxc8 elicits a corresponding switch in MN pool identity and connectivity, using RNA interference (RNAi) to eliminate Hoxc8 from caudal LMC neurons. Double-stranded RNA oligonucleotides (dsRNA) directed against *Hoxc8*, together with a marker *YFP* construct, were coelectroporated between stages 15 and 17, and motor pool identity and connectivity were assessed at stage 29. *Hoxc8* dsRNA eliminated Hoxc8 from >95% of electroporated LMC neurons (Figures 3A and 3B; Table S1). The loss of Hoxc8 was without effect on generic aspects of MN differentiation, defined by Isl1/2 expression, or on LMC differentiation, defined by Hoxc6 and RALDH2 (Figures 3C and 3D). But after *Hoxc8* dsRNA expression, the number of electroporated LMC neurons that expressed Pea3 or Scip was decreased by >95%, whereas Pea3 and Scip expression persisted in nearby nonelectroporated neurons (Figures 3G–3I; Table S1). This finding is consistent with a recent genetic study in mice (Vermot et al., 2005). Thus, Hoxc8 activity is required to specify the transcriptional identity of the Pec, ALD, and FCU motor pools.

Elimination of Hoxc8 also resulted in a caudal expansion in the domains of Hoxa5 and Hoxc5 expression (Figures 3E and 3F; Table S1), indicating that Hoxc8 activity participates in establishing the interface of exclusion between Hox5 and Hoxc8 proteins within the LMC. And ~20% of the ectopic caudal Hoxc8^{off},Hox5^{on} LMC neurons expressed Runx1 (Figure 3J; Table S1), indicating that they had acquired the transcriptional profile of the Sca motor pool. We assume that Hoxc8^{off},Hox5^{on} neurons that lack Runx1 expression have acquired other rostral pool identities not assayed here. These findings indicate that the generation of a Hoxc8^{off}, Hox5^{on} domain in the caudal region of the LMC elicits a caudal-to-rostral switch in the transcriptional identity of MN pools (Figure 3K).

We next examined whether the change in transcriptional status of motor pools elicited by elimination of Hoxc8 is accompanied by a change in muscle connectivity. We first determined if the Runx1$^+$ neurons induced at ectopic caudal positions innervated their normal muscle target in the limb. After injection of HRP into the Sca muscle of *Hoxc8* dsRNA-electroporated embryos, >50% of ectopic caudal Runx1$^+$ LMC neurons accumulated HRP (Figures 3L–3O), confirming their Sca pool identity by connectivity as well as transcriptional status. Ectopic Runx1$^+$ neurons that lacked HRP may simply not have been labeled effectively by these muscle injections. Consistent with this view, we found that all HRP-labeled LMC neurons expressed Runx1 (Figure 3N), indicating that the Sca muscle is innervated selectively by Runx1$^+$ neurons. We also examined whether the loss of Pea3 expression in caudal LMC neurons in *Hoxc8* dsRNA/*YFP*-electroporated embryos is accompanied by the loss of innervation of their normal Pec muscle target. After HRP injection into the Pec muscle, <2% of electroporated (YFP$^+$) LMC neurons accumulated HRP, whereas most nearby nonelectroporated Pea3$^+$ Pec neurons did accumulate HRP (Figures 3P–3S). These findings indicate that the caudal-to-rostral switch, from Pea3 to Runx1 expression, is accompanied by a corresponding switch in muscle connectivity.

Conversely, we examined the consequences of a rostral-to-caudal switch in the status of Hox5 and Hox8 expression within LMC neurons. To achieve this, Hoxc8 was expressed ectopically at rostral brachial levels of stage 15–17 spinal cord by in ovo electroporation, and motor pool pattern and connectivity were assessed at stages 29–34. The rostral expansion of Hoxc8 extinguished Hox5 and Runx1 expression in LMC neurons in a cell-autonomous manner (Figures 4A and 4B; Table S1; Dasen et al., 2003). Nearby neurons that lacked Hoxc8 retained both Hox5 and Runx1 (Figures 4A and 4B and data not shown), indicating the cell-autonomous influence of Hox5/8 status on motor pool identity. Of these ectopic rostral Hoxc8$^+$ MNs, ~20% expressed Pea3 (Figure 4C; Figure S6B). ~85% of these Pea3$^+$ neurons expressed Isl1 and lacked Lim1, a LIM homeodomain profile characteristic of Pec MNs, and ~15% expressed Lim1 but not Isl1, an ALD transcriptional identity (Figure S6C and data not shown). Expanding the domain of Hoxc8, however, did not result in a rostral extension in the position of Scip$^+$ MNs (data not shown), a finding we discuss below. Thus, the generation of a Hoxc8^{on},Hox5^{off} domain in the rostral region of the LMC elicits a rostral-to-caudal switch in the transcriptional identity of certain caudal MN pools (Figure 4G).

We examined whether Pea3$^+$ neurons generated at ectopic rostral positions in response to Hoxc8 innervated their normal muscle target. Tracer was injected into the Pec muscle of *Hoxc8*-electroporated embryos, and the position and transcriptional status of HRP-labeled LMC neurons was analyzed at stages 34 to 35. Between 30% and 50% of ectopic rostral Hoxc8$^+$,Pea3$^+$ MNs were labeled with HRP (Figures 4D–4F), indicative of their Pec pool connectivity. Moreover, all the rostral LMC neurons that accumulated HRP expressed Pea3 (Figure 4E), indicating that the Pec muscle is still innervated selectively by Pea3$^+$ MNs. These findings provide evidence that the rostral-to-caudal switch in the transcriptional profile of LMC neurons, from Runx1 to Pea3 expression, results in a corresponding alteration in muscle connectivity (Figure 4G).

In contrast to the changes in transcriptional profile elicited by rostral Hoxc8 expression, caudal expression of Hoxc5 or Hoxa5 did not repress Hoxc8 in LMC neurons (Figure S6D). Moreover, caudal expression of Hoxc5 or Hoxa5 did not induce the generation of Runx1$^+$ MNs (data not shown), and impaired by only ~15% the generation of Pea3$^+$ MNs (Figure S6D). Thus, the boundary of Hox5 and Hoxc8 expression within the LMC appears to be established by a unilateral repressive influence of Hoxc8.

