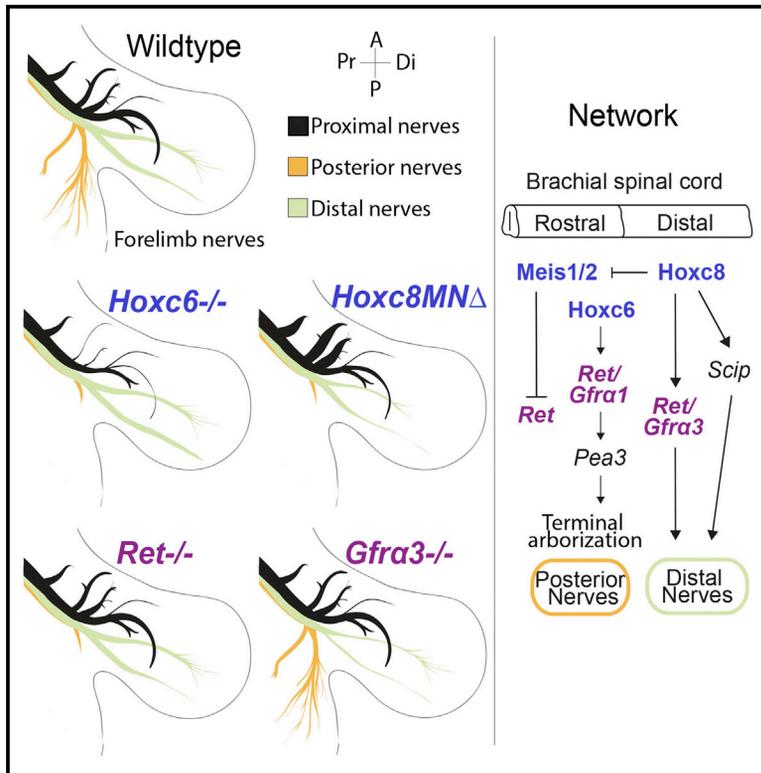


Hox Proteins Coordinate Motor Neuron Differentiation and Connectivity Programs through *Ret/Gfr α* Genes

Graphical Abstract



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In Brief

Catela et al. show that a network of Hox transcription factors governs the differentiation and connectivity of motor neurons through orchestrating the expression of *Ret* and *Gfr α* receptors. These findings indicate that Hox proteins facilitate synaptic specificity through controlling both the level and pattern of cell surface receptor expression.

Highlights

- A Hox code defines MNs projecting along the major axes of the limb
- *Ret* and *Gfr α* genes are key targets of Hox proteins in limb-innervating MNs
- Hox/Hox cofactor interactions govern *Ret* levels and *Gfr α* patterns in MNs
- Loss of *Ret* and *Gfr α 3* causes MN defects similar to *Hox* gene mutations



Hox Proteins Coordinate Motor Neuron Differentiation and Connectivity Programs through *Ret/Gfr α* Genes

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SUMMARY

The accuracy of neural circuit assembly relies on the precise spatial and temporal control of synaptic specificity determinants during development. Hox transcription factors govern key aspects of motor neuron (MN) differentiation; however, the terminal effectors of their actions are largely unknown. We show that Hox/Hox cofactor interactions coordinate MN subtype diversification and connectivity through *Ret/Gfr α* receptor genes. Hox and Meis proteins determine the levels of *Ret* in MNs and define the intrasegmental profiles of *Gfr α 1* and *Gfr α 3* expression. Loss of *Ret* or *Gfr α 3* leads to MN specification and innervation defects similar to those observed in *Hox* mutants, while expression of *Ret* and *Gfr α 1* can bypass the requirement for *Hox* genes during MN pool differentiation. These studies indicate that Hox proteins contribute to neuronal fate and muscle connectivity through controlling the levels and pattern of cell surface receptor expression, consequently gating the ability of MNs to respond to limb-derived instructive cues.

INTRODUCTION

Correct wiring of nervous systems involves both cell-intrinsic factors that contribute to neuronal subtype identities and cell surface recognition systems that facilitate the connectivity of individual neurons. Although synaptic specificity determinants have been described in many systems (Dudanova and Klein, 2013; Kolodkin and Tessier-Lavigne, 2011; Sanes and Yamagata, 2009), the regulatory mechanisms governing the expression of guidance and adhesion molecules are poorly defined. Cell fate determinants likely play a key role in orchestrating synaptic specificity programs, but there are few examples where the relationships between transcription networks and cell surface receptors have been established (Polleux et al., 2007; Santiago and Bashaw, 2014; Zarin et al., 2014b).

Progress toward defining how neuronal identity directs connectivity has emerged through the analysis of guidance decisions within the vertebrate spinal cord (Bonanomi and Pfaff, 2010; Klein and Kania, 2014). Motor neurons (MNs) targeting limb muscles are contained within the lateral motor columns (LMCs), which further differentiate into divisional and pool subtypes targeting specific limb regions (Landmesser, 2001). Neurons within the lateral division of the LMC (LMCl) innervate dorsal limb muscles, while medial LMC (LMCm) neurons project ventrally (Landmesser, 1978a; Tosney and Landmesser, 1985). MN pools positioned rostrally within the LMC typically pursue an anterior/proximal trajectory, while those residing more caudally innervate more posterior/distal limb muscles (Hollyday and Jacobson, 1990; Landmesser, 1978b). Experimental manipulations that alter MN or limb bud position indicate that motor axons can redirect their trajectories to find their appropriate targets (Ferguson, 1983; Lance-Jones and Landmesser, 1980, 1981; Stirling and Summerbell, 1988), suggesting that groups of MNs within the LMC are intrinsically programmed to select a specific pathway in response to limb-derived cues.

Depletion of factors involved in LMC subtype differentiation often disrupts the specificity of muscle target innervation, presumably due to changes in the expression of surface receptors on motor axons. For example, the Lim homeodomain (HD) transcription factors *Lhx1* and *Isl1* control expression of *Eph* guidance receptors and dictate the initial trajectories of motor axons at the base of the limb. *Lhx1* induces expression of *Epha4* in the LMCl to direct motor axons dorsally within the limb, while *Isl1* promotes *Ephb1* expression in LMCm neurons to direct axons ventrally (Helmbacher et al., 2000; Kania and Jessell, 2003; Luria et al., 2008). With the exception of this relatively simple binary decision, the mechanisms that restrict expression of surface receptors within specific MN subtypes are poorly understood.

A large family of transcription factors critical for MN subtype differentiation and connectivity are encoded by the *Hox* gene clusters, which comprise 39 genes organized in four chromosomal arrays (Philippidou and Dasen, 2013). Studies in chick have shown that *Hox* genes are differentially expressed by MNs along the rostrocaudal axis, and a network of ~20 Hox proteins defines the identities of MN pools targeting specific limb muscles (Dasen et al., 2003, 2005; Liu et al., 2001). However, whether the pattern of *Hox* gene expression in MNs is predictive of muscle target specificity is largely untested, and the precise

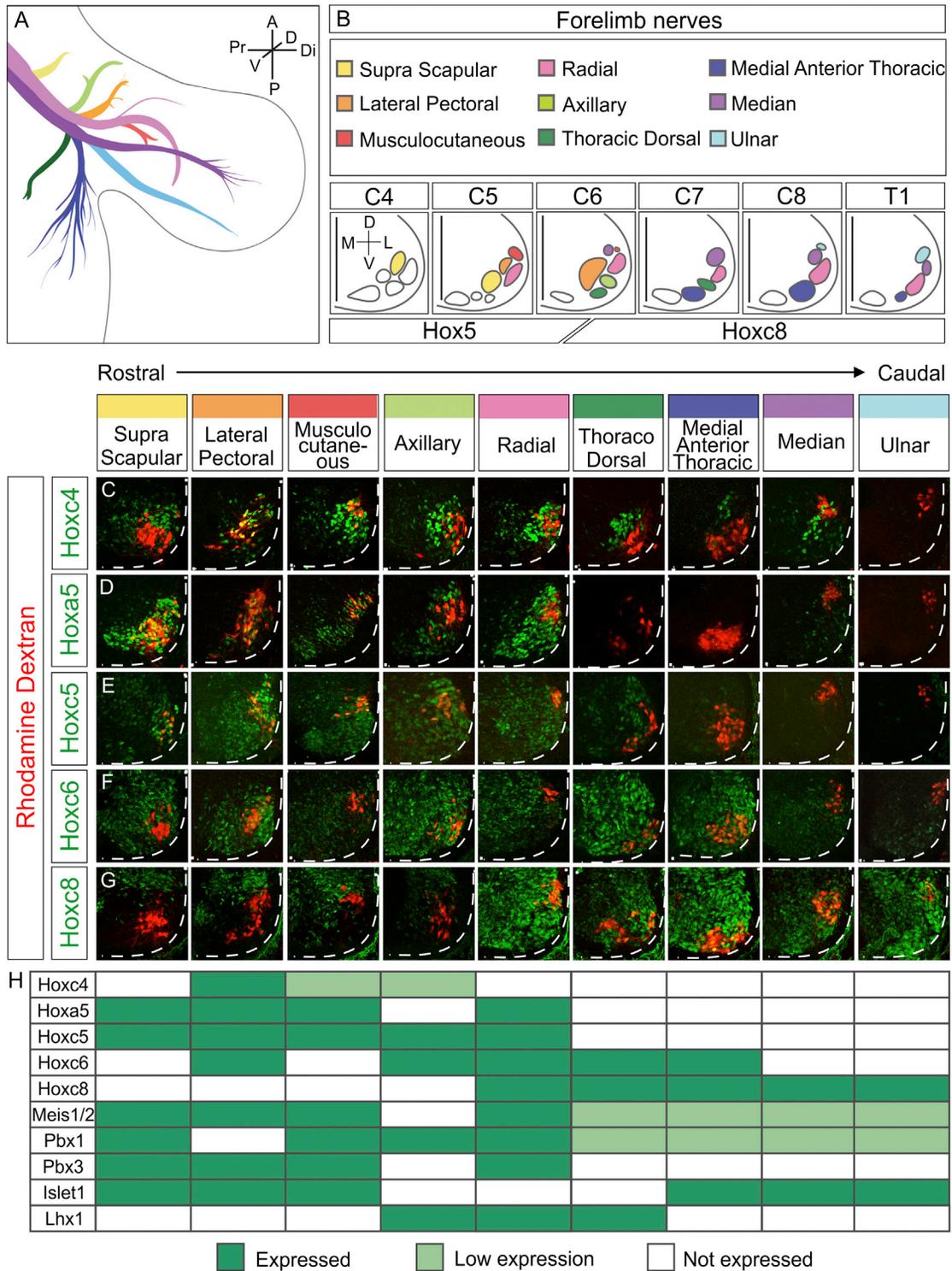


Figure 1. Combinatorial Hox Profiles Define Forelimb Innervating MNs

(A) Schematic of the primary motor nerves supplying the mouse forelimb at E12.5. Dorsal (D)-ventral (V), medial (M)-lateral (L), and proximal (Pr)-distal (Di) axes are indicated.

(B) Topographical organization of forelimb MNs. MN positions are based on retrograde labeling from nerves at E12.5 (see Figures S1A–S1I). Dorsal (D)-ventral (V) and medial (M)-lateral (L) coordinates are indicated. Segmental position of labeled LMC neurons are indicated and extend from cervical segment 4 (C4) to thoracic segment 1 (T1). Non-LMC MN populations present in these segments are also outlined.

