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Parallel *Pbx*-Dependent Pathways Govern the Coalescence and Fate of Motor Columns

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Summary

The clustering of neurons sharing similar functional properties and connectivity is a common organizational feature of vertebrate nervous systems. Within motor networks, spinal motor neurons (MNs) segregate into longitudinally arrayed subtypes, establishing a central somatotopic map of peripheral target innervation. MN organization and connectivity relies on Hox transcription factors expressed along the rostrocaudal axis; however, the developmental mechanisms governing the orderly arrangement of MNs are largely unknown. We show that *Pbx* genes, which encode Hox cofactors, are essential for the segregation and clustering of neurons within motor columns. In the absence of *Pbx1* and *Pbx3* function, Hox-dependent programs are lost and the remaining MN subtypes are unclustered and disordered. Identification of *Pbx* gene targets revealed an unexpected and apparently Hox-independent role in defining molecular features of dorsally-projecting medial motor column (MMC) neurons. These results indicate *Pbx* genes act in parallel genetic pathways to orchestrate neuronal subtype differentiation, connectivity, and organization.

Introduction

In many regions of the central nervous system groups of neurons targeting common peripheral targets are centrally organized within topographic maps. The ordered spatial

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Author Contributions

O.H. and J.S.D conceived the project, designed the experiments, and wrote the paper; R.Z. and L.S. generated *Pbx* mutants, shared them prior to publication, and helped us recover lost lines after superstorm Sandy; L.J.C. and O.H. analyzed RNAseq data; O.H., H.J., J.L., P.P., and D.H.L. performed experiments. All authors read and approved the final manuscript.

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relationship between neuronal position and target specificity is a prominent anatomical feature of primary sensory and motor systems, including the retinotectal map of the visual system and the somatotopic representation of the body surface within the cortex (Kania, 2014; Levine et al., 2012). While topographical maps appear to be critical in establishing appropriate connectivity and functionality within neural circuits, the underlying genetic mechanisms governing their formation are poorly understood.

Within the vertebrate spinal cord, the cell bodies of MNs innervating specific muscle targets are somatotopically organized within columnar, divisional, and pool subtypes (Lance-Jones and Landmesser, 1981; Landmesser, 1978a, b; Romanes, 1951). The topographical arrangement of spinal MNs appears to be a unique attribute of vertebrate motor systems, as MN subtypes of invertebrates lack somatotopic organization, although MN dendrites in *Drosophila* are highly structured (Baek and Mann, 2009; Landgraf et al., 2003; Thor and Thomas, 2002). While the purpose of MN clustering in vertebrates is not fully understood, it likely evolved to simplify the task of coordinating limb muscle activation sequences during locomotion (Fetcho, 1987), acting in part by enabling and constraining MN access to specific premotor circuits (Goetz et al., 2015; Hinckley et al., 2015; Surmeli et al., 2011).

An early step in establishing MN topographical organization involves the separation of dorsally and ventrally projecting subtypes along the mediolateral axis of the spinal cord. Neurons within the medial motor column (MMC) project dorsally to innervate axial muscles and occupy a ventromedial position. All other MN subtypes typically reside more laterally and initially pursue ventral trajectories. These highly diverse “non-MMC” populations are generated at specific segmental levels of the spinal cord as a consequence of *Hox* gene activity along the rostrocaudal axis (Philippidou and Dasen, 2013). At forelimb and hindlimb levels a network of *Hox* genes specifies the identity of the lateral motor columns (LMCs) as well as its resident ~50 MN pools targeting individual limb muscles. At thoracic levels the *Hoxc9* gene determines the identity of preganglionic motor column (PGC) neurons, and contributes to the positioning of the hypaxial motor column (HMC) (Jung et al., 2010). In contrast MMC neurons are generated at all segmental levels and appear to differentiate in a *Hox*-independent manner (Dasen et al., 2008; Dasen et al., 2003; Sharma et al., 1998).

The cell fate determinants that facilitate the clustering of MNs into columns are largely unknown. Mutation of the transcription factor *Pea3* leads to a disorganization of neurons within a subset of LMC pools (Livet et al., 2002). Downstream targets of *Pea3* include type II cadherins, which appear to be critical for the clustering of neurons within motor pools. MN pools express specific cadherin profiles, and manipulating cadherin expression alters MN settling position (Price et al., 2002). Genetic removal of α - and γ -*catenin*, which mediate signal transduction through cadherins, leads to a disorganization of neurons within the LMC (Demireva et al., 2011). Nevertheless, the separation of MMC and non-MMC populations persists in the absence of *Pea3* and *catenins*, suggesting an earlier program governs MN columnar organization.

Hox transcription factors are essential during MN subtype diversification and are plausible candidates for governing the coalescence and somatotopic organization of MMC and non-

MMC populations. Disruption of *Hox* gene function however typically leads to a transformation of ventrally-projecting MNs, while preserving their separation from the MMC. For example, in mice mutant for the *Hoxc9* gene thoracic-level specific motor columns are converted to an LMC fate, but the distribution and position of MMC neurons is unchanged (Jung et al., 2010). Similarly, the relative position of MMC and non-MMC neurons is retained after depletion of the *Foxp1* gene, which encodes an accessory factor required for Hox-dependent programs of LMC and PGC differentiation (Dasen et al., 2008; Rousso et al., 2008). Certain Hox activities in MNs are unaffected by *Foxp1* mutation, including the initial induction of the *Foxp1* gene and the cross-repressive interactions necessary to establish Hox expression boundaries (Dasen et al., 2008; Jung et al., 2014). The retention of early Hox function in *Foxp1* mutants raises the possibility that Hox-dependent pathways contribute to the segregation and clustering of spinal MNs.