These rostrocaudal transformations in MN pool identity could result from the loss or gain of Hoxc8 expression itself or from compensatory change in Hox5 expression. To determine whether Hoxc8 activity is required within its normal domain, independently of Hox5, we made use of the finding that Hox proteins have dual

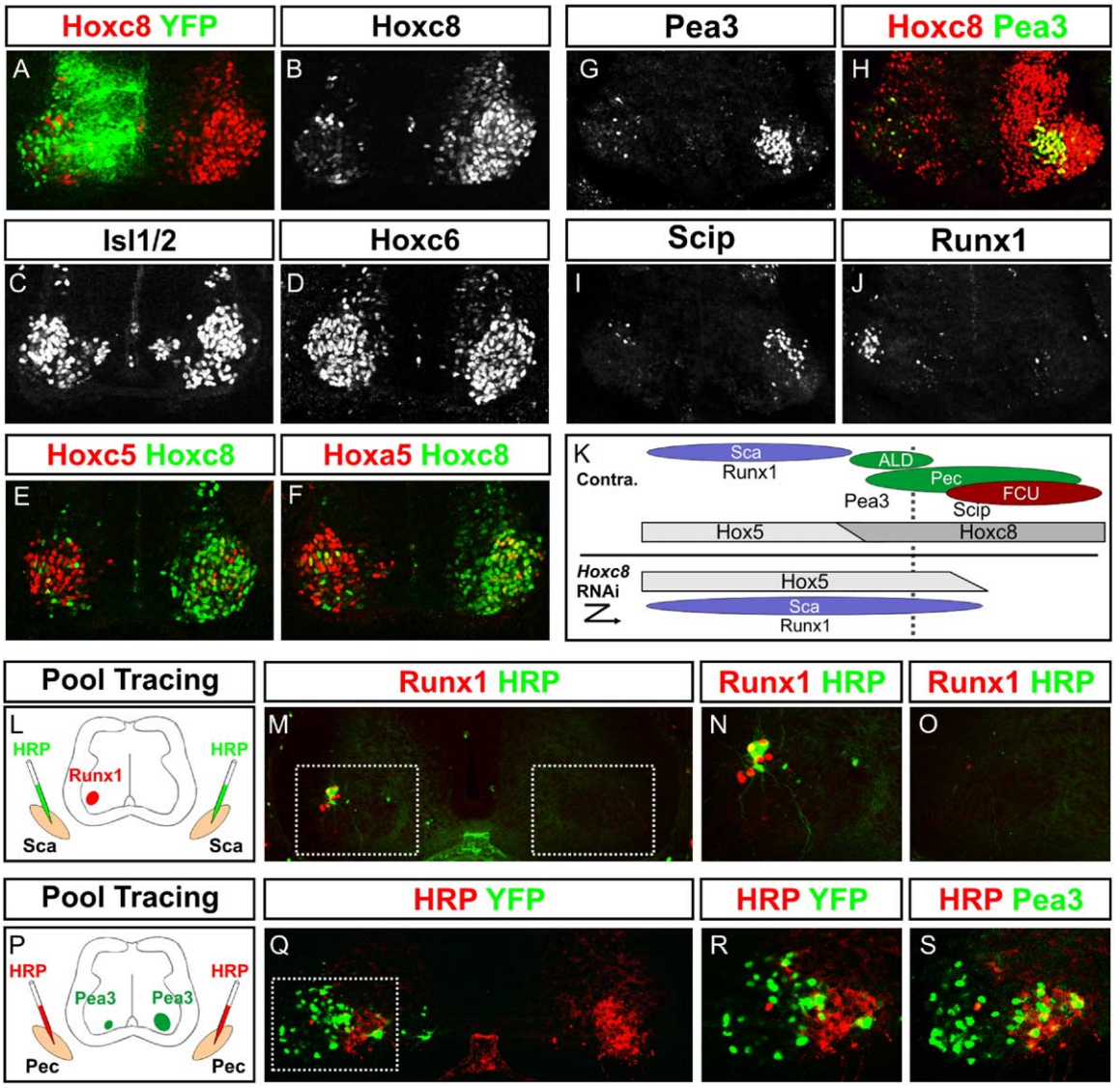

Figure 3. RNAi Elimination of Hoxc8 Elicits a Caudal-to-Rostral Switch in Motor Pool Identity and Muscle Connectivity

(A and B) Electroporation of siRNA directed against *Hoxc8* eliminates Hoxc8 protein expression in a cell-autonomous manner. Electroporated neurons are marked by coexpression of YFP.

(C and D) *Hoxc8* dsRNA does not affect Isl1/2 or Hoxc6 expression.

(E and F) LMC neurons that have lost Hoxc8 acquire expression of Hoxc5 and Hoxa5.

(G–I) Loss of Hoxc8 is accompanied by the loss of Pea3 and Scip.

(J) Loss of Hoxc8 is accompanied by the ectopic caudal expression of Runx1. The domain of caudal expansion of Hox5 and Runx1 does not extend throughout the entire region within which Hoxc8 is extinguished (Figure S6A), suggesting an additional, Hoxc8-independent constraint on the caudal extent of Hox5 and Runx1 expression.

(K) Effects of *Hoxc8* dsRNA on Hox and motor pool pattern. Segmental level of analysis is indicated by a vertical dashed line.

(L–O) HRP injection into the Sca muscle of *Hoxc8* dsRNA embryos labels ectopic caudal Runx1^{+} LMC neurons.

(P and Q) HRP injection into the Pec muscle of *Hoxc8* dsRNA embryos shows that LMC neurons that fail to express Pea3 also fail to project to the Pec muscle.

(R and S) Caudal LMC neurons that are not electroporated express Pea3 and project to the Pec muscle. Pea3^{+} YFP^{-} neurons that have not accumulated HRP project to the ALD muscle.

functions as repressors and activators in the control of MN columnar differentiation (Dasen et al., 2003). Hox repressor functions ensure the exclusion of other Hox proteins, whereas Hox activator functions induce the expression of downstream markers of MN differentiation (Dasen et al., 2003). We generated repressor forms of Hoxc8 and also of Hox5 proteins by fusing them to the repressor domain of the *Drosophila* engrailed protein (EnR). Expression of EnR-Hoxc8 at caudal levels did not impair LMC differentiation or result in caudal Hox5 expression (Figure S6E; Dasen et al., 2003) but did inhibit the generation of Pea3^{+} and Scip^{+} MNs in a cell-autonomous manner (Figures 4H and 4I). Conversely, expression of EnR-Hoxc5 or EnR-Hoxa5 at