(legend continued on next page)

requirements for the majority of *Hox* genes are not well established. Moreover, with the exception of a handful of downstream transcription factors, the target effectors of Hox proteins in MNs are yet to be determined.

In this study, we assessed the mechanisms through which *Hox* genes control expression of cell surface receptors during MN subtype differentiation. We show that MNs extending along the major nerves within the forelimb are defined by specific profiles of Hox expression and that *Hox* genes are essential in establishing the pattern and specificity of limb muscle innervation. *Hox* genes determine MN pool fates and connectivity patterns through controlling the profile of *Ret* and *Gfr α* expression and by constraining the ability of MNs to respond to limb-derived signals. We suggest that *Hox* genes govern neuronal fate and target connectivity by defining both the level and pattern of cell surface receptor gene expression.

RESULTS

Hox Profiles Define MN Groups Targeting Forelimb Muscles

To explore the relationship between profiles of Hox protein expression in MNs and peripheral target specificity, we first sought to establish a detailed molecular map of MN groups targeting forelimb and shoulder muscles in mice. We determined the position of MNs projecting along the nine major nerve pathways within the forelimb at embryonic stage (E)12.5 (Figures 1A and 1B). Consistent with studies in postnatal rat and embryonic chick (Bácskai et al., 2013; Hollyday and Jacobson, 1990; Tosolini and Morris, 2012), MNs targeting proximal forelimb muscles were located in rostral spinal segments, while MNs projecting distally were positioned caudally. Furthermore, ventrally located MNs adopted a proximal trajectory, whereas distal limb muscles were innervated by dorsally located MNs (Figure 1B; Figures S1A–S1I).

Next, we determined the profile of Hox protein expression in traced MN populations. We assessed the expression of *Hoxc4*, *Hoxa5*, *Hoxc5*, *Hoxc6*, and *Hoxc8*, as well as Hox co-factors belonging to the Meis and Pbx families. We found that *Hox5*, *Meis1*, *Meis2*, *Pbx1*, and *Pbx3* proteins were restricted to rostral brachial LMC neurons, while *Hoxc8* was expressed caudally (Figures 1D, 1E, and 1G; Figures S1J–S1L). Neurons within the *Hox5*⁺ and *Hoxc8*⁺ populations could be further distinguished by differential expression of additional Hox proteins. While all rostral LMC neurons express *Hoxc5*, *Hoxa5* was excluded from axillary MNs (Figures 1D and 1E). *Hoxc4* was present in a subset of rostral *Hox5*⁺ LMC neurons (Figure 1C), while *Hoxc6* was excluded from MNs targeting specific proximal and distal nerves (Figure 1F). Some MNs projecting along motor nerves shared identical or similar Hox codes but could be discriminated by differential expression of the Lim HD proteins *Lhx1* and *Isl1* (Figures S1M and S1N). These results indicate that the expression of Hox proteins, Hox cofactors, and Lim HD proteins mark

subsets of MNs supplying forelimb and shoulder muscles (Figure 1H).

Hox Genes Are Essential for MN Topographical Organization and Innervation Pattern

To assess the function of *Hox* genes in the organization and connectivity of MNs, we analyzed mice lacking *Hox5* (*Hoxa5* and *Hoxc5*), *Hoxc6*, and *Hoxc8*, as well as the *HoxC* gene cluster. We determined how loss of *Hox* function affects the differentiation of LMC neurons, the overall pattern of limb innervation, and the topographic relationship between MN position and target muscle specificity. Each *Hox* mutant line was crossed with *Hb9::GFP* mice to visualize motor axon projection patterns and to assist in the identification of nerves for tracing assays.

Analysis of mice lacking *Hox5* genes in MNs (*Hox5*^{MNΔ}; *Hb9::GFP* mice) (Philippidou et al., 2012) revealed grossly normal patterns of limb innervation (Figures S2A and S2B), and expression of *Hoxc4*, *Hoxc6*, *Meis*, and *Foxp1* proteins was preserved (Figure S2I; data not shown). Because assessment of axonal trajectories alone does not provide information on whether MNs make appropriate decisions to target a specific region, we performed retrograde labeling assays in *Hox5*^{MNΔ}; *Hb9::GFP* mice. Retrograde tracing from the suprascapular nerve labeled MNs that were scattered within the LMC and expressed high levels of *Hoxc4* (Figure S2J), a profile typically observed for lateral pectoral MNs. Because the profile of *Hoxc4* expression is unchanged in *Hox5*^{MNΔ} mice (Figure S2I), these results indicate that some MNs lacking *Hox5* genes adopt aberrant routes and target inappropriate muscles.

Hoxc8 is expressed by caudal brachial LMC neurons that innervate distal and posterior limb muscles. Within these segments, MN pools targeting the cutaneous maximus (CM) and anterior latissimus dorsi (ALD) muscles are defined by expression of the transcription factor *Pea3*, while median and ulnar MNs express *Scip* (Jung et al., 2010; Livet et al., 2002). In mice lacking *Hoxc8* in MNs (*Hoxc8*^{MNΔ} mice) there was a marked reduction in expression of these markers (Figures S2M–S2O). Consistent with this defect, the median and ulnar nerves were thinner, the median nerve was truncated, and terminal arbors at the CM and ALD muscles were dramatically reduced (Figures 2A, 2B, 2E, and 2F; Figures S2A, S2B, S2E, and S2F). Retrograde tracing from the ulnar, median, medial anterior thoracic, and radial nerves revealed that MNs were scattered within the LMC in *Hoxc8* mutants, in contrast to the stereotypic positioning and clustered organization in controls (Figures 2N–2P; Figure S2P).

Hoxc8 has been shown to be required for the exclusion of *Hox5* genes from caudal LMC neurons in chick (Dasen et al., 2005). In *Hoxc8*^{MNΔ} mice, we observed a caudal extension of multiple rostrally restricted factors, including *Hoxc4*, *Hoxa5*, *Meis*, and *Pbx* proteins (Figures S2Q and S2R; data not shown). The diameters of nerves originating from rostral *Hox5*⁺ LMC neurons were increased in *Hoxc8*^{MNΔ} embryos (Figure 2I),

(C–G) *Hoxc4*, *Hoxa5*, *Hoxc5*, *Hoxc6*, and *Hoxc8* expression in retrogradely labeled MNs projecting along the suprascapular, lateral pectoral, musculocutaneous, axillary, radial, thoracodorsal, medial anterior thoracic, median, and ulnar nerves.

(H) Summary of Hox expression profiles in MNs projecting along the main forelimb motor nerves.

See also Figure S1.

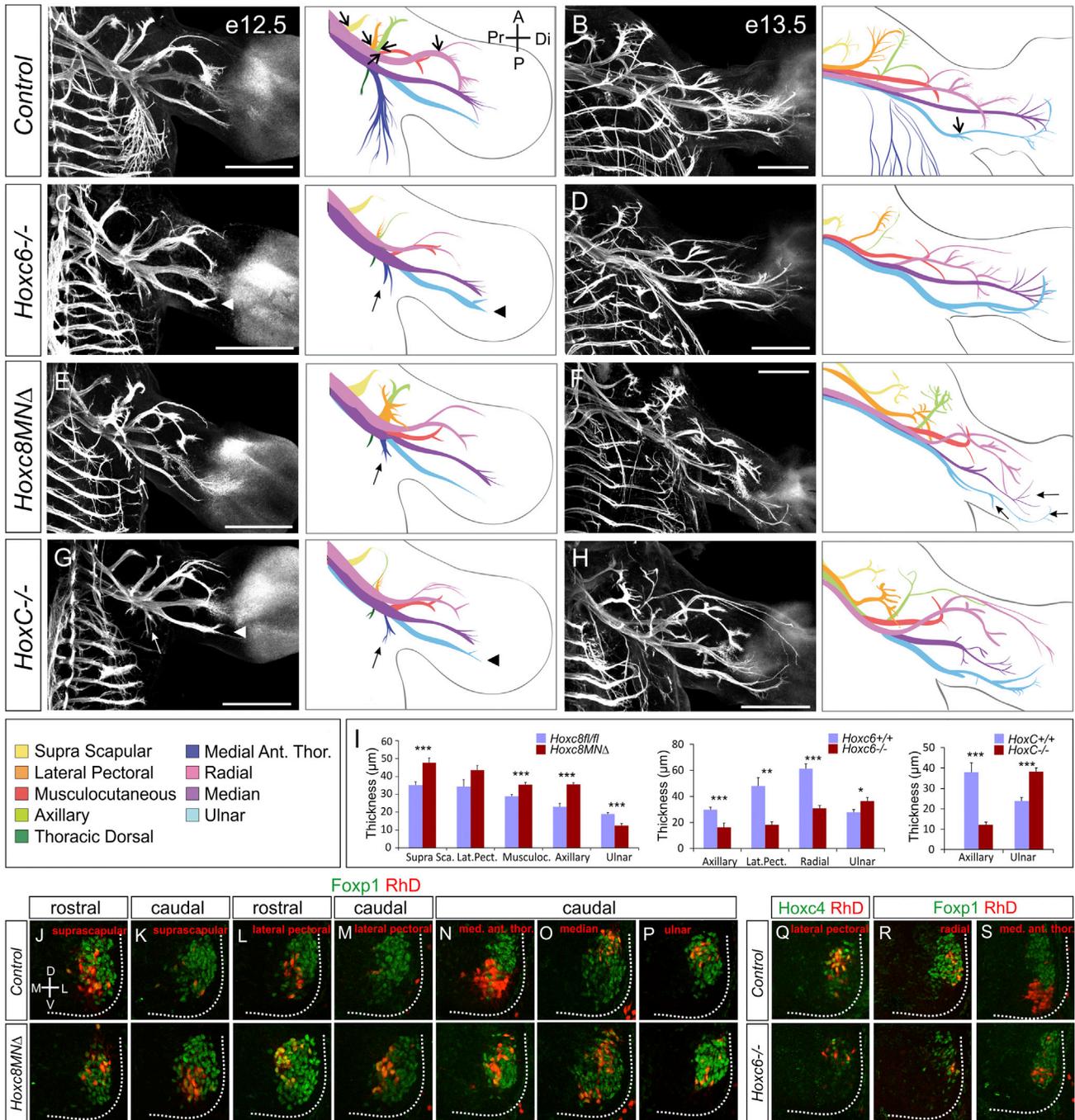


Figure 2. Hox Genes Govern Limb Innervation Pattern and MN Pool Organization

(A and B) Whole-mount GFP staining of control *Hb9::GFP* mice at E12.5 and E13.5. Schematic representations of innervation pattern are shown in right panels, and motor nerves are color coded. Arrows indicate region where nerve diameters were measured.

(C–H) Forelimb innervation patterns in *Hoxc6*, *Hoxc8*, and *HoxC* mutants at E12.5 and E13.5. Arrows and arrowheads indicate major nerve defects. Scale bars represent 500 μm . Innervation patterns in control littermates are shown in Figure S2.

(I) Quantification of muscle nerve diameters in indicated *Hox* mutant lines. Data are shown as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(J–P) Retrograde labeling of MNs in *Hoxc8* mutants. *Hoxc8* mutants display defects in the organization of suprascapular, lateral pectoral, medial anterior thoracic, median, and ulnar MNs.