We reasoned that insight into the contribution of subtype determinants during MN columnar topographic organization might emerge through manipulations that disrupt Hox activities, but otherwise preserve basic features of MN class identity. Hox proteins are known to rely on interactions with both cell type-restricted and broadly expressed cofactors (Mann et al., 2009; Moens and Selleri, 2006). In most cellular contexts, the three amino acid loop extension (TALE) class of homeodomain proteins plays prominent roles in shaping Hox protein specificity. Pbx proteins, vertebrate homologs of the *Drosophila* TALE protein extradenticle, are essential for Hox proteins to select gene targets with high affinity. Analysis of *Pbx* gene function has been constrained due to the existence of multiple gene homologs and the early lethality of mice lacking individual *Pbx* genes (Moens and Selleri, 2006). Nevertheless, studies in zebrafish and mice have demonstrated essential roles for *Pbx* genes in rostrocaudal patterning and cell type specification in the hindbrain (Cooper et al., 2003; Vitobello et al., 2011; Waskiewicz et al., 2002). Interpretation of these results is however confounded by a non-cell autonomous role of *Pbx* genes in controlling expression of morphogens during rhombomere development.

Here we investigated the role of *Pbx* genes in neuronal differentiation and topographical organization by selectively removing their activities from spinal MNs. We found that *Pbx* genes are essential for the specification and connectivity of Hox-dependent MN columnar, divisional, and pool subtypes. Unexpectedly, the remaining dorsally and ventrally projecting neurons were intermixed in *Pbx* mutants, indicating *Pbx* genes are also responsible for governing the basic program of MN clustering and columnar segregation. Identification of *Pbx* gene targets in MNs revealed an unanticipated Hox-independent role in defining molecular features of dorsally-projecting MMC neurons. These findings indicate that *Pbx* genes operate in parallel MN subtype-specific pathways to govern the formation of spinal motor columns.

Results

***Pbx* Genes Are Dispensable in Early MN Differentiation and Rostrocaudal Patterning**

To assess the involvement of *Pbx* genes in MN subtype diversification we first examined the profile of Pbx protein expression within the spinal cord. Three of the four mammalian Pbx proteins; Pbx1, Pbx2, and Pbx3, were expressed by spinal neurons at embryonic day (e) 11.5

(Figure 1A). Pbx1 was ubiquitously expressed with elevated expression in progenitors and postmitotic MNs (Figure 1A,B, S1A). Pbx2 was detected at low levels throughout the spinal cord, while Pbx3 was expressed in postmitotic neurons, with prominent expression in MNs (Figure 1A,C). Pbx3 was also restricted within Hox-dependent MN populations, including thoracic PGC neurons and subsets of limb-level LMC neurons, but was excluded from axially projecting MMC neurons (Figure 1C, S1B–D). Thus, in mice two of the four mammalian Pbx proteins, Pbx1 and Pbx3 are highly expressed by spinal MNs.

To determine the role of *Pbx* genes in MN differentiation we devised genetic strategies to inactivate their function. To circumvent the early lethality of *Pbx1* null mutant mice (Selleri et al., 2001), we bred a floxed *Pbx1* allele to *Olig2::Cre* mice to generate a MN-specific knockout line (referred to henceforth as *Pbx1^{MN}* mice) (Koss et al., 2012). *Pbx3* mutants are viable during embryogenesis (Rhee et al., 2004a), and we therefore generated *Pbx1* and *Pbx3* double mutants by introducing a *Pbx3* null line into the *Pbx1^{MN}* background. The phenotypes of *Pbx1^{MN} ; Pbx3^{-/-}* mice were not enhanced by introduction of a *Pbx2^{-/-}* allele (data not shown) (Selleri et al., 2004), and we therefore refer to *Pbx1/Pbx3* double mutants as *Pbx^{MN}* mice. In *Pbx1^{MN}*, *Pbx3^{-/-}*, and *Pbx^{MN}* lines the respective Pbx proteins were effectively depleted from MNs (Figure 1D,E), and of these alleles, only a rare *Pbx1^{MN}* mutant survived until adulthood. We also generated embryos in which both *Pbx1* and *Pbx3* were selectively eliminated from MNs by combining a floxed *Pbx3* allele (Rottkamp et al., 2008) with *Pbx1^{MN}* and *Pbx3* heterozygote alleles (Figure 1E). The phenotypes of *Pbx1 flox/flox; Pbx3^{-/flox}; Olig2::Cre⁺* embryos were indistinguishable from that of *Pbx1^{MN} ; Pbx3^{-/-}* embryos at e12.5 (data not shown).

We assessed how loss of individual and multiple *Pbx* genes affects core features of MN identity and early rostrocaudal patterning. In *Pbx1^{MN}*, *Pbx3*, and *Pbx^{MN}* embryos markers for MN neurotransmitter synthesis including choline acetyltransferase (Chat) and vesicular choline acetyltransferase transporter (Vacht) were present at e12.5, indicating *Pbx* genes are not required for generating MNs as a class (Figure 1F, S1E and data not shown). In addition the MN progenitor determinants *Olig2* and *Nkx6.1*, and the early postmitotic markers *Isl1/2*, *Hb9* and *Lhx3*, were grossly unaltered in *Pbx* mutants (Figure 1D,E, S1F). At thoracic levels the total number of MNs was similar between control and *Pbx^{MN}* mutants, although there was ~30% reduction in MNs at limb levels, similar to the loss observed in *Foxp1* mutants (Figure 4I) (Dasen et al., 2008; Rousoo et al., 2008). In *Pbx1^{MN}* mutants we also observed an expansion of the Pbx3 expression domain at caudal brachial levels (Figure S1G), although no changes in *Pbx2* and *Pbx4* expression were observed in any of the *Pbx* mutant alleles we analyzed (data not shown).