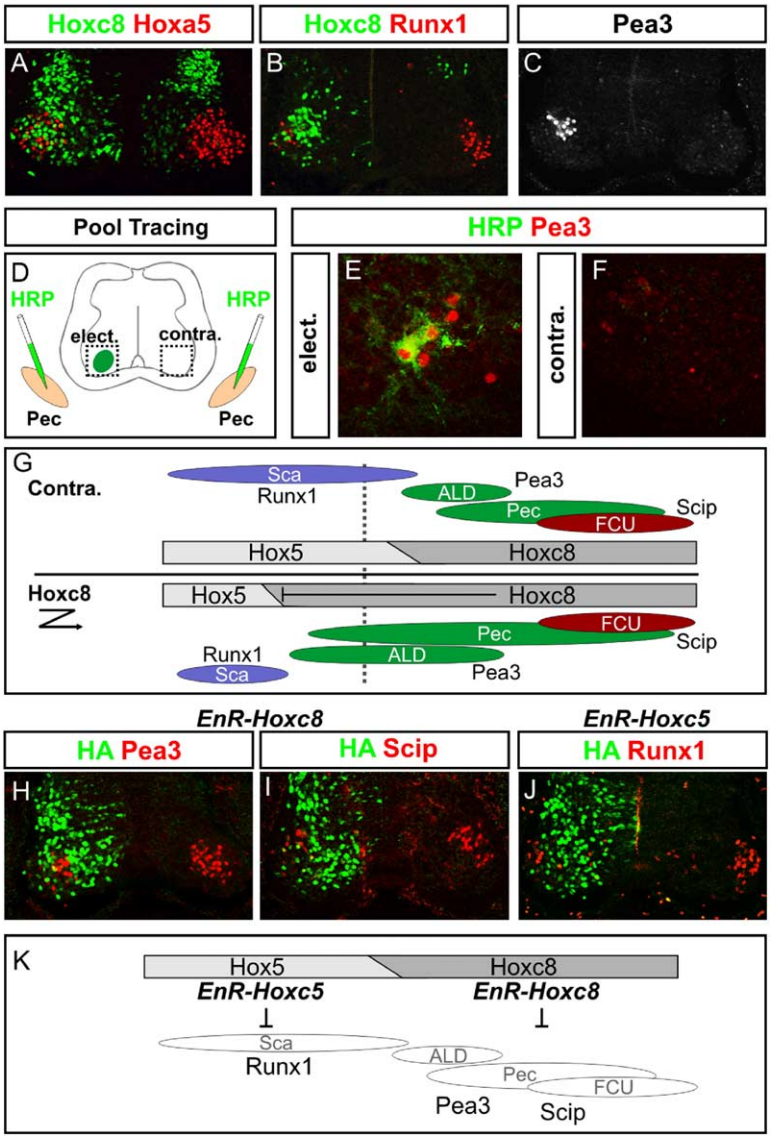

Figure 4. Expression of Hoxc8 Elicits a Rostral-to-Caudal Switch in Motor Pool Identity and Muscle Connectivity

(A) Expression of Hoxc8 in rostral LMC neurons elicits a cell-autonomous repression of Hoxa5.

(B) Rostral Hoxc8 expression represses Runx1$^+$ MNs.

(C) Rostral Hoxc8 misexpression induces Pea3$^+$ MNs.

(D–F) HRP injection into the Pec muscle reveals that ectopic rostral Pea3$^+$ MNs project to the Pec muscle. Images show electroporated and contralateral sides of the same section.

(G) Effects of rostral Hoxc8 expression on Hox pattern and motor pool identity. Segmental level of analysis is indicated by a vertical dashed line.

(H and I) Expression of EnR-Hoxc8 within the caudal LMC blocks the generation of Pea3$^+$ and Scip$^+$ MNs. Electroporated neurons in (H)–(J) are marked using an antibody against an N-terminal HA tag.

(J) Expression EnR-Hoxc5 within the rostral LMC blocks the generation of Runx1$^+$ MNs.

(K) Summary of influence of EnR-Hox derivatives on motor pool specification.

rostral levels of the LMC did not affect MN columnar differentiation or Hoxc8 pattern (Figures S6F and S6G) but did result in a cell-autonomous inhibition in the generation of Runx1$^+$ MNs (Figure 4J). These findings provide evidence that the activator functions of Hoxc8 and Hox5 are normally involved in the specification of motor pool identity (Figure 4K).

Many LMC motor pools exhibit rostral and/or caudal limits that are nested within the broader domains of Hox5 and Hoxc8 expression (Figure S4). It seems likely that the differing profiles of Hox3, Hox4, and Hox7 expression (Figures S4 and S7) define the identity and position of additional motor pools along the rostrocaudal axis of the LMC.

Intrasegmental Coding of Motor Pool Diversity by Hox and Meis Proteins

A second major feature of motor pool organization is the diversity of pools generated at a single segmental level of the LMC. We have explored whether Hox proteins also control intrasegmental aspects of motor pool diversification, focusing on the caudal, Hoxc8^{on} domain of the LMC and, in particular, on the Pec, ALD, and FCU pools.

We first assessed how the patterns of Hox expression within the LMC change over the period of intrasegmental pool diversification. The initially extensive domains of Hox3, Hox4, Hox6, and Hox7 expression evident at stages 24 to 25 (Figure S4) had become more restricted by stages 29 to 30. These restrictions occurred along the rostrocaudal axis and, in some instances, within the two divisions of the LMC (Figure S7). For example, by stage 29, Hoxa7 is restricted to a caudal set of lateral, Lim1$^+$ LMC neurons that abut the caudal end of the ALD pool, whereas Hoxc4 is restricted to largely complementary sets of LMC neurons (Figure S7). In addition, Hoxc6 protein expression became restricted to subsets of LMC neurons by stage 29 (see below). We document below that these restrictions in Hox4, Hox6, and Hox7 expression have a critical influence on the assignment of motor pool identity.

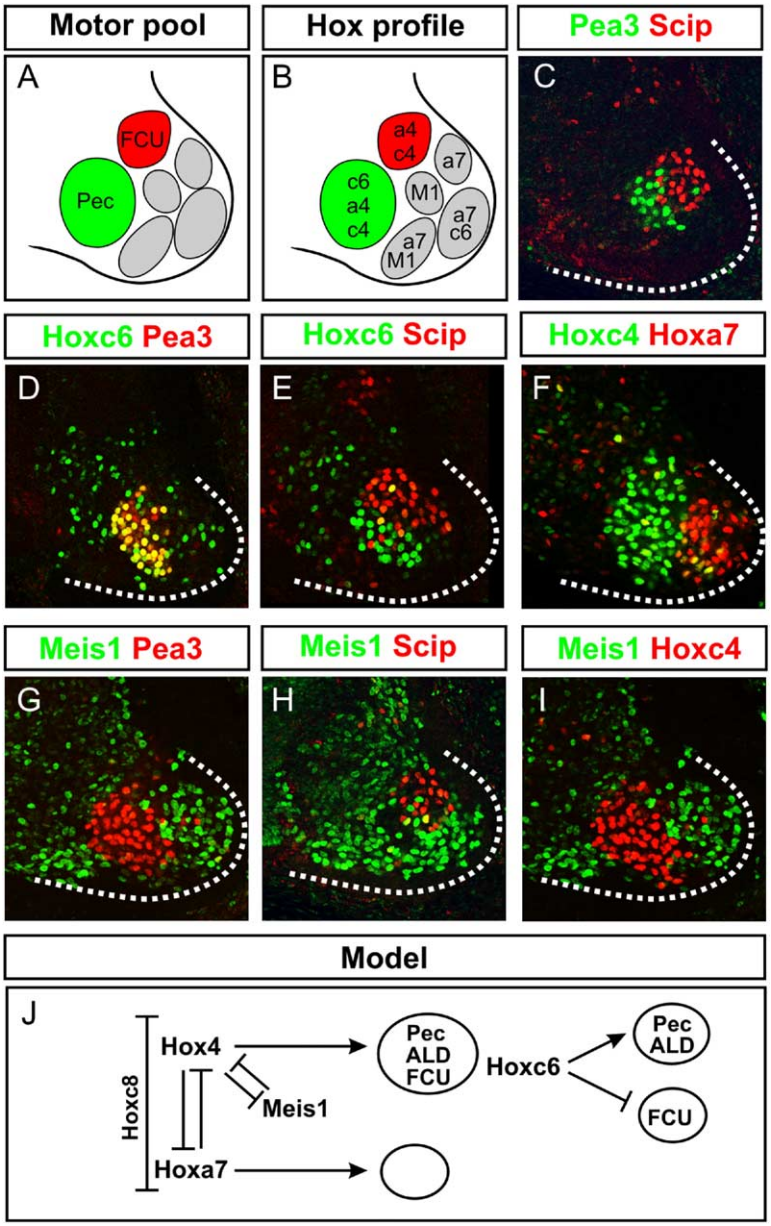

Figure 5. Patterns of Hox and Meis Expression by MNs at Caudal Levels of the LMC

(A) Simplified motor pool map at caudal LMC level, showing Pec and FCU motor pools within the medial LMC.