(Q–S) Retrograde labeling of MNs projecting along the lateral pectoral, medial anterior thoracic, and radial nerves in *Hoxc6* mutants. The position and organization of MNs projecting along the suprascapular and musculocutaneous nerves are not affected in *Hoxc6* mutants (Figures S2K and S2L).

See also Figure S2.

suggesting that some caudal LMC neurons are redirected to targets of Hox5⁺ MNs. To test this, we performed retrograde tracing assays from the suprascapular and lateral pectoral nerves, revealing a caudal extension of Hox5⁺ MN pools (Figures 2J–2M). To further assess whether a fate switch occurred in *Hoxc8* mutants, we took advantage of a *LacZ* reporter inserted into the *Hoxc8* locus. Staining for β -galactosidase (β Gal) indicated that MNs within the caudal LMC coexpressed *Hoxa5*, *Meis2*, and β Gal in *Hoxc8* mutants (Figures S2S and S2T). Furthermore, MNs projecting along the suprascapular and lateral pectoral nerves expressed β Gal in *Hoxc8* mutants (Figures S2U and S2V). These studies suggest that in *Hoxc8* mutants there is a partial transformation of caudal LMC neurons to a Hox5⁺ fate.

Hoxc6 is required for the expression of *Pea3* and the innervation of the CM and ALD muscles (Figures 2C and 2D; Figures S2C and S2D) (Lacombe et al., 2013). Further analysis of *Hoxc6*^{-/-}; *Hb9::GFP* mice revealed additional defects not present in either *Hox5* or *Hoxc8* mutants. The diameters of the axillary, lateral pectoral, and radial nerves were reduced by ~50% in *Hoxc6* mutants, while ulnar nerve diameters were increased by 31% (Figures 2C, 2D, and 2I). Retrograde labeling assays revealed that MNs projecting along the lateral pectoral, radial, and median anterior thoracic nerves were disorganized (Figures 2Q–2S; Figures S2K and S2L). Mice lacking the *HoxC* cluster displayed MN phenotypes that were a composite of defects in *Hoxc6* and *Hoxc8* mutants, including altered nerve diameters and a lack of intramuscular arborization at the CM and ALD muscles (Figure 2G–2I; Figures S2G and S2H). Collectively, these analyses reveal essential roles for *Hox* genes in establishing the appropriate pattern of forelimb innervation and MN pool organization.

Ret and Gfr α Receptors Are Selectively Expressed by Subsets of LMC Neurons

What are the effectors of Hox proteins in MNs, how are they regulated, and how do they contribute to LMC topographic organization and connectivity? To address these questions, we screened for surface receptors whose expression patterns correlated with Hox profiles along the rostrocaudal axis or within a single segment. One group of genes that met these criteria included the receptor tyrosine kinase *Ret* and members of its *Gfr α* coreceptors. Analysis of *Ret* in brachial LMC neurons revealed striking differences in expression levels along the rostrocaudal axis (Figures 3A and 3B; Figures S3A and S3B). In rostral Hox5⁺ LMC neurons, MNs expressed low levels of *Ret* mRNA and protein relative to the caudal Hoxc8⁺ domain, which expressed high levels at E12.5 (Figures 3A, 3B, 3E, and 3F).

Ret signaling is mediated by *Gfr α* receptors, which, upon ligand binding, dimerize and activate specific downstream signaling cascades. Selective expression of *Gfr α* genes has been reported in subsets of MNs (Gould and Oppenheim, 2004; Gu and Kania, 2010; Homma et al., 2003); therefore, we assessed the expression pattern of each of the four murine *Gfr α* receptors. *Gfr α 1* expression was broadly expressed by brachial MNs at E12.5, with elevated levels observed within the caudal LMC (Figure 3C). *Gfr α 3* was restricted to a caudal subpopulation of LMC neurons that overlapped with the position

of *Scip*⁺ MNs (Figures 3D and 3G; Figure S3C). *Gfr α 2* was weakly expressed by brachial MNs, while *Gfr α 4* was not detected (Figure S3D; data not shown).

Comparison of *Ret* and *Gfr α* expression between mouse and chick revealed largely conserved profiles, with a few notable differences (Figures 2O and 2P; Figure S3F). As in mouse, high levels of *Ret* mRNA and protein were detected in caudal Hoxc8⁺ LMC neurons in chick, while lower levels were present in rostral Hox5⁺ MNs (Figures 3H, 3I, 3L, and 3M). *Gfr α 1* displayed a more restricted pattern in chick than in mouse, with high levels of expression overlapping with the position of *Pea3*⁺ MNs (Figures 3J and 3N; Figure S3F). In contrast to mouse, *Gfr α 2* expression was present in a lateral population of brachial MNs in chick, while *Gfr α 3* was not detected (Figure S3E; data not shown). In chick, *Gfr α 4* expression was similar to mouse *Gfr α 3*, occupying a position overlapping with *Scip*⁺ LMC neurons (Figures 3K and 3N; Figure S3F).

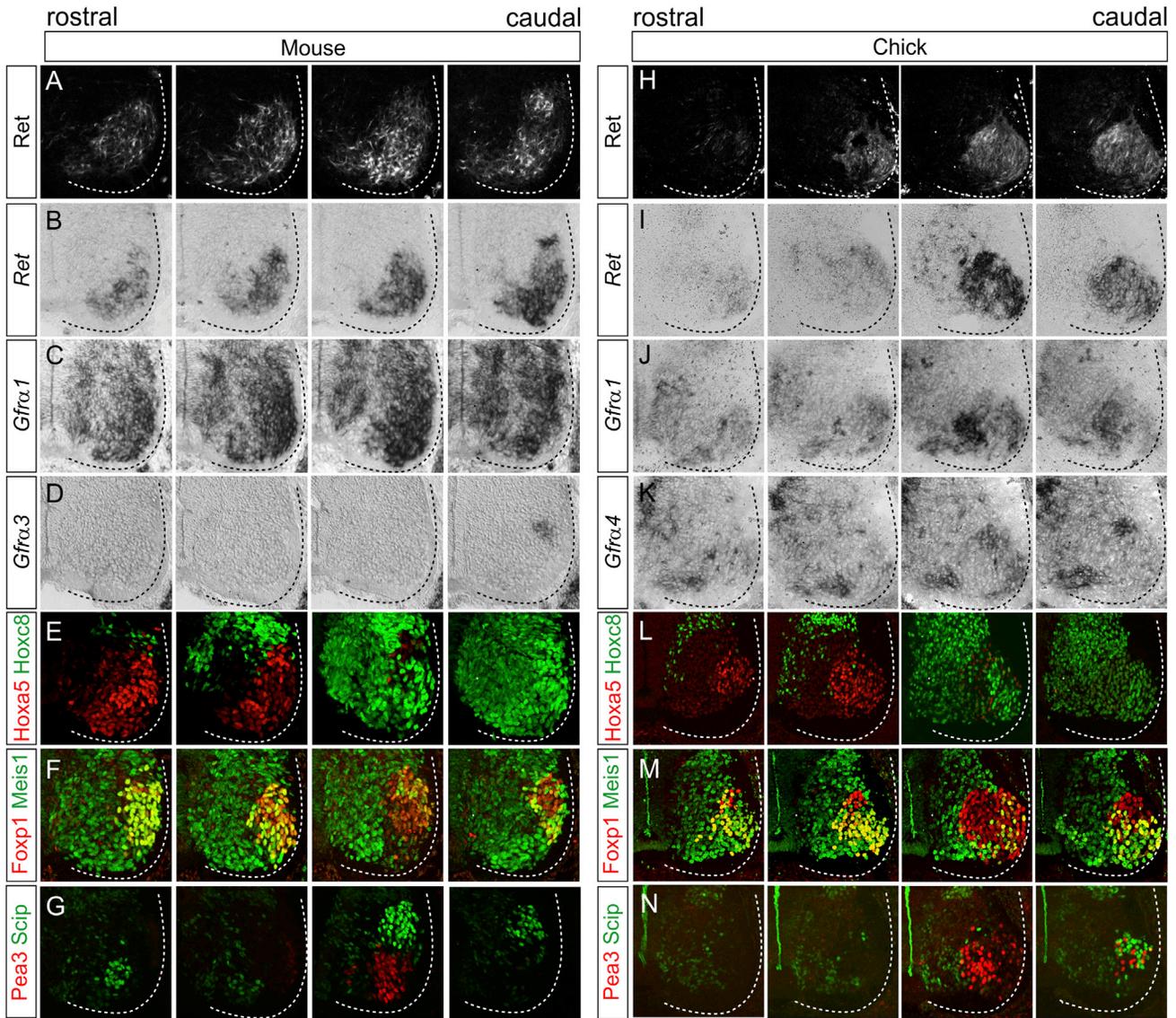
Ret and Gfr α 3 Are Downstream Targets of Hox Proteins in LMC Neurons

To determine whether the MN organization and connectivity defects observed in *Hox* mutants are due to loss of *Ret*/*Gfr α* signaling components, we analyzed *Hox* mutant mice for changes in *Ret* and *Gfr α* expression. We assessed *Ret* mRNA in *Hoxc6*, *Hoxc8*^{MN Δ} , and *HoxC* mutant animals and compared expression with control littermates at E12.5. We also quantified the levels of *Ret* protein in mutant lines and control littermates. This analysis revealed a pronounced reduction in both *Ret* mRNA and protein levels in the posterior Hoxc8⁺ region in *Hoxc6*, *Hoxc8*, and *HoxC* cluster mutants (Figures 4A–4I). *Ret* expression was present in caudal LMC neurons but reduced to levels comparable to those observed in rostral Hox5⁺ neurons.

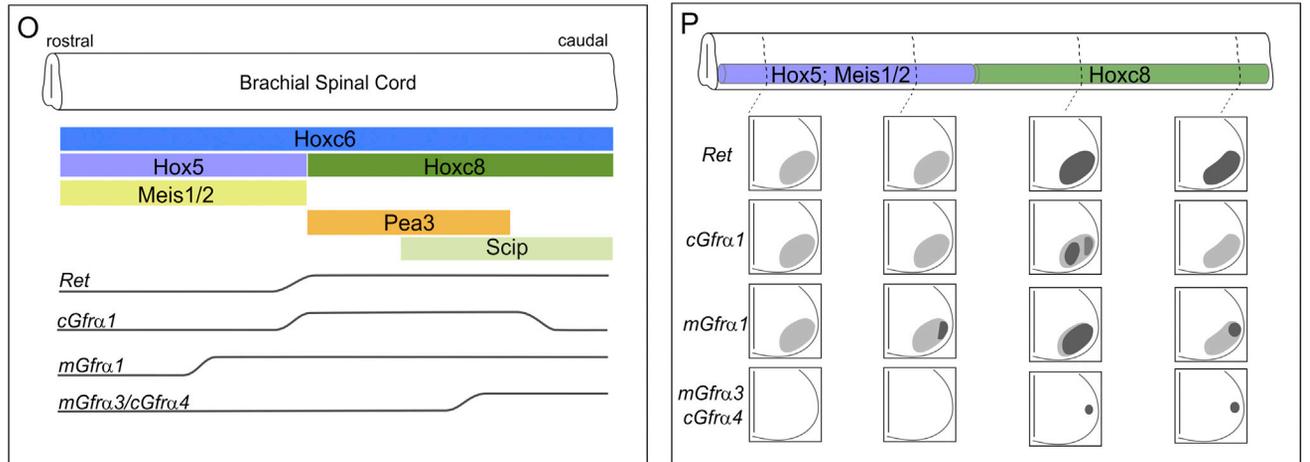
We also examined the expression of *Gfr α* genes in *Hox* mutants. Expression of *Gfr α 3* was markedly decreased in *Hoxc8*^{MN Δ} and *HoxC*^{-/-} embryos but was grossly normal in *Hoxc6* mutants (Figures 4J–4O; Figure S4B). Expression of *Gfr α 1* was largely unaffected in *Hoxc6*, *Hoxc8*^{MN Δ} , and *HoxC* mutants at E12.5 (Figures S4A and S4B). These results show that *Hox* genes contribute to the differential expression of *Ret* in LMC neurons along the rostrocaudal axis and are essential for the intrasegmental pattern of *Gfr α 3*.