In the hindbrain *Pbx* function has been implicated in positive autoregulatory interactions that maintain expression of *Hox* genes in specific rhombomeres (Tumpel et al., 2009). At spinal levels a major determinant of *Hox* gene expression is cross-repressive interactions between Hox proteins and *Hox* genes (Philippidou and Dasen, 2013). We therefore investigated a possible function of *Pbx* genes in controlling the pattern of *Hox* expression within the spinal cord. In *Pbx^{MN}* mutants the rostrocaudal boundaries between *Hoxc6/Hoxc9* (brachial/thoracic boundary) and *Hoxa5/Hoxc8* (rostral brachial/caudal brachial boundary) were preserved (Figure 1G–I, S1I–K). In thoracic segments *Hoxc9* protein levels were reduced in

MNs of *Pbx^{MN}* mice at e12.5, while the overall patterns of *Hoxa5*, *Hoxc6*, and *Hoxc8* were similar to controls (Figure 1H,I,S1I–K). These observations indicate that Pbx proteins contribute to sustaining the levels of certain Hox proteins, but are dispensable for cross-repressive interactions in spinal MNs.

To further assess whether Hox cross-repressive interactions can occur independent of Pbx function, we determined the effects of misexpressing Hox mutant derivatives in which the canonical Pbx interaction motifs have been mutated. We generated mutations within motifs N-terminal to the homeodomain which are necessary for high affinity interactions with Pbx proteins on DNA. Misexpression of a *Hoxc9* derivative with a mutated Pbx interaction motif (*Hoxc9IM* NW→AA) in chick neural tube was able to repress *Hoxc6*, *Hoxa5* and LMC specification at brachial levels (Figure 1J,K, S1L). However, unlike wildtype *Hoxc9*, this mutation failed to induce PGC neurons at brachial levels (Figure S1L). As previously reported *Hoxc6IM* repressed *Hoxc9* at thoracic levels (Figure 1L,M). *Hoxc6IM* can induce an LMC fate at thoracic levels (Lacombe et al., 2013), likely as a consequence of *Hoxc9* attenuation. Although we cannot rule out the possibility that Hox proteins interact with Pbx proteins through additional domains (Merabet and Mann, 2016), these results indicate that *Pbx* genes are not required for establishing the overall rostrocaudal pattern of *Hox* expression in MNs.

***Pbx* Genes are Essential for the Differentiation of Hox-Dependent MN Subtypes**

We next examined the function of *Pbx* genes in the diversification of segmentally-restricted MN subtypes. At limb levels LMC neurons are defined by expression of the transcription factor *Foxp1* and the retinoic acid (RA) synthetic enzyme *Raldh2* (Dasen et al., 2008). In *Pbx3* mutants *Foxp1* and *Raldh2* expression was unaltered at brachial levels (Figure S2A). In *Pbx1^{MN}* embryos we observed a marked reduction in the number of brachial *Foxp1⁺*, *Raldh2⁺* neurons (Figure S2D,E), although the penetrance of this phenotype was apparently offset by the upregulation of *Pbx3* within this region (Figure S1G). Analysis of mice lacking both *Pbx1* and *Pbx3* revealed a dramatic reduction in *Foxp1* and *Raldh2* expression (Figure 2A,B). *Raldh2*-dependent RA synthesis in LMC neurons provides a feed-back signal that promotes the proliferation of MN progenitors (Sockanathan and Jessell, 1998), and the loss of this signal likely accounts for the decrease in brachial MNs in *Pbx* mutants. These results indicate *Pbx* genes are essential to establish the identity and normal number of LMC neurons.

Hox genes are critical for the further differentiation of LMC neurons into divisional and pool subtypes which determine how motor axons select muscle targets in the limb (Dasen et al., 2005). Expression of *Lhx1* in the lateral division of the LMC defines MNs that project to the dorsal limb compartment (Kania, 2014), and this population of *Lhx1⁺*, *Foxp1⁺* neurons was depleted in *Pbx^{MN}* mutants (Figure 2C,D). Within the LMC, neurons targeting individual limb muscles segregate into MN pools, some of which can be defined by expression of specific transcription factors. In *Pbx^{MN}* mice, we observed a loss of MN pools marked by *Scip*, *Nkx6.1* and *Nkx6.2* expression (Figure 2I–K). Expression of the pool marker *Pea3* was preserved in *Pbx^{MN}* mutants (Figure 2L), consistent with studies suggesting Pbx-independent regulation of its expression (Catela et al., 2016; Lacombe et al., 2013), and

reliance on peripheral signaling to control its induction (Figure S2F–H) (Haase et al., 2002). *Pbx* genes are therefore essential for the appearance of multiple molecular features of LMC subtypes.

At thoracic levels *Hox* genes are necessary for the differentiation of PGC neurons that innervate the sympathetic chain ganglia. PGC neurons express neuronal nitric oxide synthase (nNos), phospho (p) Smad1/5/8, Isl1, low levels of Foxp1, and settle dorsolaterally (Dasen et al., 2008). In *Pbx^{MN}* mutants nNos and pSmad expression is lost from MNs and Isl1⁺ MNs fail to migrate to a dorsolateral position (Figure 2E–H). Interestingly, the differentiation of PGC neurons also relied on the net level of *Pbx* expression in MNs as *Pbx1^{MN}*; *Pbx3^{+/-}* mutants (which retain a single copy of *Pbx3*) also display dramatically reduced numbers of PGC neurons (Figure S2B,C). The dose-dependent phenotypes of *Pbx* mutant alleles likely reflect differences in the level and pattern of *Pbx1* and *Pbx3* within specific MN subtypes (Figure S1A–D).

In addition to these well-characterized Hox-dependent MN populations, other segmentally-restricted subtypes were affected in *Pbx* mutants. At rostral cervical levels two non-LMC (Foxp1⁻) populations of MNs can be defined by the expression of the transcription factors Sox5 and Scip (Philippidou et al., 2012). Sox5 is expressed by a laterally-positioned Lhx3⁺, Hb9⁺, Isl1⁺ MN pool, and this population is lost in *Pbx^{MN}* mice (Figure 2M). Motor neurons within the phrenic motor column (PMC) rely on the activities of *Hox5* genes and coexpress Scip and Isl1. In *Pbx^{MN}* embryos this population is reduced, disorganized, and shifted to a more medial position (Figure 2N). Interestingly both Scip⁺ PMC and Sox5⁺, Lhx3⁺ MNs are retained in *Foxp1* mutants (Dasen et al., 2008; Rousso et al., 2008), indicating that loss of *Pbx* genes affects most segmentally-restricted MNs subtypes.