(B) Hox and Meis protein-expression profiles at a level containing Pec and FCU pools. M1, Meis1; Hoxc6 is shown as c6, etc.

(C) Pea3 and Scip mark adjacent but distinct MN nuclei in a transverse section of the caudal LMC at stage 29.

(D–I) Hox and Meis expression by caudal LMC neurons at stages 29–31.

(D) Hoxc6 expression is detected in Pea3$^+$ MNs.

(E) Scip$^+$ MNs do not express high levels of Hoxc6.

(F) Hoxc4 and Hoxa7 expression in LMC neurons is mutually exclusive in caudal LMC neurons at stage 31.

(G–I) Meis1 is excluded from the domain of Pea3, Scip, and Hox4 expression.

(J) Potential regulatory interactions inferred from Hox profiles in Pec and FCU pools.

At caudal levels of the LMC, the profile of Hoxa4, a6, and a7; Hoxc4, c6, and c8; and Meis1 expression was informative in distinguishing the Pec, ALD, and FCU pools from each other and from adjacent caudal pools (Figures 5A–5I). All three pools expressed Hox4 proteins, whereas most other caudal motor pools lacked Hox4 proteins (Figure 5F and Figure S8). Conversely, these three pools were characterized by the exclusion of Meis1 and Hoxa7 (Figures 5F–5I). Hoxc6 was maintained in the Pea3$^+$ Pec and ALD pools but extinguished from the Scip$^+$ FCU pool (Figures 5D and 5E and data not shown), whereas Hoxa6 was excluded from all three pools (data not shown). We note that the Pec and ALD pools resemble each other in their expression of Hox4 and Hox6 proteins and their exclusion of Meis1 and Hoxa7 proteins (Figures S7 and S8 and data not shown), an intriguing finding, given that both pools express Pea3. This analysis therefore reveals that the differential profile of expression of Hox4 and Hox6 proteins distinguishes the Pec and FCU pools.

The profiles of Hox and Meis expression make three predictions about the regulatory interactions that control the formation of the Pec and FCU motor pools (Figure 5J). First, the generation of Pec, ALD, and FCU MNs might involve the exclusion of Meis1 and/or Hoxa7. Second, the formation of Pec, ALD, and FCU MNs is dependent on Hox4 activity. Third, the differential expression of Hoxc6 directs the diversification of the Pec and FCU pools. We tested these predictions by changing the profile of Hox and Meis expression using ectopic expression and RNAi strategies.

Meis1 Exclusion and Motor Pool Specification

To test whether the exclusion of Meis1 is required for the specification of the Pec, ALD, or FCU pools, we

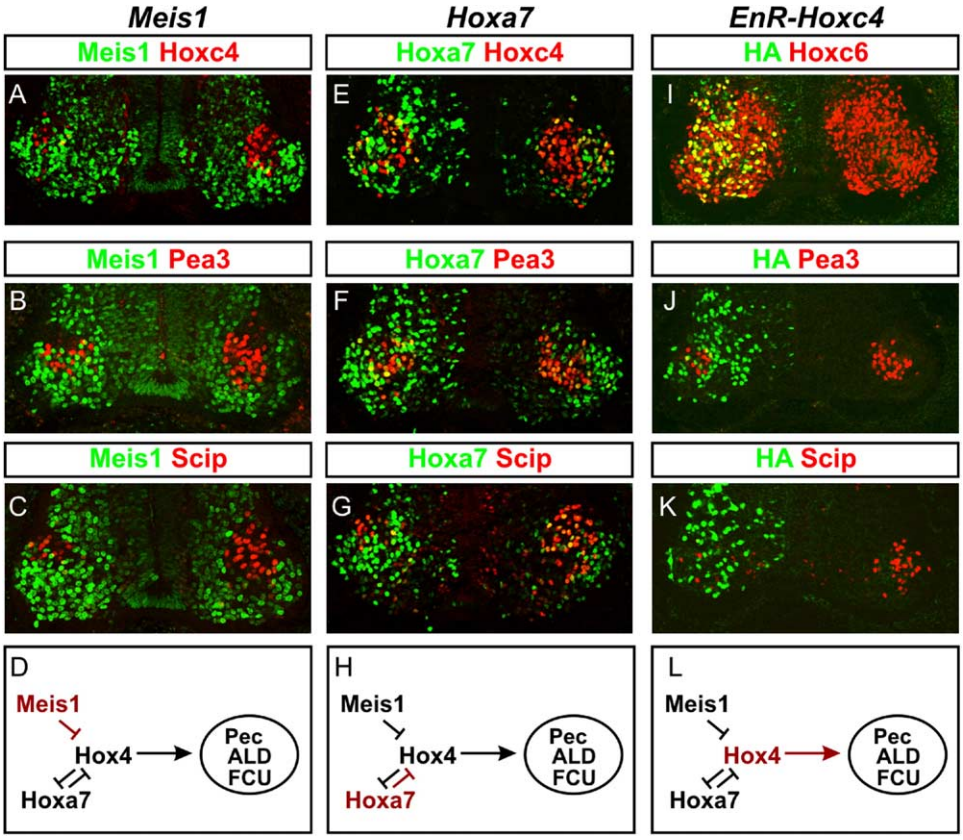

Figure 6. Activities of Meis1, Hoxa7, and Hox4 in Motor Pool Specification

(A–D) Ectopic expression of Meis1 represses Hoxc4, Pea3, and Scip. Meis1-electroporated neurons were identified by coelectroporation with *CMV-GFP* plasmids (data not shown). (D), (H), and (L) summarize interactions of Meis1, Hox7 and Hox4 relevant to the formation of Pec and FCU pools.

(E–H) Ectopic Hoxa7 expression represses Hoxc4, Pea3, and Scip.

(I–L) Ectopic expression of EnR-Hoxc4 does not affect Hoxc6 but represses Pea3 and Scip.

examined the consequences of ectopic Meis1 expression at caudal levels of the LMC. Misexpression of Meis1 did not affect caudal LMC specification, assessed by the persistence of early expression of Hox6 and Hoxc8 proteins and of RALDH2 at stage 29 (Figure S9A and data not shown). Expression of Meis1 did, however, repress the expression of Hox4 proteins in brachial LMC neurons in a cell-autonomous manner (Figure 6A; Table S1). Expanded Meis1 expression also inhibited the differentiation of Pea3$^+$ and Scip$^+$ MNs in a cell-autonomous manner, indicative of a block in Pec, ALD, and FCU pool formation (Figures 6B and 6C; Table S1). Thus, the exclusion of Meis1 appears necessary for the specification of Pec, ALD, and FCU pools (Figure 6D).