Hox Network Interactions Determine the Profiles of Ret/ Gfr α Genes in MNs

Our analyses indicate that multiple *Hox* genes are necessary to define *Ret*/*Gfr α* expression in LMC neurons but also raise the question of how interactions among Hox proteins contribute to their specific patterns. We first determined whether *Hox* genes are sufficient to induce *Ret* and *Gfr α* expression outside their normal domains. We misexpressed *Hox* genes in the neural tubes of stage (st)13 chick embryos using the *chicken β -actin* (CAGGs) promoter, and we analyzed *Ret*, *Gfr α 1*, and *Gfr α 4* expression at st27. We found that both *Hoxc6* and *Hoxc8* are sufficient to induce high levels of *Ret* at thoracic levels, where *Ret* levels are normally low (Figures 5A, 5D, 5E, and 5H; Figures S5A and S5C). In contrast, misexpression of the rostrally expressed *Hox* genes, *Hoxc4* and *Hoxa5*, did not induce *Ret* at any level (Figures S5I–S5L).



Summary



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Both *Hoxc6* and *Hoxc8* can impose an LMC fate on thoracic MNs through induction of the transcription factor *Foxp1* (Dasen et al., 2008; Lacombe et al., 2013). The elevated levels of *Ret* observed upon thoracic *Hoxc6* and *Hoxc8* misexpression, therefore, could be indirect, due to the actions of *Foxp1*. To determine whether *Foxp1* can elevate *Ret* expression independent of *Hox* genes, we misexpressed *Foxp1* at rostral cervical and thoracic levels. After misexpression of *Foxp1* elevated *Ret* expression was not observed at either level (Figures S5G and S5H; data not shown). These results indicate that *Foxp1* alone is insufficient to promote the high levels of *Ret* observed in caudal LMC neurons.

Hoxc6 is expressed in rostral *Hox5*⁺ LMC neurons, yet it is presumably incapable of promoting high *Ret* levels in this region. We tested whether factors present in rostral LMC neurons contribute to the reduced levels of *Ret*. Misexpression of *Meis1* in caudal *Hoxc8*⁺ LMC neurons, where *Ret* levels are relatively high, resulted in a marked inhibition of *Ret* expression (Figures 5I and 5L; Figures S5E and S5F). In contrast, misexpression of *Hoxc4*, *Hoxa5*, *Pbx1*, and *Pbx3* did not alter *Ret* expression in caudal LMC neurons (Figures S5M–S5P; data not shown).

Although *Hoxc6* and *Hoxc8* were both capable of elevating *Ret* levels, they each promoted distinct patterns of *Gfra* expression and MN pool identities. *Hoxc6*, but not *Hoxc8*, induced expression of *Gfra1* in cervical MNs, where expression of this receptor is normally low (Figure 5B; Figure S5D). This result is consistent with the observation that, in chick, *Gfra1* is restricted to the *Hoxc6*⁺, *Pea3*⁺ MN pool but excluded from *Scip*⁺ MNs. In addition, *Hoxc8* was able to induce expression of *Gfra4* at thoracic levels (Figure 5F; Figure S5B), while *Meis1* repressed both *Gfra1* and *Gfra4* at caudal brachial levels (Figures 5J and 5K). *Hoxc6* and *Hoxc8* also promoted distinct MN pool fates, with *Hoxc6* inducing *Pea3* expression and *Hoxc8* inducing *Scip* at thoracic levels (Figures 5C and 5G) (Lacombe et al., 2013).

These results indicate that regulatory interactions among *Hoxc6*, *Hoxc8*, and *Meis1* define the patterns of *Ret*, *Gfra1*, and *Gfra4* in brachial LMC neurons (Figure 5Y). *Hoxc6* and *Hoxc8* are sufficient to induce elevated *Ret* levels, while *Meis1* dampens *Ret* expression in rostral LMC neurons. *Hoxc6* promotes expression of *Gfra1* within MN pools defined by *Pea3* expression, while *Hoxc8* promotes *Gfra4* and a *Scip*⁺ MN pool identity.

Hox and Meis Regulatory Interactions Control Ret Expression in Lumbar LMC Neurons

Our analyses of *Ret* gene regulation at brachial levels are seemingly at odds with studies in lumbar LMC neurons, where *Ret* is essential during dorsoventral projection decisions (Kramer

et al., 2006). Dorsally projecting LMCI neurons express high levels of *Ret*, while LMCm neurons express low levels. In the absence of *Ret*, LMCI motor axons fail to pursue a dorsal trajectory along the peroneal nerve (Bonanomi et al., 2012; Kramer et al., 2006). To determine whether *Ret* expression in lumbar LMC neurons is defined by similar Hox-regulatory interactions, we assessed whether *Hox* genes expressed at lumbar levels can induce *Ret* expression. We found that, after misexpression of the lumbar LMC determinants *Hoxc10* and *Hoxd10*, elevated levels of *Ret* were detected in thoracic MNs (Figures 5S–5V).

Because *Meis1* is involved in dampening *Ret* expression in rostral brachial MNs, we asked whether it is also involved in restricting *Ret* to lumbar LMCI neurons. In both mouse and chick, *Meis1* expression was restricted to the LMCm at lumbar levels and only weakly detected in LMCI neurons (Figures 5M–5R). Moreover, misexpression of *Meis1* at lumbar levels repressed *Ret* expression in LMCI neurons (Figures 5W and 5X). These observations indicate that the pattern of *Ret* expression in brachial and lumbar LMC neurons is defined by a common Hox/*Meis* regulatory strategy (Figures 5Y and 5Z).

Ret and Gfra1 Activities Bypass the Requirement for Hox Genes in Pea3 Induction

What is the functional significance of the profile of *Ret* and *Gfra* genes during MN differentiation? The ligand for *Ret*/*Gfra1* receptors, *Gdnf*, is broadly expressed in the periphery (Haase et al., 2002; Jung et al., 2010), suggesting that MN responsiveness to peripheral signals is constrained by LMC-intrinsic factors. To test this, we asked whether elevating *Ret* and *Gfra1* outside their normal domains affects MN pool differentiation. We expressed *Ret* and *Gfra1* by in ovo electroporation at rostral brachial and thoracic levels, where expression of these genes is normally low, and assessed expression of *Pea3*, a known target of *Gdnf*-*Ret*/*Gfra1* signaling (Haase et al., 2002). Elevating *Ret* expression at thoracic levels, but not in rostral LMC neurons, generated ectopic *Pea3*⁺ MNs (Figures 6A–6D; Figure S6E). The selective induction of *Pea3* at thoracic levels appears to stem from the fact that thoracic MNs in chick also express *Gfra1* (Figure S6A), creating a condition where MNs now express both *Ret* and *Gfra1*. Consistent with this idea; upon coexpression of *Ret* and *Gfra1* in either rostral brachial or thoracic MNs, we observed induction of *Pea3* expression (Figures 6I–6N and 6Q). In contrast, expression of *Gfra1* in either rostral LMC or thoracic segments was insufficient to induce *Pea3* (Figures 6E–6H).

Induction of *Pea3* after misexpression of *Ret* and *Gfra1* did not lead to ectopic expression of LMC-associated transcription factors such as *Hoxc6*, *Hoxc8*, or *Foxp1* (Figures S6B–S6D). These observations indicate that *Pea3* induction can occur