Peripheral Innervation Defects in *Pbx* Mutants

Because molecular signatures of Hox-dependent MN subtypes are lost in *Pbx^{MN}* mice, we next determined the impact of *Pbx* mutation on the trajectory and target selectivity of motor axons. We bred *Pbx^{MN}* mutant mice to *Hb9::GFP* mice, in which all motor axons are GFP labeled, and analyzed the overall pattern of peripheral innervation. Projections into the limb were detectable in *Pbx^{MN}*; *Hb9::GFP* mutants at e12.0, but at subsequent time points the distal nerve branches were thinner, and specific target regions lacked innervation (Figure 3A,B). Nerve branches to muscles in the proximal forelimb were missing or stunted in *Pbx^{MN}* mutants, including a severe reduction in the density of projections along the radial nerve (Figure 3A,B). *Pbx^{MN}*; *Hb9::GFP* mice also displayed pronounced innervation defects within the hindlimb. The most striking defect was observed along the tibialis anterior nerve which was completely absent at in *Pbx^{MN}* mice at e13.5 (Figure 3C,D)

At thoracic levels MNs within the PGC pursue a ventromedial trajectory towards sympathetic chain ganglia (scg), and subsequently send collateral projections that extend along the rostrocaudal axis. In wholemount staining of control *Hb9::GFP* mice, these projections are visible as a medial GFP⁺ band that extends parallel to the spinal cord (Figures 3E, S3C,D). In *Pbx^{MN}* mutants projections towards and between sympathetic chain ganglia were dramatically reduced (Figures 3G, S3A,B,G,H). Similarly, in *Pbx1* conditional mutants retaining one copy of *Pbx3* there was a pronounced decrease in

sympathetic chain ganglia innervation (Figures 3F, S3E,F). In contrast motor nerves projecting to axial and hypaxial muscles (which derive from MMC and thoracic HMC neurons, respectively) were preserved in *Pbx^{MN}* mutants (Figure 3G).

One of the most severely affected nerve branches in *Pbx* mutants derives from phrenic MNs that extend to the diaphragm muscle. At e12.5 the phrenic nerve was visible in *Pbx^{MN} ; Hb9::GFP* mutants, but was dramatically thinner and shorter than in control littermates (Figure S3I,J). By e16.5 there was a severe loss of synapses at the diaphragm, with the majority of muscle fibers lacking innervation and postsynaptic acetyl choline receptor clusters (Figure 3H–K). The severe defects in diaphragm innervation likely account for the perinatal lethality of *Pbx^{MN}* mutants.

Motor Columns Are Disorganized in *Pbx* Mutants

What are the fates of the remaining MN populations after deletion of *Pbx* genes? In mice mutant for the Hox accessory factor *Foxp1*, LMC and PGC neurons acquire the identity of thoracic HMC neurons, while axially projecting MMC neurons are unaffected (Dasen et al., 2008). In *Pbx* mutants the number of *Lhx3⁺*, *Hb9⁺* MMC neurons was not significantly altered at brachial and thoracic levels (Figure 4A–I). The remaining populations consisted predominantly of MNs with an HMC-like molecular profile (*Hb9⁺*; *Isl1/2⁺*) as well a smaller group that expressed *Isl1/2* alone (Figure 4I, S4A–D). These results indicate that in the absence of *Pbx* function, the remaining MNs display molecular features of MMC and HMC subtypes.

Analysis of the distribution of MNs in *Pbx* mutants revealed a striking disorganization in their settling position. In *Pbx^{MN}* mice *Lhx3⁺*, *Hb9⁺* (MMC-like); *Hb9⁺*, *Isl1/2⁺* (HMC-like) and *Isl1/2⁺* MNs were intermixed at e12.5 (Figure 4A–H). This phenotype was observed at all rostrocaudal levels of the spinal cord, and was particularly prominent at thoracic levels. Comparison of serial sections in *Pbx* mutants revealed no positional preference of MMC and non-MMC neurons indicating they are stochastically positioned within the ventral spinal cord (Figure 4A–H). In contrast, the organization and specification of ventral interneurons was not affected in *Pbx^{MN}* mice (Figure S4G). To quantify the degree of MN intermixing in *Pbx* mutants, we calculated a columnar mixing index (Cmi) for thoracic MMC and HMC neurons in control and mutant mice at e12.5 (Demireva et al., 2011). This allowed us to determine the extent to which MMC neurons invade the confines of the HMC, and HMC invasion into the MMC. In control thoracic sections Cmi values for MMC→HMC and HMC→MMC averaged 0.14 and 0.15 respectively. In *Pbx* mutants this value increased to 0.90 for MMC→HMC and 0.85 for HMC→MMC (Figure 4J).

Because the transcription factors used to discriminate columnar identities (*Lhx3*, *Hb9*, and *Isl1/2*) are expressed by the precursors to all MN subtypes, we considered the possibility that the observed intermixing is not due to migratory or clustering defects, but rather a failure of MNs to fully differentiate in *Pbx^{MN}* mice. If, however the remaining columnar subtypes are properly differentiated, they would be predicted to target muscles appropriate for their molecular identity. Because loss of an LMC identity leads to a random targeting of limb muscles by MNs (Dasen et al., 2008; Roussot et al., 2008), we assessed the targeting of MMC and HMC neurons at thoracic levels, where these two populations are present

normally. We injected tracers into the intercostal nerves (targets of HMC neurons) or axial muscles (targets of MMC neurons) in control and *Pbx^{MN}* mutants at e12.5 and monitored the transcriptional profile of retrogradely labeled MNs. After tracer injection into the intercostal nerves of *Pbx^{MN}* mutants, labeled MNs exhibited an HMC profile (*Isl1/2⁺*, *Hb9⁺*) and lacked *Lhx3* (Figure 4K). After injection into axial muscles, labeled MNs exhibited an MMC profile (*Lhx3⁺*, *Hb9⁺*, *Isl1/2^{low}*) (Figure 4K). In agreement with the analysis of MMC and HMC molecular profiles, retrogradely labeled neurons lacked any clear columnar organization. These results indicate that despite their altered position, the remaining thoracic MMC and HMC neurons in *Pbx^{MN}* mice differentiate and select appropriate muscle targets. In contrast, retrograde labeling from the forelimb ulnar nerve labeled HMC-like profile (*Isl1⁺*, *Lhx3⁻*) that were dispersed within the spinal cord (Figure S4H), similar to the targeting defects observed in *Foxp1* mutants (Dasen et al., 2008).