Hoxa7 Exclusion and Motor Pool Specification

We also tested whether the exclusion of Hoxa7 is required for the specification of the Pec, ALD, or FCU pools. Expression of Hoxa7 in caudal LMC neurons did not affect the pattern of Hox6 and Hoxc8 expression or of RALDH2 (Figure S9C and data not shown). Yet Hoxa7 repressed Hox4 proteins and inhibited the differentiation of Scip$^+$ FCU, Pea3$^+$,Isl1$^+$ Pec, and Pea3$^+$,Lim1$^+$ ALD MNs (Figures 6E–6G; Table S1). Hoxa7 expression did not influence the pattern of expression of Meis1, nor did Meis1 influence Hoxa7, suggesting that these two factors act independently in their restrictive influence on Hox4 expression (Figures S9B and S9D). Thus, the exclusion of Hoxa7 permits the formation of Pec, ALD, and FCU MNs (Figure 6H).

Hox4 Activity and Motor Pool Specification

The ability of ectopic Meis1 and Hoxa7 to suppress Hox4 expression and block the differentiation of Pea3$^+$ and Scip$^+$ MNs raised the possibility that Hox4 activity might be required to generate Pec, ALD, and FCU motor pools. To test the requirement for Hoxc4 activity in motor pool specification, we ectopically expressed an EnR-Hoxc4 fusion protein within caudal LMC neurons. EnR-Hoxc4 did not block LMC formation, as assessed by Hoxc6 and RALDH2 (Figure 6I and data not shown), but it did block the differentiation of Pea3$^+$ and Scip$^+$ MNs in a cell-autonomous manner (Figures 6J and 6K; Table S1). These observations provide evidence that the transcriptional-activator function of Hox4 proteins is required for Pec, ALD, and FCU pool formation (Figure 6L). Furthermore, expanding the domain of Hoxc4 repressed expression of Hoxa7 (Figure S9E), indicating that the emergence of largely exclusive LMC domains

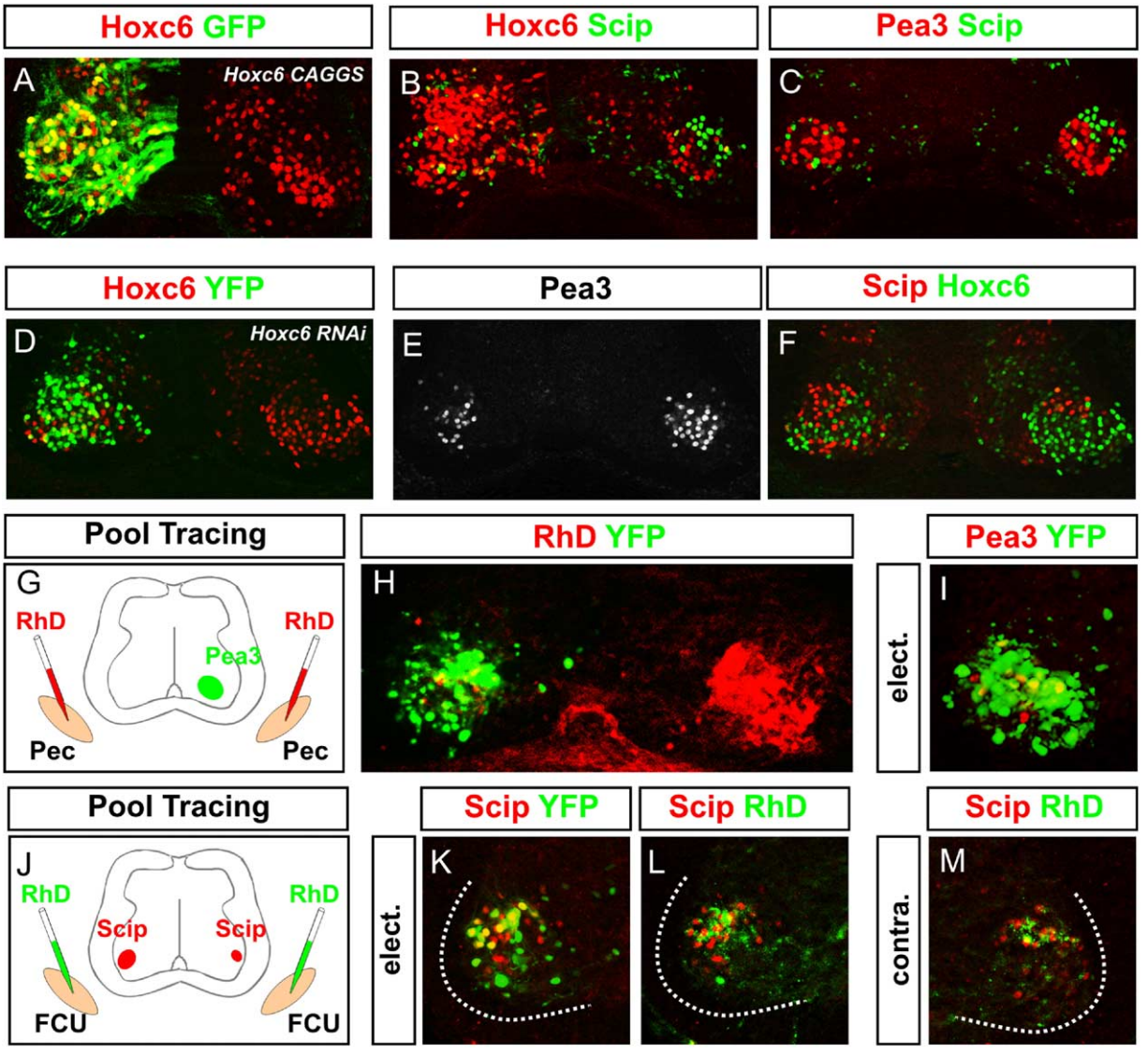

Figure 7. The Status of Hoxc6 Expression Regulates Pec and FCU Pool Specification

(A–C) Expanded expression of Hoxc6 represses Scip$^+$ MNs and increases the number of Pea3$^+$ MNs. The increase in the number of Pea3$^+$ neurons typically corresponded to ~40% of the number of lost Scip$^+$ neurons, suggesting that the gain of Hoxc6 converts some Scip$^+$ neurons to alternative, Pea3$^-$ motor pool fates.

(D–F) Effect of *Hoxc6* RNAi on Scip and Pea3 expression.

(D) MNs electroporated with *Hoxc6* siRNAs (marked by nuclear YFP) fail to express Hoxc6.

(E and F) *Hoxc6* RNAi blocks the generation of Pea3$^+$ MNs and expands the number of Scip$^+$ MNs.

(G–I) After *Hoxc6* RNAi, few if any electroporated (YFP$^+$) neurons at caudal LMC levels express Pea3, and very few are labeled with RhD after injection into the Pec muscle.