Figure 3. Expression of Ret and Gfra Genes in LMC Neurons

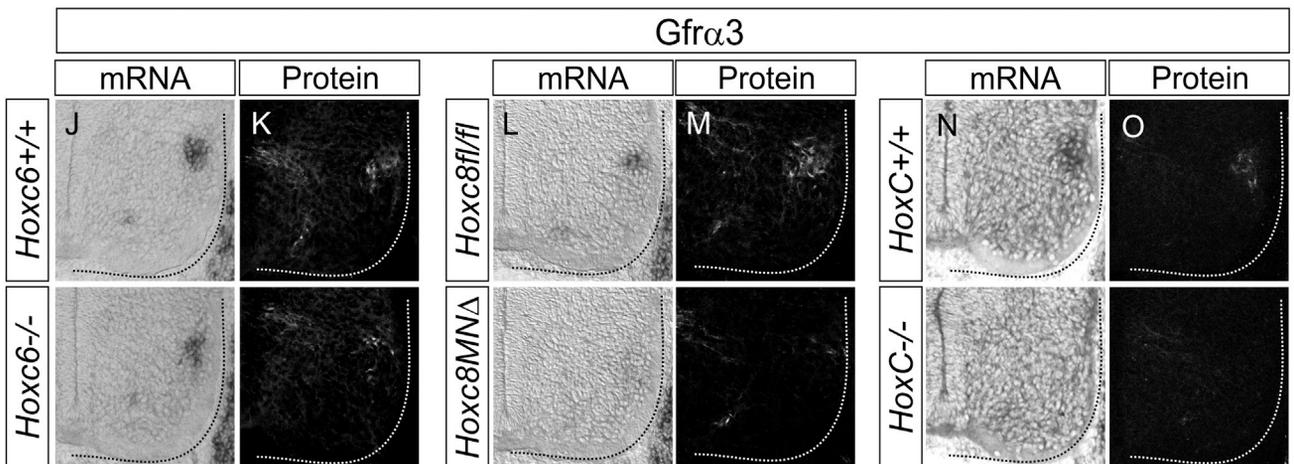
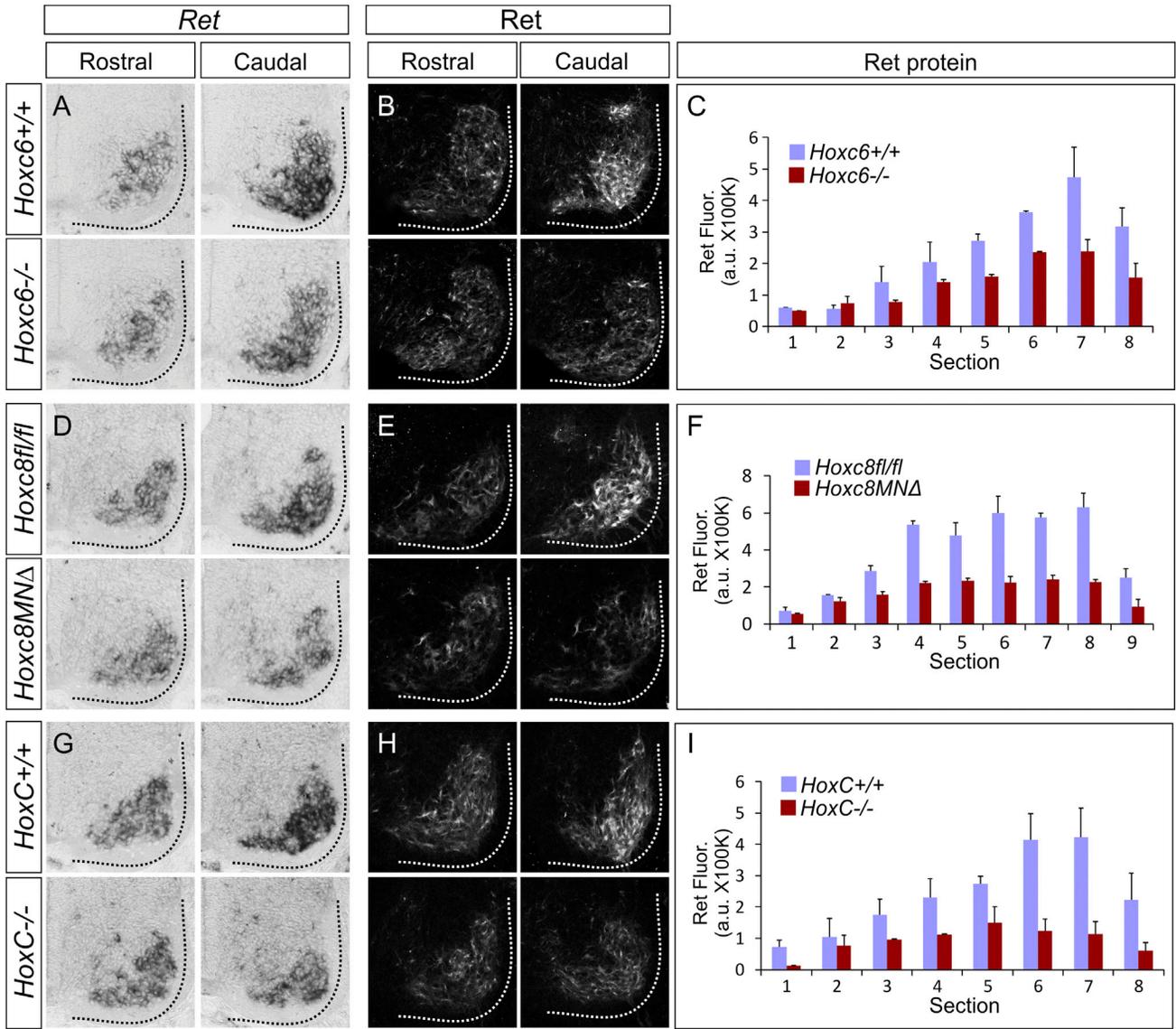
(A–G) Expression patterns of *Ret*, *Gfra1*, and *Gfra3* in MNs along the rostrocaudal axis, aligned with expression of *Hoxa5*, *Hoxc8*, *Meis1/2*, *Foxp1*, *Pea3*, and *Scip* at E12.5.

(H–N) Expression of *Ret*, *Gfra1*, and *Gfra4* in MNs aligned with *Hoxa5*, *Hoxc8*, *Meis1/2*, *Foxp1*, *Pea3*, and *Scip* in chick at st29.

(O) Summary of the expression profiles of mouse (m) and chick (c) *Ret*, *mGfra1*, *cGfra1*, *mGfra3*, and *cGfra4* along the rostrocaudal axis. Expression domains of *Hoxa5*, *Hoxc8*, *Meis1/2*, *Hoxc6*, *Pea3*, and *Scip* are depicted for positional comparison.

(P) Summary of the intrasegmental expression profile of *Ret*, *mGfra1*, *cGfra1*, *mGfra3*, and *cGfra4* in brachial MNs.

See also Figure S3.



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independently of an earlier LMC specification program, implying that a key function of *Hox* genes is to establish the domain in which the *Pea3*⁺ MN pool can be specified. High levels of *Ret* in combination with *Gfra1* expression appears, therefore, to define the competence of MNs to express *Pea3* (Figure 6R). Given that in *Pea3* mutants there is a selective loss in terminal branches at the CM muscle (Livet et al., 2002), these results suggest that the CM innervation defects observed in *Hoxc8* and *Hoxc6* mutants are due to the loss of *Pea3* induction by *Ret*/*Gfra1* signaling.

Ret and Gfra3 Mutants Display Innervation Defects Similar to Those of Hox Mutants

To assess whether attenuation of *Ret*/*Gfra* signaling contributes to the MN defects observed in *Hox* mutants, we analyzed mice lacking *Ret* and *Gfra3*. Previous analysis of *Ret* mutants revealed essential roles in the innervation of dorsal hindlimb muscles (Bonanomi et al., 2012; Kramer et al., 2006), and *Gdnf* and *Gfra1* mutants have been shown to be required for the induction of *Pea3* at brachial levels (Haase et al., 2002). However the role of *Ret* in the specification and connectivity of LMC neurons has not been fully established. In *Ret* mutants, expression of *Hoxc6*, *Hoxc8*, and *Foxp1* was preserved at E12.5 and E13.5, consistent with a function downstream of *Hox* genes (Figures S7A–S7C; data not shown). However, there was a marked reduction in the number of MNs expressing *Pea3* (Figures 7A and 7B). Interestingly, the total number of *Scip*⁺ MNs was increased by 38% in *Ret*^{-/-} embryos, leading to a ventral expansion of this pool (Figures 7C and 7D). Conversely, generating ectopic *Pea3* neurons, via misexpression of *Ret* and *Gfra1*, led to a marked reduction in the number of *Scip*⁺ neurons (Figures 6O–6Q). These results indicate that the establishment of the normal distribution of *Pea3*⁺ and *Scip*⁺ MNs relies on *Ret* function.

Next, we analyzed the pattern of limb innervation in *Ret* mutants. Similar to *Hoxc6*^{-/-}, *Hoxc8*^{MNΔ}, and *Pea3*^{-/-} mice (Livet et al., 2002), *Ret*^{-/-} embryos displayed a marked loss in axonal arbors at the CM and ALD muscles (Figures 7E–7H). Projections along the ulnar and median nerves were present in *Ret*^{-/-}:*Hb9::GFP* mice but displayed aberrant branches along the ulnar nerve, resulting in thinning of this nerve distally (Figures 7G, 7H, and 7N). Retrograde tracing of MNs projecting along the median and ulnar nerves labeled MNs positioned more ventrally, as observed in *Hoxc8* mutants (Figures 7J and 7K). In contrast, tracing from the medial anterior thoracic nerve in *Ret* mutants labeled MNs positioned more dorsally, similar to *Hoxc6* and *Hoxc8* mutants (Figure 7I).

We next analyzed mice lacking *Gfra3*, which is restricted to *Scip*⁺ MNs. In *Gfra3*;*Hb9::GFP* mice, both the median and ulnar nerves were thinner at E13.5 (Figures 7L–7N), similar to *Hoxc8*^{MNΔ} mice. However, in contrast to *Hoxc8*^{MNΔ} and *Ret* mutant mice, retrograde labeling from the median and ulnar

nerves in *Gfra3* mutants indicated a grossly normal organization (Figures S7D and S7E). Given that in *Hoxc8* mutants median and ulnar MNs are disorganized, these results indicate that *Gfra3* mutation does not phenocopy loss of *Hoxc8* and that *Hox* proteins have additional targets required for motor pool clustering. The number of *Scip*⁺; *Foxp1*⁺ MNs was unchanged in *Gfra3* mutants (Figure S7F), suggesting that the innervation defects are due to an inability of motor axons to fully extend within the limb. Collectively, these results indicate that *Ret* and *Gfra3* mutants display limb innervation defects similar to *Hox* mutants.

DISCUSSION

Networks of transcription factors are critical for neuronal subtype diversification, but how cell fate determinants orchestrate expression of surface receptors is poorly understood. In this study, we found that *Hox* genes are essential for the organization and peripheral connectivity of MNs targeting the forelimb and identified *Ret* and *Gfra* genes as key targets of their actions. We discuss these findings in the context of transcriptional and signaling networks conferring MN-muscle target specificity and consider the possible mechanisms contributing to the diversification of limb innervation programs in tetrapods.

Hox Genes Govern MN Diversity and Peripheral Innervation Pattern

Hox genes are essential for neuronal specification in the hindbrain and spinal cord (Philippidou and Dasen, 2013; Tümpel et al., 2009), but the extent to which they define the connectivity of limb-innervating MNs is unclear. LMC neurons segregate into ~50 MN pools, each pool defined by its connectivity to a single limb muscle. While certain MN pools express subtype-restricted factors, whether a selective transcriptional code defines each subtype is unknown. We found that MNs extending along the nine major forelimb nerve pathways are defined by restricted patterns of *Hox* proteins and *Hox* cofactors. *Hox5* genes define MNs that target proximal/anterior limb regions, and the *Hoxc8* gene defines MNs that target distal/posterior regions, with additional *Hox* genes and cofactors contributing to MN diversity and connectivity within these broad domains. In principle, additional layers of target specificity could be imparted by differences in *Hox* protein levels between MN pools, which may grade surface receptor expression. In support of this idea, level-dependent activities of *Hox* proteins in MNs have been demonstrated during the innervation of leg muscles in *Drosophila* (Baek et al., 2013).

Consistent with roles in establishing the identity and connectivity of LMC neurons, the removal of *Hox* genes leads to defects in the pattern of limb innervation and dissolves the normal topographic relationship between MN position and target specificity. Mutation of individual *Hox* genes causes a deterioration of MN

Figure 4. Multiple Hox Genes Establish Ret and Gfra Profiles in LMC Neurons

(A–I) Analysis of *Ret* expression in *Hox* mutant mice at E12.5 is shown (A–H). *Ret* levels are attenuated in *Hoxc6*, *Hoxc8*, and *HoxC* mutants within caudal LMC neurons. *Ret* mRNA and protein levels are shown in the indicated mutants and control littermates. (C), (F), and (I) show quantification of *Ret* antibody immunofluorescence (Fluor.) from serial sections along the rostrocaudal axis (see Experimental Procedures). Data are shown as mean ± SEM.

(J–O) *Gfra3* expression is markedly downregulated in *Hoxc8* and *HoxC* mutants but preserved in *Hoxc6* mutants.

See also Figure S4.