Several lines of evidence indicate that the intermixing of HMC and MMC neurons reflect a unique function of *Pbx* genes during MN differentiation. Analysis of *Foxp1* mutants revealed a loss of Hox-dependent subtypes at limb and thoracic levels, with the remaining MNs consisting predominantly of HMC and MMC neurons (Dasen et al., 2008; Rousso et al., 2008). Despite the similarity in the loss of MN identities in *Foxp1* and *Pbx* mutants, in the absence of *Foxp1* the remaining HMC and MMC neurons were clustered and well segregated (Figures 4L, S4E). In mice lacking the *HoxA* and *HoxC* gene clusters, Hox-dependent MN subtypes are similarly lost at brachial and thoracic levels (Jung et al., 2014). The *HoxA* and *HoxC* clusters encode the majority of Hox proteins expressed at these levels, and therefore approximate a Hox-less ground state. In combined *HoxA* and *HoxC* mutants, the clustering and segregation of the remaining subtypes (MMC, HMC, and *Isl1/2⁺* MNs) was unaffected (Figures 4M, S4F) (Jung et al., 2014). Calculation of columnar mixing indices in *Foxp1* and *HoxA/C* mutants revealed no increase in thoracic MMC/HMC intermixing relative to Cmi values of control littermates (Figure 4J). These observations suggest a unique, and possibly Hox-independent, function for Pbx proteins in the organization of columnar subtypes projecting to epaxial and hypaxial muscles (Figure 4N).

Identification of Genes Selectively Depleted in *Pbx* Mutants

The intermixing of the remaining MMC and non-MMC neurons in *Pbx^{MN}* mice prompted us to consider whether *Pbx* genes might selectively regulate target effectors present in these populations. To explore this possibility, we analyzed a panel of genes demonstrated to be downstream of MN fate determinants. We reasoned genes which facilitate the segregation and clustering of MMC and non-MMC populations would be specifically lost in *Pbx* mutants, but maintained under conditions where columnar segregation is preserved, such as in *Foxp1* mutants. We screened over two dozen genes, including members of the *cadherin* and *ephrin/Eph* genes families, which have been implicated in neuronal migration and clustering within the hindbrain and spinal cord (Kania, 2014). This analysis identified a number of motor pool-restricted genes that are diminished in *Pbx^{MN}* mice, although the majority of these genes were also downregulated in *Foxp1* mutants (Figure S5A–E and data not shown).

These results encouraged us to initiate an unbiased screen to identify genes selectively lost in *Pbx^{MN}* mice. We compared gene expression profiles in MNs isolated from control and *Pbx^{MN}* embryos at e12.5. We purified MNs from *Pbx^{MN} ; Hb9::GFP* and control *Hb9::GFP* embryos by fluorescence-activated cell sorting (FACS) (Figure 5A). Due to the distinct molecular profiles of MNs generated at brachial and thoracic levels, we independently profiled both populations. We extracted RNA from MNs purified from 9 *Pbx^{MN} ; Hb9::GFP* and 9 *Hb9::GFP* embryos at brachial and thoracic levels, pooled 3 RNA samples of each genotype, and prepared 12 bar coded libraries. We then performed expression profiling by RNA-seq. The samples were mixed into two pools and run on two 50-nucleotide paired-end read rapid run flow cell lanes with the Illumina HiSeq 2500 sequencer.

To evaluate the quality of this screen we examined the expression of genes known to be differentially expressed between brachial and thoracic levels. Comparison of expression profiles between purified control samples yielded known cervical/brachial- (*Aldh1a2/Raldh2, Etv4/Pea3, Runx1, Lypd1/Lynx2, Hoxc6*) and thoracic- level (*nNos, Etv1/Er81, Hoxc9*) restricted MN determinants (Figure 5B, Table S1). Read counts of *HoxC* cluster genes were similar between control and *Pbx^{MN}* mice at both brachial and thoracic levels (Figure S6E), reinforcing the conclusion that the *Hox* patterns are grossly preserved in *Pbx^{MN}* mice.

Because loss of *Pbx* genes affects MN organization at all rostrocaudal levels, we focused on genes whose profiles were altered at both brachial and thoracic levels. Comparison of gene profiles within brachial and thoracic MNs identified 31 transcripts (27 downregulated and 4 upregulated genes) that were common to both populations and were differentially expressed between control and *Pbx* mutant mice (Figure 5C,D, S6A–D, Table S2,S3). To validate these targets, we used *in situ* hybridization to compare expression patterns of candidates between control and *Pbx^{MN}* mice at e12.5. Analysis of the 27 downregulated candidates identified 13 that were expressed by MNs in control animals (Figures 6A–M, data not shown). Each of these genes was undetectable or markedly downregulated in MNs of *Pbx^{MN}* mice, confirming them as *Pbx* targets (Figure 6A–M). Novel genes that were upregulated in *Pbx^{MN}* mice and validated by *in situ* hybridization included *Cpne5* and *Cdkn1a* (Figure 6N,O). In control embryos *Cpne5* was weakly expressed by MNs with elevated expression in MMC neurons (Figure 6N). In *Pbx^{MN}* mice *Cpne5* expression was markedly upregulated in all remaining MNs (Figure 6N). Additional genes from this list were either not detected by *in situ* hybridization or expressed by non-MN populations (data not shown).