(J–M) After *Hoxc6* RNAi, a high proportion of YFP$^+$, Scip$^+$, Hoxc6$^-$ MNs are labeled with RhD after injection into the FCU muscle.

of Hox4 and Hoxa7 result from their crossrepressive interactions.

Hox6 Activity and Motor Pool Specification

We next examined whether the late restriction of Hoxc6 underlies the diversification of the Pea3$^+$ Pec and Scip$^+$ FCU pools. As a first test, we used electroporation to expand the domain of Hoxc6 expression in caudal LMC neurons. Expansion of Hoxc6 produced a cell-autonomous inhibition in the generation of Scip$^+$ MNs and an increase in the total number of Pea3$^+$ MNs (Figures 7A–7C; Table S1). We next examined the consequences of RNAi-mediated elimination of Hoxc6 expression on Pec and FCU pool fates. At early stages of LMC differentiation, the profiles of Hoxa6 and Hoxc6 overlap, and both proteins possess similar LMC-inducing activity (Dasen et al., 2003). We reasoned, therefore, that the loss of Hoxc6 might influence motor pool fate without impairing earlier aspects of LMC specification. Consistent with this idea, coelectroporation of dsRNA directed against *Hoxc6* and a marker *YFP* construct eliminated Hoxc6 from many LMC neurons without eliminating Hoxa6 or preventing LMC differentiation, assessed by

RALDH2 (Figures S9G–S9I). The loss of Hoxc6 also resulted in a cell-autonomous decrease in the generation of Pea3$^+$ MNs and a complementary increase in the formation of Scip$^+$ MNs (Figures 7D–7F; Table S1; Figure S9F). Thus, the status of Hoxc6 expression in caudal LMC neurons that express Hox4 and exclude Meis1 proteins appears to underlie the selection of Pea3 or Scip expression by motor pools.

We also examined whether the change in Pea3 and Scip status of LMC neurons is accompanied by an alteration in muscle connectivity. We determined the muscle target of the expanded population of Scip$^+$ MNs generated at caudal LMC levels after Hoxc6 elimination. Injection of RhD into the Pec muscle of *Hoxc6* dsRNA/*YFP*-electroporated embryos revealed that <2% of electroporated neurons expressed Pea3 or accumulated HRP (Figures 7G–7I). Thus, neurons that have lost Pea3 fail to project to their normal muscle target. Conversely, injection of RhD into the FCU muscle of *Hoxc6* dsRNA/*YFP*-electroporated embryos resulted in retrograde labeling of ~70% of Scip$^+$,YFP$^+$ MNs (Figures 7J–7M). This finding, taken together with the ~80% increase in the number of Scip$^+$ MNs (Table S1), suggests that many, and possibly all, of the extra Scip$^+$ neurons generated after elimination of Hoxc6 project to the FCU muscle, as do their normal Scip$^+$ counterparts. Thus, the Hoxc6-dependent switch in the generation of Pea3$^+$ and Scip$^+$ neurons is accompanied by a corresponding change in the pattern of muscle-target innervation.

With this insight into the Hox network that directs the intrasegmental diversification of motor pools, we returned to our earlier observation that ectopic expression of Hoxc8 in the rostral LMC induces Pea3$^+$ MNs of Pec and ALD character but not Scip$^+$ neurons of FCU character. We examined whether this restriction reflects the status of Hox4 and Hoxc6 expression in Hoxc8-recipient cells. In support of this idea, we observed that >95% of the rostral LMC neurons that acquired Hoxc8 coexpressed Hoxc6 and Hox4 proteins and had extinguished Meis1 expression (Figures S6B and S10)—a Hox profile that promotes Pec/ALD and inhibits FCU pool differentiation. Thus, the rostrocaudal and intrasegmental programs of motor pool specification appear to obey a coherent Hox regulatory logic.

Discussion

MNs acquire specialized pool identities that direct their axons to target muscles in the limb, and the specificity of these connections is critical to locomotor behavior. We have found that three classical attributes of MN pools—their remarkable diversity, their stereotyped position, and their connectivity—are established by a Hox regulatory network, the details of which are discussed below.

A Hierarchy of Hox Regulatory Interactions Specifies Motor Pool Identity

The selectivity with which spinal MNs innervate target muscles in the developing forelimb depends on three programs of MN subtype specification (Figures 1A–1C). A program of columnar specification assigns MNs with a LMC identity that directs their axons toward the limb. A program of divisional specification assigns LMC neurons with medial or lateral identities that direct motor axons into the ventral- or dorsal-limb mesenchyme. And a program of pool specification confers LMC neurons with diverse identities that direct motor axons to individual muscle targets.

Our findings indicate that each of these programs of MN subtype specification is controlled through the coordinate activities of Hox proteins. The emergence of MN columnar identities depends on the spatial distribution of Hox6, Hox9, and Hox10 proteins along the rostrocaudal axis of the spinal cord (Dasen et al., 2003; Shah et al., 2004). The expression of Hoxa6 and Hoxc6 by newly generated MNs assigns brachial LMC columnar identity, and this transcriptional profile directs the axons of LMC neurons into the limb (Dasen et al., 2003). Hox6 proteins also initiate the program of divisional specification of LMC neurons by activating the expression of RALDH2, thus establishing LMC neurons as a source of retinoids (Sockanathan and Jessell, 1998; Solomin et al., 1998). In turn, the exposure of late-born LMC neurons to retinoids induces the patterned expression of LIM homeodomain proteins that define LMC divisional identity and direct motor axon trajectories along the dorsoventral axis of the limb mesenchyme (Kania and Jessell, 2003; Sockanathan and Jessell, 1998).

Within the columnar constraint provided by Hox6 expression, individual LMC neurons also acquire one of ~50 pool identities. This extreme instance of neuronal diversification appears to be established by the regulatory interactions of Hox4, Hox5, Hox6, Hox7, and Hox8 proteins (Figure 8A). One set of Hox interactions, exemplified by the activities of the Hox5 and Hox8 proteins, constrains motor pools to specific rostrocaudal levels of the LMC, and a second set, involving Hox4, Hox6, Hox7, and Meis1 proteins, drives the intrasegmental diversification of motor pools (Figure 8B). Moreover, the Hox regulatory rules that normally drive motor pool diversification at caudal levels of the LMC are preserved when Hox proteins are expressed at rostral levels of the LMC, indicating that these two aspects of motor pool diversification obey a unified and consistent regulatory logic. Over the period that this Hox regulatory network operates, MNs destined to populate an individual pool are still scattered within the LMC, interspersed with the inhabitants of other pools (Lin et al., 1998, Price et al., 2002). Thus, the clustering of MNs into coherent pools emerges only as a late consequence of the early assignment of their Hox identity.