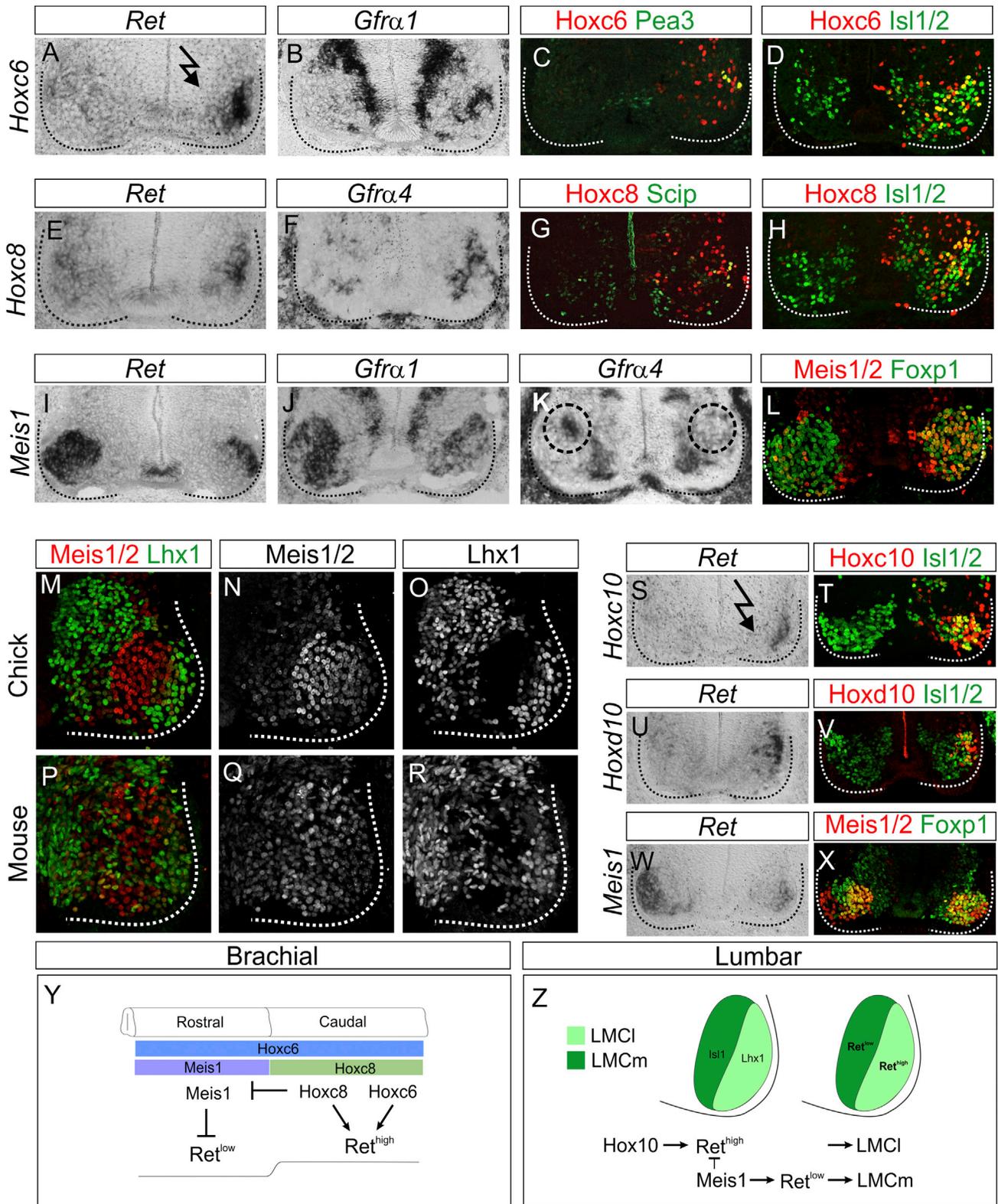


Figure 5. Hox and Meis Interactions Govern Ret Expression in LMC Neurons

(A–D) Electroporation of *Hoxc6*-pCAGGs induces *Ret* and *Pea3* expression at thoracic levels and *Gfra1* at rostral cervical levels. *Hoxc6* and *Isl1/2* staining is shown to indicate electroporated MNs. Arrow in (A) indicates the electroporated side of the embryo.

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organization and peripheral target specificity along multiple nerves. However, with the exception of the CM and ALD muscles, most targets appear to receive at least some innervation in the absence of *Hox* genes. These observations are in agreement with analysis of *Foxp1* mutants, where *Hox*-dependent LMC programs are lost, and MNs target limb muscles in a stochastic manner (Dasen et al., 2008; Rousso et al., 2008). Therefore, in the absence of *Hox* genes, motor axons can continue to pursue trajectories along the available paths within the limb but appear to lose the ability to select appropriate targets.

The most severe deficits after the removal of *Hox* genes are apparent upon assessment of the relationship between MN position and target specificity. *Hox* mutants display specific defects in the normal topographic organization and position of MN pools. In *Hoxc8* mutants, MNs targeting the distal forelimb are disorganized within the LMC, and a subset of MNs lacking *Hoxc8* target proximal forelimb muscles normally supplied by *Hox5*⁺ populations. These observations suggest that in *Hoxc8* mutants, there is a partial transformation of LMC neurons to a *Hox5*⁺ fate and connectivity pattern. Although it is unclear whether similar fate transformations occur in each of the *Hox* mutants we analyzed, their phenotypes likely reflect a loss of guidance systems that dictate the selection of one peripheral innervation pathway over another.

Hox Target Effectors in Neuronal Fate Specification and Axon Guidance

The ability of motor axons to navigate toward and innervate peripheral targets with precision relies on the selective expression of cell surface receptors. Combinatorial sets of transcription factors are known to be essential for neuronal subtype diversification, but how intrinsic factors shape MN identities and innervation pattern is poorly defined. Studies in *Drosophila* have shown that the transcription factor Hb9 acts in ventrally projecting MNs to regulate expression of the *Robo2* guidance receptor, while in dorsally projecting MNs, *Eve2* controls expression of the *Unc5* gene (Santiago et al., 2014; Zarin et al., 2014a). In vertebrates, motor axon guidance in the limb bud is known to require the activities of a variety of genes, including *Ret*, *Epha4*, *Ephb1*, *Frizzled3*, *Npn1*, and *Celsr3* (Bonanomi and Pfaff, 2010; Hua et al., 2013; Santiago and Bashaw, 2014). However, beyond the contribution of Lim HD proteins to the selection of dorsal and ventral limb trajectories, how fate determinants orchestrate MN connectivity is poorly understood.

We found that *Hox*/*Hox* cofactor interactions define the spatial profiles of *Ret* and *Gfra* genes in MNs, and signaling through *Ret*/*Gfra* receptors is essential in *Hox*-dependent programs of MN differentiation and connectivity. During MN pool specification, activation of *Ret*/*Gfra1* signaling by *Gdnf* is required for *Pea3*

expression in a subset of LMC neurons (Haase et al., 2002). Application of *Gdnf* throughout the spinal cord, however, induces *Pea3* only within the caudal LMC populations that would have expressed it normally. This suggests the existence of intrinsic systems that limit MN responses to *Gdnf*. Our results indicate that *Hox*/*Meis* interactions define the pattern of *Ret* and *Gfra1* in caudal LMC neurons, thereby restricting the ability of peripheral cues to induce *Pea3* expression. In support of this idea, elevating *Ret* and *Gfra1* throughout the spinal cord is sufficient to activate *Pea3*, bypassing the requirement for *Hox* genes. *Hox* genes, therefore, appear to constrain MN responsiveness to peripheral cues by selectively regulating expression of surface receptors.

In addition to contributing to MN pool diversification, signaling through *Ret* is essential during motor axon guidance decisions. In the hindlimb, *Ret* is required for the selection of a dorsal trajectory by LMC axons, acting in part by modulating signaling through Eph receptors (Bonanomi et al., 2012). We found that *Hox*/*Meis* regulatory interactions determine the spatial profiles of *Ret* expression in LMC neurons and that silencing *Ret* and *Gfra3* causes innervation defects similar to those observed after selective removal of *Hox* genes. In the absence of *Ret*, projections toward posterior and distal muscle groups are deteriorated, similar to the innervation defects observed in *Hoxc6* and *Hoxc8* mutants. These phenotypes appear to be due, in part, to the loss of *Pea3*, as *Pea3* mutants display a severe reduction in intramuscular branches at the CM muscle (Livet et al., 2002). Our studies show that loss of *Ret* and *Gfra3* also leads to innervation defects in the distal forelimb, raising the possibility that *Ret*/*Gfra* signaling contributes to guidance decisions independent of *Pea3* regulation.

Hox Genes and the Evolution of Limb Innervation Programs

The musculoskeletal system of the forelimb varies significantly among vertebrates, raising the question of how MN specification programs evolved to accommodate different motor behaviors. The basic topographical organization of MNs is conserved among all tetrapods that have been examined (Fetcho, 1992), and evolutionary modification of *Hox* activities appears to have contributed to changes in LMC position relative to the limbs (Jung et al., 2014). During the evolution of paired appendages, the appearance of new muscle groups enabled species to achieve greater complexity in the range of limb movements. Presumably, this process required an expansion of MN subtype identities from a more simplified ancestral population. The Lim HD code governing dorsoventral projections appears to be present in pectoral fins of zebrafish (Uemura et al., 2005), suggesting that this program appeared prior to land invasion. As the

(E–H) Electroporation of *Hoxc8-pCAGGs* induces *Ret*, *Gfra4*, and *Scip* expression at thoracic levels.

(I–L) Misexpression of *Meis1* represses *Ret*, *Gfra1*, and *Gfra4* in caudal LMC neurons.

(M–R) Expression of *Meis1/2* and *Lhx1* in chick and mouse lumbar LMC neurons. In both chick and mouse, *Meis1/2* expression is reduced in lateral *Lhx1*⁺ MNs, which express high levels of *Ret*.

(S–V) Electroporation of *Hoxc10* and *Hoxd10* induces expression of *Ret* at thoracic levels.

(W and X) *Meis1* misexpression represses *Ret* in lumbar LMC neurons.

(Y) Summary of *Ret* regulation by *Hox*/*Hox* co-factors in brachial LMC neurons.

(Z) Summary showing regulation of *Ret* by *Hox*/*Hox* co-factors at lumbar levels.

See also Figure S5.