Among the confirmed downregulated genes in *Pbx* mutants were a number that were restricted to ventral spinal populations. Included in this group were the cell surface proteins *Lifr* and *Megf11*, the secreted protein *Dkk3*, the intracellular protein *Ezr*, and the transcription regulators *Mecom*, *Ebf2*, *Ldb2*, *Hlf*, and *Creb5* (Figure 6A–D,G–I,J,L). Expression of *Ldb2*, *Hlf*, *Lifr*, and *Creb5* was not detectable in the spinal cords of *Pbx^{MN}* mice at e12.5, while expression of *Mecom*, *Dkk3*, *Ezr*, and *Megf11* was selectively lost from subsets of MNs (Figure 6). *Mecom* was expressed in ventromedial MN populations as well as a dorsal interneuron population at all rostrocaudal levels. In *Pbx^{MN}* mutants expression of *Mecom* was markedly diminished in MNs, while its pattern in dorsal interneurons was

unaffected (Figure 6A). Expression of *Mecom*, *Dkk3*, *Ebf2*, *Ldb2*, *Hlf*, *Lifr*, and *Cyp26b1* were also maintained in *Foxp1* mutants, while *Cpne5* and *Cdkn1a* were not upregulated (Figure S5F–T), suggesting regulation of these targets independent of the Hox/Foxp1-dependent programs acting in non-MMC neurons.

***Mecom* Defines a Pbx-Dependent Population of MNs Targeting Axial Muscle**

The identification of MN subtype-restricted genes that are selectively downregulated in *Pbx* mutants enabled us to further assess a potential Hox-independent function of Pbx proteins during MN specification. Due to its restricted expression in ventromedial MN populations we chose *Mecom* for further analysis. We characterized the pattern of *Mecom* expression relative to MN determinants between e9.5–e12.5. *Mecom* protein was first detected at e9.5 as the first brachial postmitotic MNs appear, and was subsequently maintained in a subset of medial MNs (Figures 7A,B, S7A). At e12.5 *Mecom* colocalized with markers for MMC identity at all rostrocaudal levels of the spinal cord, but was excluded from *Foxp1*⁺ LMC and PGC neurons (Figure 7A,B). In addition, *Mecom* was absent from rostral brachial *Sox5*⁺, *Lhx3*⁺ MNs, but was present in a small subset of thoracic *Lhx3*[−], *Foxp1*[−] MNs (Figure 7B, S7B). The MMC-restricted pattern of *Mecom* was also conserved in MNs of chick embryos (Figure S7C). *Mecom* therefore defines a novel postmitotic marker labeling MMC neurons projecting to dorsal axial muscles.

We next investigated the regulation of *Mecom* by Pbx proteins and other MN subtype determinants. In *Pbx*^{MN} mutants the level of *Mecom* protein expression was markedly reduced, with low levels detected in the remaining scattered *Lhx3*⁺ MMC cells (Figure 7C,M). In contrast the pattern of *Mecom* expression was unaffected in *Foxp1* and *HoxC* cluster mutants, and its restriction to MMC neurons was retained (Figure 7C). These observations provide additional *in vivo* evidence that Pbx proteins function independently of the Hox/Foxp1 program to regulate expression of MMC-restricted genes such as *Mecom*.

If *Mecom* is regulated by Pbx proteins independent of Hox function, its expression could reflect an output of an earlier MMC-specific differentiation program. We therefore tested if determinants of MMC identity can induce expression of *Mecom* in non-MMC neurons. Misexpression of the Hb9 transcription factor has been shown to impose a MN fate on interneurons, while expression of *Lhx3* in all MNs directs an MMC fate (Sharma et al., 2000; William et al., 2003). Because expression of *Lhx3* in non-MNs produces predominantly V2a interneurons, while expression of Hb9 generates MNs with either MMC or HMC-like properties (Dasen et al., 2008; Thaler et al., 2002), we coexpressed both factors to produce ectopic MMC neurons. We found that misexpression of *Lhx3* and Hb9 induced ectopic *Mecom*⁺ MNs (Figure 7D,E, S7D). *Lhx3* and Hb9 coexpression also extinguished expression of *Foxp1*, a known target of Hox proteins in MNs (Figure 7F).

To test whether the supernumerary *Mecom*⁺ MNs induced by *Lhx3* and Hb9 requires Pbx function, we expressed these factors in conjunction with a dominant-negative Engrailed-repressor fusion with Pbx1 (EnPbx1). Expression of EnPbx1 alone repressed *Mecom* and *Foxp1*, but not Hb9, consistent with Pbx1 activity being required for the differentiation of MMC and LMC neurons (Figure 7G–I). In contrast, coexpression of *Lhx3*, Hb9, and EnPbx1 failed to generate *Mecom*⁺ MMC neurons (Figure 7J–L). Collectively, these results

show that *Pbx* genes are essential for the normal expression of *Mecom* in MNs, and act in concert with *Lhx3* to determine its MMC-restricted pattern (Figure 7N). *Pbx* proteins therefore appear to act in parallel Hox-dependent and independent programs to control the subtype differentiation and organization of MN subtypes.

Discussion

The clustering of motor neurons into longitudinally arrayed columnar groups is a defining feature of topographical maps within tetrapod motor systems, but the underlying genetic mechanisms governing their formation has remained elusive. We found that *Pbx* genes are essential for the formation and differentiation of spinal motor columns. Consistent with roles as Hox cofactors, *Pbx* genes are required for the specification of MN subtypes along the rostrocaudal axis and the establishment of appropriate patterns of peripheral innervation. Unexpectedly, our studies show that *Pbx* genes are also critical for the coalescence of MNs into columns, revealing a novel molecular program mediating the partitioning of dorsally projecting MMC neurons from all other MN subtypes. These studies could provide a foundation for resolving the role of MN position in locomotor circuit connectivity and exploring the origins of topographic organization within motor systems.