This analysis of motor pool specification reinforces the view that repressive interactions between Hox proteins direct MN diversification in the spinal cord (Dasen et al., 2003). But there are distinctions in the interactions of these Hox proteins. The Hox6/Hox9 protein pair exhibits mutually repressive interactions during MN columnar specification (Dasen et al., 2003), whereas the Hox5 and Hox8 interaction that occurs during motor pool specification is asymmetric (Figure 8A). And the repressive interaction between Hox4 and Hoxa7 proteins that occurs during the intrasegmental diversification of pools appears not to be absolute and unfolds only gradually. This reliance on Hox repressive interactions to allocate identities to spinal MNs is in apparent

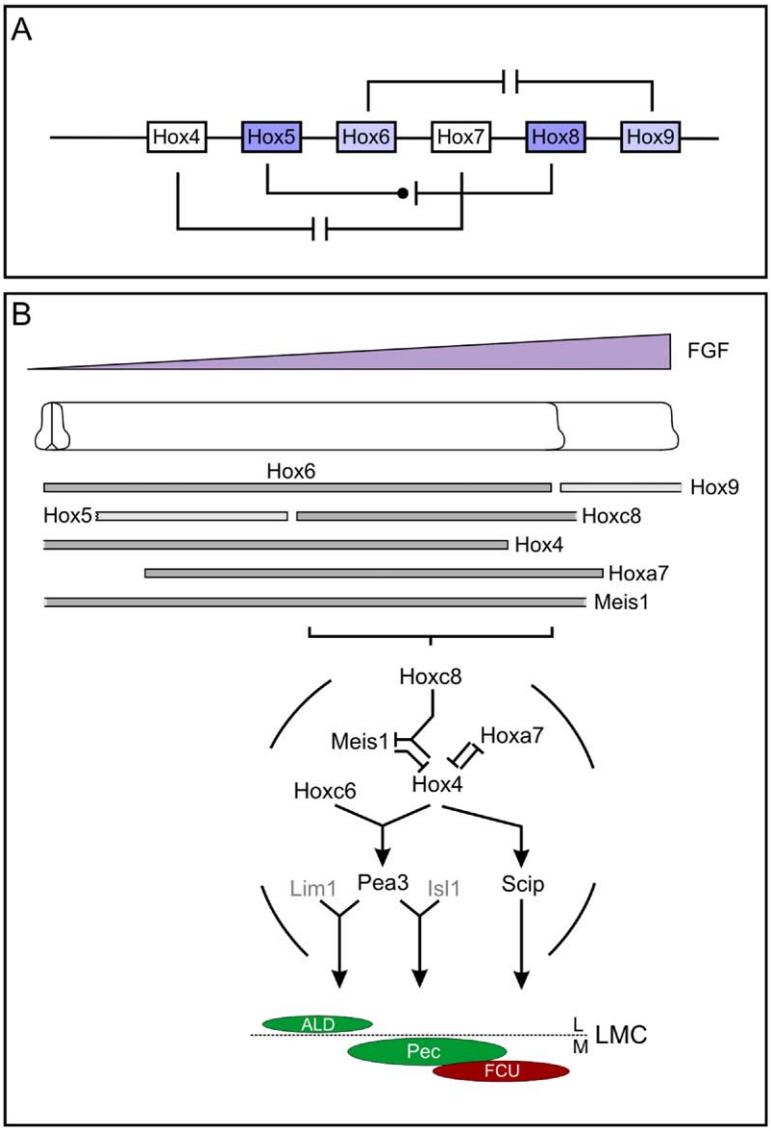

Figure 8. A Hox Regulatory Network that Specifies MN Pool Identity

(A) Schematic arrangement of chromosomal Hox clusters, showing the regulatory interactions that operate during the assignment of MN columnar and pool fates at brachial levels of the spinal cord. The Hox interactions that direct columnar and pool identities at brachial and thoracic levels adhere to a "nΔ2" specificity rule: selective repressive interactions are observed between Hox paralogs separated by two intervening genes.

(B) Sequential steps in the assignment of pool identity within the brachial LMC. A gradient of FGF signaling at neural-tube stages establishes the initial rostrocaudal limits of Hox protein expression within brachial MNs. The caudal limit of the brachial LMC maps to the boundary of Hox6 and Hox9 expression, and the rostral limit is defined by Hoxc6 expression. Within the caudal domain of the brachial LMC, defined by the region of overlap of Hox6 and Hoxc8 expression, Hox and Meis interactions resolve to generate LMC neurons that express different combinations of Hox4, Hox6, Hox7, and Meis1 proteins. This Hox profile directs the expression of Pea3 and Scip and, in combination with LIM homeodomain proteins, specifies the identity of three MN pools that innervate the ALD, Pec, and FCU muscles.

contrast with the Hox circuitry involved in hindbrain patterning, where positive regulatory interactions have been emphasized (Gavalas et al., 2003; Nonchev et al., 1997).

Extrinsic and Intrinsic Determinants of Motor Pool Identity

The generic and columnar fates of spinal MNs are assigned by extrinsic signals that operate along the dorsoventral and rostrocaudal axes of the neural tube (Jessell, 2000; Dasen et al., 2003). The strategy for assigning pool identity and position along the rostrocaudal axis of the LMC appears to be a variant of that used to direct MN columnar differentiation. The initial rostrocaudal positioning of the Hox6 proteins that assign brachial LMC fate is established by the early exposure of neural-tube cells to graded FGF signaling (Bel-Vialar et al., 2002; Dasen et al., 2003; Liu et al., 2001). Similarly, elevating the level of FGF signaling elicits a coordinate positional shift in the pattern of each of the Hox3 to Hox8 proteins that define aspects of brachial LMC character. Thus, the patterns of Hox expression that determine motor pool identity along the rostrocaudal axis of the LMC appear to be set by extrinsic FGF-mediated signals that act during the early phase of motor pool specification.

The mechanisms that drive the intrasegmental program of motor pool diversification are less clear. We find that the specification of motor pool identity by Hox proteins occurs in a cell-autonomous manner, suggesting that this intrasegmental program has its origins in an intrinsic Hox regulatory network. One model for such a network invokes the idea that all LMC neurons generated at a specific segmental level of the LMC initially express the same set of Hox proteins as a reflection of their rostrocaudal position. But within this cohort of equivalent neurons, the persistent coexpression of certain Hox proteins is prohibited by virtue of their mutually repressive interactions. As a consequence, minor fluctuations in starting Hox conditions within individual

MNs will result in a "winner-take-all" extinction of expression of one or the other of two opponent Hox proteins on a largely stochastic basis. The final complement of Hox proteins expressed within any given LMC neuron will therefore represent only a small subset of the starting repertoire. The existence of mechanisms that impart a bias to the otherwise stochastic outcome of Hox crossregulatory interactions could account for the observation that MNs are allocated to distinct pools in different numbers in anticipation of the size of their muscle target (Lin et al., 1998). Potential sources of such a bias include asymmetries in the strength of Hox repression or initial differences in the level or onset of Hox expression within individual neurons. We note that this stochastic view of motor pool diversification has elements in common with the workings of transcriptional-repressor networks that have been engineered de novo in bacterial model systems (Elowitz and Leibler, 2000).

Hox Proteins Direct Motor Pool Transcriptional Identity and Target Connectivity

The proposal that developing MNs possess intrinsic pool identities that direct target-muscle connectivity emerged first through embryological manipulations that revealed that the axons of specific LMC neurons project to their targets with high precision (Landmesser, 1978), even when forced to enter the limb from aberrant positions (Landmesser, 2001). Our findings show that experimental alteration in Hox profile reliably predicts the change in transcriptional identity of motor pools and the subsequent specificity of target-muscle connections. Thus, a Hox regulatory network appears to lie at the heart of selective MN connectivity with limb muscles, providing a molecular basis for interpretation of these classic studies.