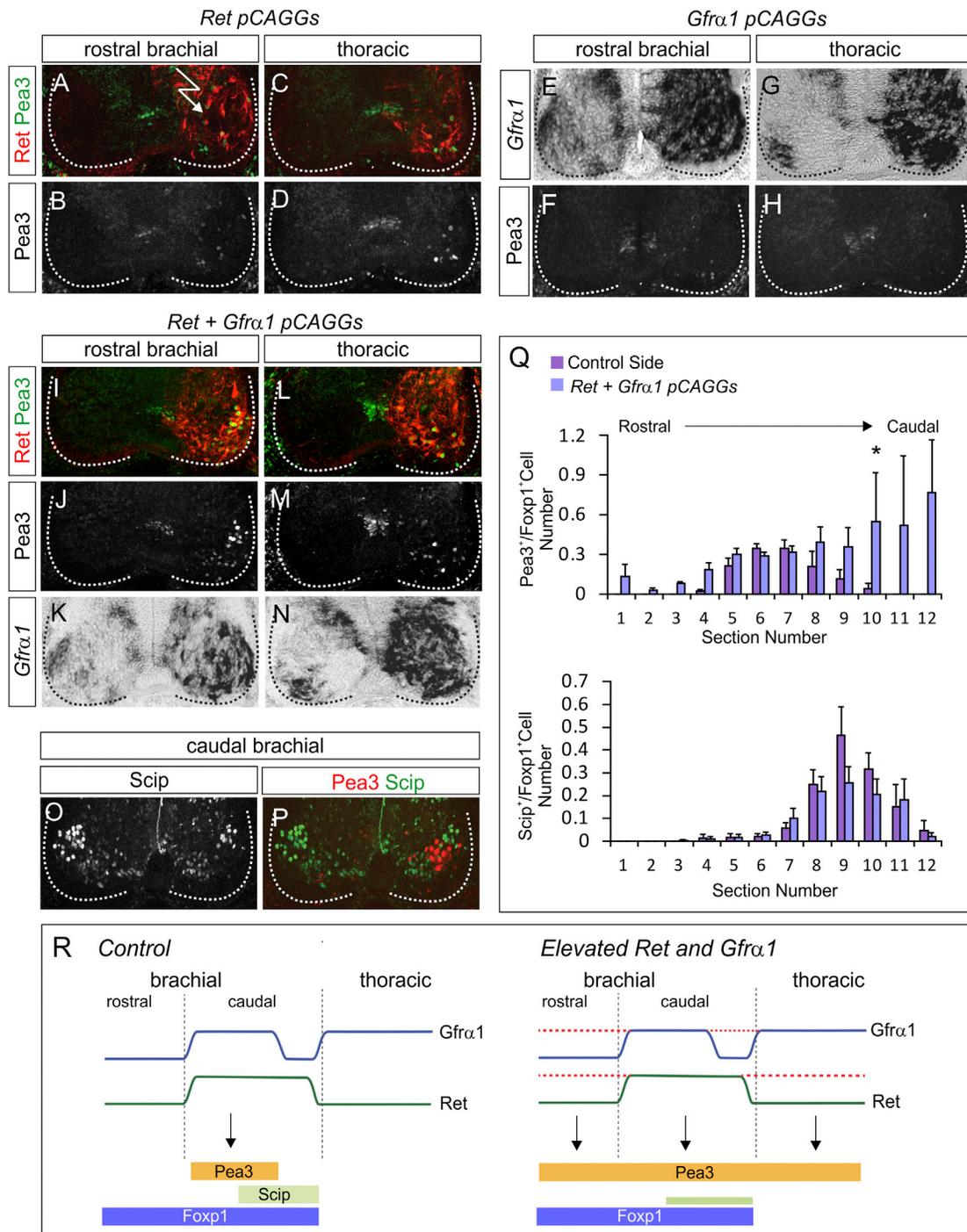


Figure 6. Ret and Gfra1 Patterns Facilitate Pea3 Induction in MN Pools

(A–D) Misexpression of *Ret* under pCAGGs induces *Pea3* expression in thoracic MNs, but not rostral brachial LMC neurons.

(E–H) *Gfra1* misexpression fails to induce *Pea3* expression in rostral brachial and thoracic MNs.

(I–N) Co-expression of *Ret* and *Gfra1* is sufficient to induce *Pea3* at both rostral brachial and thoracic levels.

(O and P) After induction of *Pea3* by *Ret* and *Gfra1*, there is a reduction in the number of *Scip*⁺ LMC neurons.

(Q) Quantification showing increased numbers of *Pea3*⁺ and reduced *Scip*⁺ MNs along the rostrocaudal axis after *Ret* and *Gfra1* misexpression. Numbers are representative of electroporation experiments with efficiencies of greater than 50%. Data indicate mean ± SEM. Star indicates $p < 0.05$.

(R) Diagram summarizing the relationship between *Ret*, *Gfra1*, *Scip*, and *Pea3* expression, as well as the effects of *Ret* and *Gfra1* misexpression on the differentiation of *Pea3*⁺ and *Scip*⁺ MN pools.

See also Figure S6.

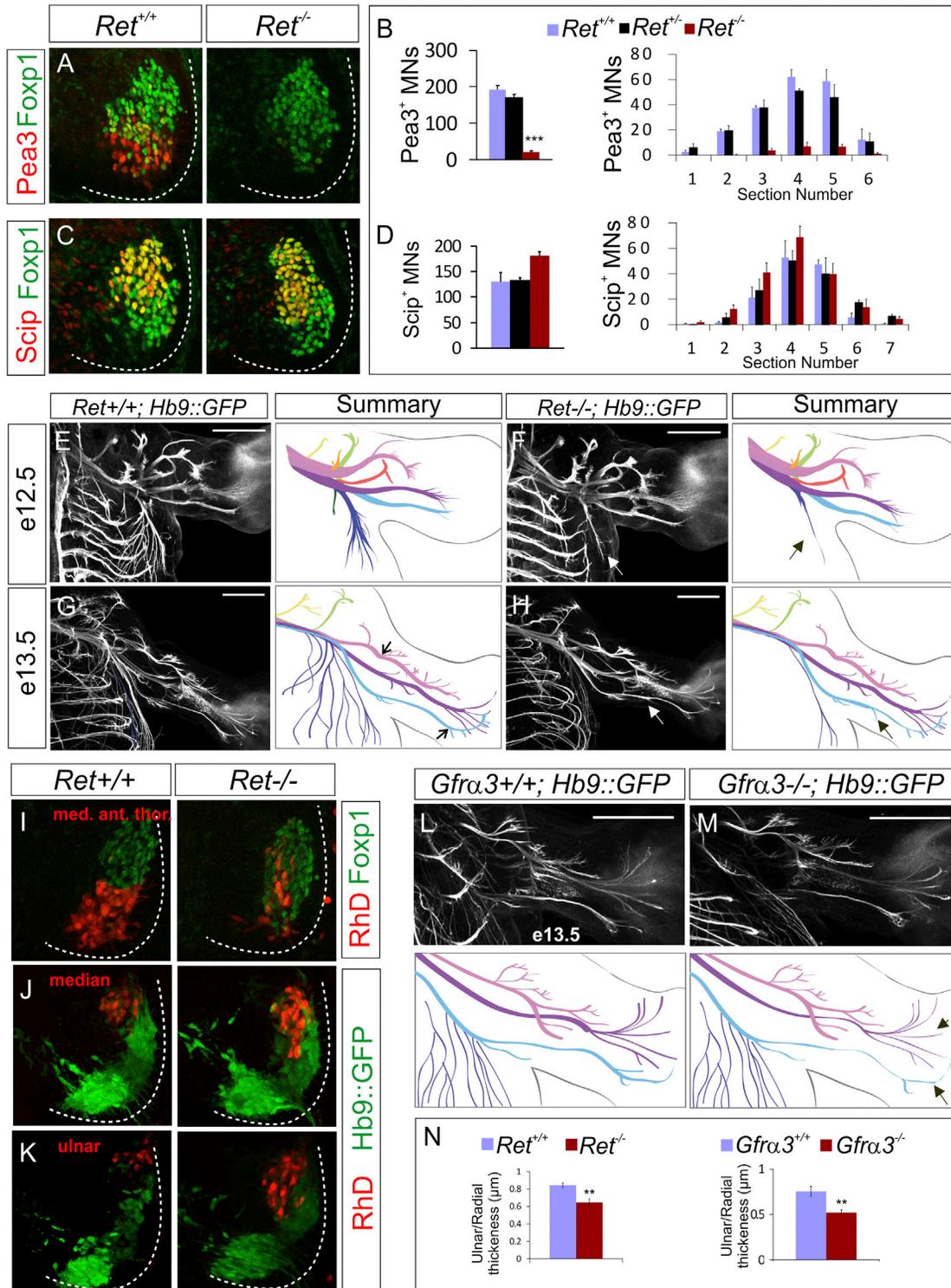


Figure 7. *Ret* and *Gfra3* Are Required for MN Pool Differentiation and Connectivity

(A) Pea3 expression is reduced in brachial LMC neurons in *Ret* mutants at E12.5.

(B) Quantification of the number and distribution of Pea3⁺ MNs in *Ret*^{-/-} and control mice.

(C) The number of Scip⁺ MNs increases in *Ret*^{-/-} mice.

(D) Quantification of the number and distribution of Scip⁺ MNs.

(E and F) Whole-mount GFP staining showing defects in CM muscle innervation in *Ret*^{-/-} embryos (arrow) at E12.5.

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number of muscle groups expanded in early vertebrates, *Hox5* and *Hox8* paralogs could have been co-opted to govern the innervation of proximal/anterior and distal/posterior muscles, respectively. Subsequently, the actions of *Hox4*, *Hox6*, and *Hox7* genes further diversified MN pools to allow for more selective targeting of limb muscles.

Comparison of limb innervation programs in mice and chick suggests the existence of diversification programs acting downstream of *Hox* genes. Both birds and rodents appear to share a common *Hox/Ret/Gfra*-based regulatory strategy for specifying *Pea3*⁺ MN pools. However, there are significant functional differences between the muscle groups targeted by these neurons. In birds, *Pea3*⁺ MNs target the pectoralis (Pec), a large muscle providing the major driving force for flexing the wing during flight (Biewener, 2011; Hollyday and Jacobson, 1990). By contrast, in mice, most *Pea3*⁺ MNs target the cutaneous maximus, a subcutaneous muscle present in fur-bearing mammals, with no known role in locomotion. We found that, in chick, *Gfra1* is restricted to the *Pea3*⁺ pool, while in mice, *Gfra1* is broadly expressed by LMC neurons. Differences in the regulation of *Gfra* genes by *Hox* proteins may have contributed to the evolutionary divergence of muscle-specific connectivity programs in birds in mammals.

In summary, these studies indicate that *Ret* and *Gfra* genes are key targets of *Hox* proteins during MN specification and the establishment of limb innervation pattern. Given that one feature of *Hox* activity is to define *Ret* levels in MNs, it is plausible that additional target effectors are under similar graded control mechanisms. Regulation of the levels of receptor gene expression could represent a common regulatory strategy through which networks of transcription factors establish neuronal subtype identities and determine target specificity.

EXPERIMENTAL PROCEDURES

Animals

All mouse procedures were approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine.

In Situ Hybridization and Immunohistochemistry

Embryos were harvested between E11.5 and E14.5, fixed in 4% paraformaldehyde for 1.5–2 hr, and processed for in situ hybridization or immunohistochemistry. For whole-mount immunohistochemistry, embryos were processed for GFP staining, as previously described (De Marco Garcia and Jessell, 2008). Confocal images were obtained with a Zeiss (LSM 700) microscope and analyzed with ImageJ software.

Retrograde Labeling of MNs

Embryos were harvested at E12.5 and eviscerated, and forelimb nerves were visualized using a MVX10 wide-field fluorescent microscope (Olympus). The nerve of interest was cut and injected with lysine-fixable rhodamine-dextran (Molecular Probes). Embryos were incubated for 3.5–4.5 hr in oxygenated DMEM/F12 (50:50) solution at 30°C–34°C, fixed, and processed for immunohistochemistry.

In Ovo Chick Embryo Electroporations

Electroporations were performed on Hamburger and Hamilton (HH) st12–14 chick embryos as previously described (Dasen et al., 2003). Plasmid concentrations ranged from 100 to 500 ng/μl, and pBluescript II KS(+) (pBKS) was used as carrier DNA to achieve a final concentration of 1 μg/μl. Results for each experiment are representative of five or more embryos in which the electroporation efficiency in MNs was >50%.