***Pbx* Genes as Cofactors for Hox-Dependent Steps in MN Differentiation**

Hox genes are essential for the specification of neuronal classes along the rostrocaudal axis where they contribute to the diversification and connectivity of MN columnar, divisional, and pool subtypes. *Pbx* cofactors are well known to enhance the affinity and binding selectivity of Hox proteins to target sites, but their precise roles during neuronal subtype specification are poorly defined. In the hindbrain, mutation of *Pbx* genes disrupts expression of extrinsic signaling factors, leading to non-cell autonomous defects in neuronal specification and connectivity (Cooper et al., 2003; Vitobello et al., 2011). By eliminating *Pbx* genes selectively from MNs, we found that *Pbx* proteins are cell autonomously required for the differentiation of limb and thoracic-specific MN subtypes. In contrast to the role of the Hox accessory factor *Foxp1*, which is necessary for subtype differentiation of LMC and PGC neurons (Dasen et al., 2008; Rousso et al., 2008), loss of *Pbx* genes affects all ventrally-projecting MN subtypes. These results indicate that *Pbx* genes are essential for the differentiation of the majority of Hox-dependent subtypes.

While *Pbx* genes are necessary for the differentiation of MNs, not all Hox activities are lost in their absence. In spinal MNs Hox cross-repressive interactions define the position of columns and pools along the rostrocaudal axis, as exemplified by the phenotype of *Hoxc9* mutants, where brachial-level *Hox* genes are derepressed and thoracic MNs are transformed to an LMC fate (Jung et al., 2010). We find that *Pbx* genes are dispensable for this repressive activity, as in their absence *Hox* boundaries are preserved. Moreover, expression of the majority of *Hox* genes is not affected by loss of *Pbx* genes, suggesting positive-autoregulatory interactions are not critical in most spinal MNs. Thus while the ability of *Hox* genes to promote neuronal diversity relies on *Pbx* activity, early Hox patterns appear to be established in a *Pbx*-independent manner.

Hox-Independent Roles of *Pbx* Genes in MN Columnar Organization

In tetrapods MNs projecting to functionally-related peripheral targets cluster into columnar and pool groups, establishing a central somatotopic map of peripheral innervation (Kania, 2014; Levine et al., 2012). The topographical organization of MNs can be revealed at a molecular level by expression of certain classes of transcription factors including Hox, Lim HD, and Foxp1 proteins (Dasen et al., 2008; Tsuchida et al., 1994). While Lim HD proteins define many MN subtypes, their specific role in establishing columnar organization is unclear. In mice lacking Lhx3/4 and Isl1/2 Lim HD proteins, MNs lose basic features of their identity or are transformed to an interneuron fate (Sharma et al., 1998; Thaler et al., 2004), confounding any potential role in MN clustering. In the absence of *Hox* genes or *Foxp1*, MNs still retain core features of their identity, and the remaining columnar subtypes are well clustered. In contrast, *Pbx* genes appear to have a relatively specific role in segregating MMC from non-MMC populations. In the absence of *Pbx* genes, MNs still retain general features of their identity but the remaining MMC and HMC populations are intermixed. These observations indicate that pathways acting within MMC and non-MMC populations ensure MN coalescence and appropriate settling position (Figure 8A).

In principle the segregation of MMC and non-MMC populations could be governed by specific molecular programs acting within these groups, or a consequence of the migratory paths of MNs as a function of their relative birth order. Our analysis of *Pbx* gene targets suggest the absence of columnar organization is due to the combined loss of molecular signatures of MMC and non-MMC neurons. We found that *Pbx* genes are essential to regulate a set of MMC-restricted genes, including the transcription factor *Mecom*. *Mecom* is selectively lost in *Pbx* mutants but retained in the absence of *Foxp1*, suggesting *Mecom* is regulated independently of Hox protein activity. Consistent with this idea misexpression of determinants of MMC fates such as Lhx3 can induce expression of *Mecom* at all rostrocaudal levels. Lhx3 is known to suppress Hox-dependent programs in MNs (Dasen et al., 2008), providing further evidence that *Mecom* induction does not rely on specific Hox proteins. In the cortex, a CNS region that lacks *Hox* gene expression, *Pbx1* has been recently shown to bind within regions of the *Mecom* locus, suggesting direct regulation of *Mecom* by *Pbx* proteins (Golonzhka et al., 2015). These observations are in agreement with studies in *Drosophila* and mice, showing *Pbx* proteins have essential functions independent of their roles as Hox cofactors (Merabet and Mann, 2016). Our studies indicate that *Pbx* proteins can act within a single neuronal class to facilitate both Hox-dependent and Hox-independent programs of neuronal organization and connectivity.

MN Clustering and Topographic Organization Within the Motor System

While multiple classes of transcription factors contribute to the formation of MN topographical maps, the developmental mechanisms through which columnar organization is achieved are not well understood. *Reelin* and its receptor *Disabled* play essential roles in the migration and final positioning of LMC and PGC subtypes, and are downstream targets of Foxp1 and Lhx1 (Palmesino et al., 2010). In mice lacking *Reelin* or *Disabled* neurons occupy inappropriate positions within the spinal cord but are otherwise well clustered (Kania, 2014). The transcription factor *Pea3* is necessary for the organization of MNs targeting the cutaneous maximus (CM) muscle, and in *Pea3* mutants CM neurons are

interspersed with MN pools occupying the same segment. Targets of *Pea3* include *cadherin8*, and type II cadherins have been implicated in the clustering of MN pools (Demireva et al., 2011; Livet et al., 2002). However, the early genetic pathways that ensure the clustering and segregation of MMC and non-MMC neurons are not known.

Our results indicate that *Pbx* genes operate in parallel pathways to govern MMC and non-MMC differentiation, and that these two programs coordinate the coalescence and organization of motor columns. In non-MMC neurons, including limb-innervating LMC populations, loss of *Pbx* genes prevents the differentiation of Hox-dependent divisional and motor pool subtypes. These phenotypes are highly reminiscent of mutation in the Hox accessory factor *Foxp1*, where LMC neurons are transformed to an HMC fate, and the position of LMC pools is scrambled, likely as a consequence of altered *cadherin* expression (Figure 8B). Nevertheless the segregation of MMC and HMC neurons persists in *Foxp1* mutants, due to the preservation of organizational systems acting within MMC neurons.