These findings also provide insight into the way Hox activities coordinate motor axon trajectory in the developing limb. On arriving at the base of the limb, the axons of LMC neurons select a ventral or dorsal trajectory in the limb mesenchyme and then establish specific anteroposterior and proximodistal trajectories that take them to the position of newly cleaved muscle masses (Tosney and Landmesser, 1985). The Hox6-activated program of LIM homedomain protein expression within the LMC determines the dorsoventral trajectory of motor axons through regulation of Eph expression and signaling (Kania and Jessell, 2003). In a complementary way, pool-specific profiles of Hox protein expression are likely to determine muscle-nerve trajectory along the anteroposterior and proximodistal axes of the limb (Stirling and Summerbell, 1988). Hox proteins are also expressed by limb mesenchymal cells (Izpisua-Belmonte and Duboule, 1992) and could contribute to the establishment of motor axon trajectory by positioning guidance cues at specialized "decision regions" within the limb mesenchyme (Tosney and Landmesser, 1985).

The selection of specific muscle-nerve trajectories may be determined through the activities of the pool-restricted transcription factors that are induced by Hox proteins. Nkx6 homeodomain proteins are expressed by LMC neurons in a pool-specific pattern that is controlled by Hox proteins (Dasen et al., 2003), and, in *Nkx6.1* mutant mice, these MN pools innervate foreign muscle targets (N.V. De Marco and T.M.J., unpublished data). Moreover, Pea3 expression is critical for later aspects of motor pool differentiation that occur after motor axons have reached their targets, most notably the clustering of MNs into pools (Lin et al., 1998; Livet et al., 2002). Thus, the output of MN Hox regulatory circuits appears to be mediated through the expression of downstream pool transcription factors, which in turn direct target-muscle connectivity and motor pool clustering.

The central role of Hox proteins in assigning LMC divisional identity raises the issue of whether divisional status is a factor in the generation of pool diversity. Evidence that LIM homeodomain proteins control LMC divisional identity and axonal trajectory without influencing the Hox network that assigns pool identity emerges from an analysis of the transcriptional profile of the Pec and ALD motor pools. Pec MNs are located in the medial division of the LMC and express Isl1, whereas ALD MNs are located in the lateral division of the LMC and express Lim1. Yet both pools possess a common Hox profile and share expression of Pea3 (Figure 8B). Thus, the same Hox profile can be used to assign a common pool character to sets of MNs located in the two divisions of the LMC, potentially halving the numerical challenge inherent in motor pool diversification.

Hox Genes and Neural-Circuit Assembly

The task of specifying over 50 MN classes, each projecting to a specific target cell group, appears to have been met by deploying the regulatory interactions of members of a structurally related and chromosomally clustered set of 39 Hox proteins. Hox genes are also involved in establishing MN subtype identity in the hindbrain (Bell et al., 1999; Gaufo et al., 2003; Studer et al., 1996). Yet the informational content resident in the combinatorial use of Hox proteins far exceeds the requirements for MN diversification. Since Hox proteins are also expressed by spinal interneurons and sensory neurons (Belting et al., 1998; Ensini et al., 1998), they could have a more extensive role in the assembly of spinal locomotor circuits. Finally, the early requirement for coordinated locomotor function in many vertebrate species demands that the assembly of motor circuits proceeds without extensive refinement through sensory experience (Frank and Wenner, 1993). The self-organizing features inherent in the Hox transcriptional regulatory network described here may help to endow developing MNs and motor circuits with their apparent high degree of genetic determination.

Experimental Procedures

Expression Constructs

Hox cDNAs were isolated by RT-PCR and confirmed by sequencing. cDNAs were cloned into *pCAGGS* vectors by standard procedures. For generation of HA-tagged *Drosophila* EnR (aa 2–229) derivatives, cDNAs were cloned by PCR to generate in-frame fusion proteins.

In Ovo Electroporation

Neural-tube electroporation of DNA constructs was performed on stage 12–17 chick embryos (Dasen et al., 2003). For misexpression

of Hox genes, plasmids were titrated (typically 100–500 ng/μl *pCAGGS* vector, using *CMV-GFP* plasmid as carrier DNA) to generate levels of ectopic protein expression qualitatively similar to endogenous levels. Electroporation resulted in transgene expression in the spinal cord, with no detectable expression in surrounding paraxial or lateral plate mesoderm or their derivatives. In each experiment, ~140 embryos were electroporated, with a survival efficiency of ~25%, such that each set of results reflects an analysis of ~35 manipulated embryos. Electroporation efficiencies (e.e.) in individual embryos ranged from 30% to 90% of LMC neurons at the segmental level under analysis, and results reported derive from embryos with an e.e. of >50%.

RNA Interference

RNA interference was performed by electroporation of 21 nucleotide dsRNA (Dharmacon, Option A4). To identify electroporated neurons, dsRNA (final concentration 5 mg/ml) was combined with *CMV-eGFP* or *CMV-nYFP* plasmids (final concentration 0.7 mg/ml). Targets sequences used: *Hoxc8* 5′-CTACGACTGCAGATTTCCA-3′, *Hoxc6* 5′-GCCGAGGACCTTATGACTA-3′.

In Situ Hybridization Histochemistry and Immunohistochemistry

In situ hybridization was performed as described (Dasen et al., 2003). Many *Hox* probes were a gift from Cliff Tabin. Antibodies against Hoxc5, Hoxc6, Hoxc8, and LIM homeodomain proteins have been described (Liu et al., 2001; Tsuchida et al., 1994). Antibodies against other Hox proteins are described in the Supplemental Data. Additional antibodies: mouse anti-HA 1:10,000 (Covance), goat anti-Meis1 (1:500, Santa Cruz Biotech, SC-10599), rabbit anti-Pbx1/2/3 (Santa Cruz Biotech, SC-888), goat anti-HRP 1:2000 (Jackson ImmunoResearch Inc.). Rabbit anti-Scip antibody was a gift from Deis Meijer.

Retrograde Labeling of Neurons

Retrograde labeling and analysis of MNs was performed as in Ensini et al. (1998) and Lin et al. (1998). HRP (Roche) or 3000 molecular weight rhodamine dextran (RhD) (Molecular Probes) was injected into wing muscles of stage 34–36 embryos, which were incubated at 33°C for 5–6 hr in oxygenated Tyrode's and fixed in 4% paraformaldehyde. In control, nonelectroporated embryos, the number of neurons within a specific motor pool that accumulated retrograde tracer varied between 25% and 90% depending on experiment and muscle position. For analysis of the target projections of ectopic MNs in Hox-electroporated embryos, the success of muscle injection and retrograde labeling was confirmed by evidence of tracer accumulation in host MNs located at their normal rostrocaudal position within the LMC.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and ten figures and can be found with this article online at http://www.cell.com/cgi/content/full/123/3/477/DC1/.

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