Quantifications and Statistical Analyses

Cell counting was performed using ImageJ software in at least three embryos per genotype. Cells stained for *Foxp1*, *Pea3*, or *Scip* were counted and aligned by comparison of LMC position between littermates. Nerve thickness was analyzed by measuring the length of a line running perpendicular to the long axis of the nerve. For consistent comparison, measurements were taken in similar regions of the nerves between littermates. *Ret* fluorescence intensities were measured using ImageJ software, as described previously (Gavet and Pines, 2010), with some modifications. For fluorescence measurements, we used the following formula for each section of at least three animals per genotype: *Foxp1*⁺ cell signal = sum of the intensity of pixels for *Foxp1*⁺ cells. Background signal = average signal per pixel for a region selected just beside *Foxp1*⁺ cells. *Foxp1*⁺ *Ret* fluorescence signal = *Foxp1*⁺ cell signal – surface selected (number of pixels for the selected area) × background. Statistical significance was determined with the unpaired two-tailed Student's *t* test using Microsoft Excel.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.01.067>.

AUTHOR CONTRIBUTIONS

C.C., M.M.S., and J.S.D. conceived and designed the study. D.H.L. provided technical assistance, and J.-P.L. generated reagents essential for the studies. C.C. and J.S.D. wrote the manuscript.

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REFERENCES

- Bácskai, T., Fu, Y., Sengul, G., Rusznák, Z., Paxinos, G., and Watson, C. (2013). Musculotopic organization of the motor neurons supplying forelimb and shoulder girdle muscles in the mouse. *Brain Struct. Funct.* *218*, 221–238.
- Baek, M., Enriquez, J., and Mann, R.S. (2013). Dual role for *Hox* genes and *Hox* co-factors in conferring leg motoneuron survival and identity in *Drosophila*. *Development* *140*, 2027–2038.
- Biewener, A.A. (2011). Muscle function in avian flight: achieving power and control. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* *366*, 1496–1506.

(G and H) Forelimb innervation at E13.5 showing failure of ulnar MNs to project distally in *Ret*^{-/-} embryos (arrow). Arrows in the control diagram indicate region where nerve diameters were measured.

(I–K) Disorganization of MNs in *Ret*^{-/-} mice shown by retrograde labeling of MNs projecting along the medial anterior thoracic, median, and ulnar nerves.

(L and M) Forelimb innervation pattern in *Gfra3*^{+/+} and *Gfra3*^{-/-} mice at E13.5 showing thinning of the median and ulnar nerves.

(N) Quantification of muscle nerve diameters expressed as a ratio between the radial and ulnar nerves in *Ret* and *Gfra3* mutants and controls at E13.5.

Data are shown as mean ± SEM. Scale bars represent 500 μm. **p < 0.01; ***p < 0.001.

See also Figure S7.

- Bononomi, D., and Pfaff, S.L. (2010). Motor axon pathfinding. *Cold Spring Harb. Perspect. Biol.* 2, a001735.
- Bononomi, D., Chivatakarn, O., Bai, G., Abdesselem, H., Lettieri, K., Marquardt, T., Pierchala, B.A., and Pfaff, S.L. (2012). Ret is a multifunctional coreceptor that integrates diffusible- and contact-axon guidance signals. *Cell* 148, 568–582.
- Dasen, J.S., Liu, J.P., and Jessell, T.M. (2003). Motor neuron columnar fate imposed by sequential phases of Hox-c activity. *Nature* 425, 926–933.
- Dasen, J.S., Tice, B.C., Brenner-Morton, S., and Jessell, T.M. (2005). A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. *Cell* 123, 477–491.
- Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., and Jessell, T.M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* 134, 304–316.
- De Marco Garcia, N.V., and Jessell, T.M. (2008). Early motor neuron pool identity and muscle nerve trajectory defined by postmitotic restrictions in Nkx6.1 activity. *Neuron* 57, 217–231.
- Dudanova, I., and Klein, R. (2013). Integration of guidance cues: parallel signaling and crosstalk. *Trends Neurosci.* 36, 295–304.
- Ferguson, B.A. (1983). Development of motor innervation of the chick following dorsal-ventral limb bud rotations. *J. Neurosci.* 3, 1760–1772.
- Fetcho, J.R. (1992). The spinal motor system in early vertebrates and some of its evolutionary changes. *Brain Behav. Evol.* 40, 82–97.
- Gavet, O., and Pines, J. (2010). Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Dev. Cell* 18, 533–543.
- Gould, T.W., and Oppenheim, R.W. (2004). The function of neurotrophic factor receptors expressed by the developing adductor motor pool in vivo. *J. Neurosci.* 24, 4668–4682.
- Gu, W.X., and Kania, A. (2010). Examining the combinatorial model of motor neuron survival by expression profiling of trophic factors and their receptors in the embryonic Gallus gallus. *Dev. Dyn.* 239, 965–979.
- Haase, G., Dessaud, E., Garcès, A., de Bovis, B., Birling, M., Filippi, P., Schmalbruch, H., Arber, S., and deLapeyrière, O. (2002). GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. *Neuron* 35, 893–905.
- Helmbacher, F., Schneider-Maunoury, S., Topilko, P., Tiret, L., and Charnay, P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* 127, 3313–3324.
- Hollyday, M., and Jacobson, R.D. (1990). Location of motor pools innervating chick wing. *J. Comp. Neurol.* 302, 575–588.
- Homma, S., Yaginuma, H., Vinsant, S., Seino, M., Kawata, M., Gould, T., Shimada, T., Kobayashi, N., and Oppenheim, R.W. (2003). Differential expression of the GDNF family receptors RET and GFR α 1, 2, and 4 in subsets of motoneurons: a relationship between motoneuron birthdate and receptor expression. *J. Comp. Neurol.* 456, 245–259.
- Hua, Z.L., Smallwood, P.M., and Nathans, J. (2013). Frizzled3 controls axonal development in distinct populations of cranial and spinal motor neurons. *eLife* 2, e01482.
- Jung, H., Lacombe, J., Mazzoni, E.O., Liem, K.F., Jr., Grinstein, J., Mahony, S., Mukhopadhyay, D., Gifford, D.K., Young, R.A., Anderson, K.V., et al. (2010). Global control of motor neuron topography mediated by the repressive actions of a single hox gene. *Neuron* 67, 781–796.
- Jung, H., Mazzoni, E.O., Soshnikova, N., Hanley, O., Venkatesh, B., Duboule, D., and Dasen, J.S. (2014). Evolving Hox activity profiles govern diversity in locomotor systems. *Dev. Cell* 29, 171–187.
- Kania, A., and Jessell, T.M. (2003). Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. *Neuron* 38, 581–596.
- Klein, R., and Kania, A. (2014). Ephrin signalling in the developing nervous system. *Curr. Opin. Neurobiol.* 27, 16–24.
- Kolodkin, A.L., and Tessier-Lavigne, M. (2011). Mechanisms and molecules of neuronal wiring: a primer. *Cold Spring Harb. Perspect. Biol.* 3, a001727.
- Kramer, E.R., Knott, L., Su, F., Dessaud, E., Krull, C.E., Helmbacher, F., and Klein, R. (2006). Cooperation between GDNF/Ret and ephrinA/EphA4 signals for motor-axon pathway selection in the limb. *Neuron* 50, 35–47.
- Lacombe, J., Hanley, O., Jung, H., Philippidou, P., Surmeli, G., Grinstein, J., and Dasen, J.S. (2013). Genetic and functional modularity of Hox activities in the specification of limb-innervating motor neurons. *PLoS Genet.* 9, e1003184.
- Lance-Jones, C., and Landmesser, L. (1980). Motoneuron projection patterns in the chick hind limb following early partial reversals of the spinal cord. *J. Physiol.* 302, 581–602.
- Lance-Jones, C., and Landmesser, L. (1981). Pathway selection by embryonic chick motoneurons in an experimentally altered environment. *Proc. R. Soc. Lond. B Biol. Sci.* 214, 19–52.
- Landmesser, L. (1978a). The development of motor projection patterns in the chick hind limb. *J. Physiol.* 284, 391–414.
- Landmesser, L. (1978b). The distribution of motoneurons supplying chick hind limb muscles. *J. Physiol.* 284, 371–389.
- Landmesser, L.T. (2001). The acquisition of motoneuron subtype identity and motor circuit formation. *Int. J. Dev. Neurosci.* 19, 175–182.
- Liu, J.P., Laufer, E., and Jessell, T.M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* 32, 997–1012.
- Livet, J., Sigrist, M., Stroebel, S., De Paola, V., Price, S.R., Henderson, C.E., Jessell, T.M., and Arber, S. (2002). ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. *Neuron* 35, 877–892.
- Luria, V., Krawchuk, D., Jessell, T.M., Laufer, E., and Kania, A. (2008). Specification of motor axon trajectory by ephrin-B:EphB signaling: symmetrical control of axonal patterning in the developing limb. *Neuron* 60, 1039–1053.
- Philippidou, P., and Dasen, J.S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. *Neuron* 80, 12–34.
- Philippidou, P., Walsh, C.M., Aubin, J., Jeannotte, L., and Dasen, J.S. (2012). Sustained Hox5 gene activity is required for respiratory motor neuron development. *Nat. Neurosci.* 15, 1636–1644.
- Polleux, F., Ince-Dunn, G., and Ghosh, A. (2007). Transcriptional regulation of vertebrate axon guidance and synapse formation. *Nat. Rev. Neurosci.* 8, 331–340.
- Roussou, D.L., Gaber, Z.B., Wellik, D., Morrisey, E.E., and Novitsch, B.G. (2008). Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. *Neuron* 59, 226–240.
- Sanes, J.R., and Yamagata, M. (2009). Many paths to synaptic specificity. *Annu. Rev. Cell Dev. Biol.* 25, 161–195.
- Santiago, C., and Bashaw, G.J. (2014). Transcription factors and effectors that regulate neuronal morphology. *Development* 141, 4667–4680.
- Santiago, C., Labrador, J.P., and Bashaw, G.J. (2014). The homeodomain transcription factor Hb9 controls axon guidance in Drosophila through the regulation of Robo receptors. *Cell Rep.* 7, 153–165.
- Stirling, R.V., and Summerbell, D. (1988). Specific guidance of motor axons to duplicated muscles in the developing amniote limb. *Development* 103, 97–110.
- Tosney, K.W., and Landmesser, L.T. (1985). Development of the major pathways for neurite outgrowth in the chick hindlimb. *Dev. Biol.* 109, 193–214.
- Tosolini, A.P., and Morris, R. (2012). Spatial characterization of the motor neuron columns supplying the rat forelimb. *Neuroscience* 200, 19–30.
- Tümpel, S., Wiedemann, L.M., and Krumlauf, R. (2009). Hox genes and segmentation of the vertebrate hindbrain. *Curr. Top. Dev. Biol.* 88, 103–137.
- Uemura, O., Okada, Y., Ando, H., Guedj, M., Higashijima, S., Shimazaki, T., Chino, N., Okano, H., and Okamoto, H. (2005). Comparative functional genomics revealed conservation and diversification of three enhancers of the *Isl1* gene for motor and sensory neuron-specific expression. *Dev. Biol.* 278, 587–606.
- Zarin, A.A., Asadzadeh, J., Hokamp, K., McCartney, D., Yang, L., Bashaw, G.J., and Labrador, J.P. (2014a). A transcription factor network coordinates attraction, repulsion, and adhesion combinatorially to control motor axon pathway selection. *Neuron* 81, 1297–1311.
- Zarin, A.A., Asadzadeh, J., and Labrador, J.P. (2014b). Transcriptional regulation of guidance at the midline and in motor circuits. *Cell. Mol. Life Sci.* 71, 419–432.