In *Pbx* mutants there is a selective depletion in a subset of MMC-restricted genes, and loss of these factors likely contributes to their disorganization. Importantly, loss of the Pbx-dependent program does not affect the ability of the remaining MMC and HMC neurons to select appropriate muscle targets, suggesting a unique function of Pbx targets in governing MN columnar organization. It is unlikely that this MMC-specific program governs MN coalescence alone, but rather acts in concert with the Pbx/Hox-dependent network. Consistent with this idea, a preliminary analysis of existing *Mecom* mutants indicated a grossly normal segregation of MMC and non-MMC neurons (Hanley, unpublished observations), likely due to the preservation of Hox-dependent clustering programs. The disordering of MMC and non-MMC neurons therefore appears to be due to the loss of both Pbx-dependent programs, a condition that is achieved through removal of *Pbx* genes from all MN subtypes.

Columnar Organization and the Evolution of Motor Circuits

What is the purpose of organizing MNs into columns? The segregation of MNs into columnar groups appears to be a unique organizational feature of vertebrates, and is conserved in all tetrapod classes that have been examined including birds, reptiles and mammals (Jung and Dasen, 2015). A basic step in establishing MN topography involves the separation of dorsally-projecting MMC neurons from ventrally projecting subtypes. In contrast, MNs targeting dorsal and ventral axial muscle compartments in zebrafish are largely intermixed with each other (Ampatzis et al., 2013; Menelaou and McLean, 2012). Nevertheless, axial MNs of zebrafish appear to be functionally organized along the dorsoventral axis, where specific “pools” of MMC-like neurons are recruited at distinct locomotor speeds (Ampatzis et al., 2014; McLean et al., 2007). This organizational feature may have evolved to coordinate the activation of axial MNs that drive specific types of undulatory locomotor behaviors, such as slow swimming or predator escape responses. In contrast, in tetrapods MMC neurons are typically associated with postural stabilization, and locomotion is driven predominantly by LMC neurons. Although the origin of the Pbx-dependent MMC program in tetrapods is unclear, it may have appeared during the transition

of vertebrates to terrestrial habitats, or was selectively lost in lineages adapted to undulatory forms of locomotion.

The organization of MNs into columnar groups could impact the assembly and function of motor networks by restricting the neuronal populations that a MN has access to. It has been demonstrated that LMC and MMC neurons engage distinct populations of spinal premotor interneurons (Goetz et al., 2015). LMC neurons receive a preponderance of inputs from ipsilaterally located inhibitory interneurons, while MMC neurons connect with premotor populations that are evenly distributed across both sides of the spinal cord. The medial location of MMC neurons could enable access to the contralateral side of the spinal cord, allowing the MMC to capture a greater proportion of inputs from commissural interneurons. Similarly, the inputs that MNs receive from proprioceptive sensory neurons appears to be shaped by the relative position of motor pools within the LMC (Surmeli et al., 2011). The *Pbx*-dependent pathways described here may have evolved as a means to separate MMC premotor circuits required for postural stabilization from the LMC-directed networks that govern locomotion.

Experimental Procedures

Mouse Genetics

Pbx1 flox (Koss et al., 2012), *Pbx3*^{-/-} (Rhee et al., 2004b), and *Pbx3 flox* (Rottkamp et al., 2008), have been described previously. *Pbx3 flox* and *Hb9::eGFP* mice were obtained from Jackson Laboratories. Animal procedures were performed in accordance with the US National Institutes of Health Animal Protection Guidelines and approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine.

Wholemout, Immunohistochemistry and *In Situ* Hybridization

Immunohistochemistry was performed on 16 μ m cryostat sections as described (Dasen et al., 2005). Primary antibodies were generated as described (Dasen et al., 2008; Dasen et al., 2005; Tsuchida et al., 1994). Additional antibodies are described in Supplemental Experimental Procedures. Wholemount antibody staining was performed as described (Dasen et al., 2008) and GFP-labeled motor axons were visualized in projections of confocal Z-stacks (400–600 μ m). Dissections and wholemounts of diaphragm muscles from E14–18.5 mice were stained as described (Philippidou et al., 2012). Unless indicated otherwise, immunohistological data shown in figures are representative of n>3 mutants analyzed and are taken from animals which are *Pbx1*^{ΔMN}; *Pbx3*^{-/-}. Images for control animals are from age matched littermates that are Cre⁻, and either *Pbx3*^{+/+}, or *Pbx3*^{+/-}. Further information on histological analyses are described in Supplemental Experimental Procedures.

In Ovo Chick Embryo Electroporation

Chick neural tube electroporations were performed at Hamburger and Hamilton (HH) st12–14 and analyzed at st27–28 as previously described (Dasen et al., 2003). Plasmid concentrations ranged from 100–500 ng/ μ l and pBKS was used as carrier DNA to achieve a final concentration of 1 μ g/ μ l. Results for each experiment are representative of five or more embryos in which the electroporation efficiency in MNs was >50%. The *Hoxc9IM*-

pCAGGS construct was generated by mutation in the conserved Pbx interaction domain (ANWI→AAAI), and *Hoxc6IM-pCAGGS* has been described previously (Lacombe et al., 2013).

RNAseq and Computational Analysis

Details on acquisition of RNAseq data are described in Supplemental Experimental Procedures. The alignment program, Bowtie (version 1.0.0) was used with reads mapped to the Ensemble NCBIM37/mm9 (iGenome version) with two mismatches allowed. The uniquely-mapped reads were subjected to subsequent necessary processing, including removal of PCR duplicates, before transcripts were counted with htseq-count. Counts files were imported into the R statistical programming environment and analyzed with the DESeq2 R/Bioconductor package (Love et al., 2014). Analyses were done on the NYULMC high performance computing cluster. Reproducible pipeline scripts are available: <https://github.com/dasenlab/Pbx-Neuron-Paper>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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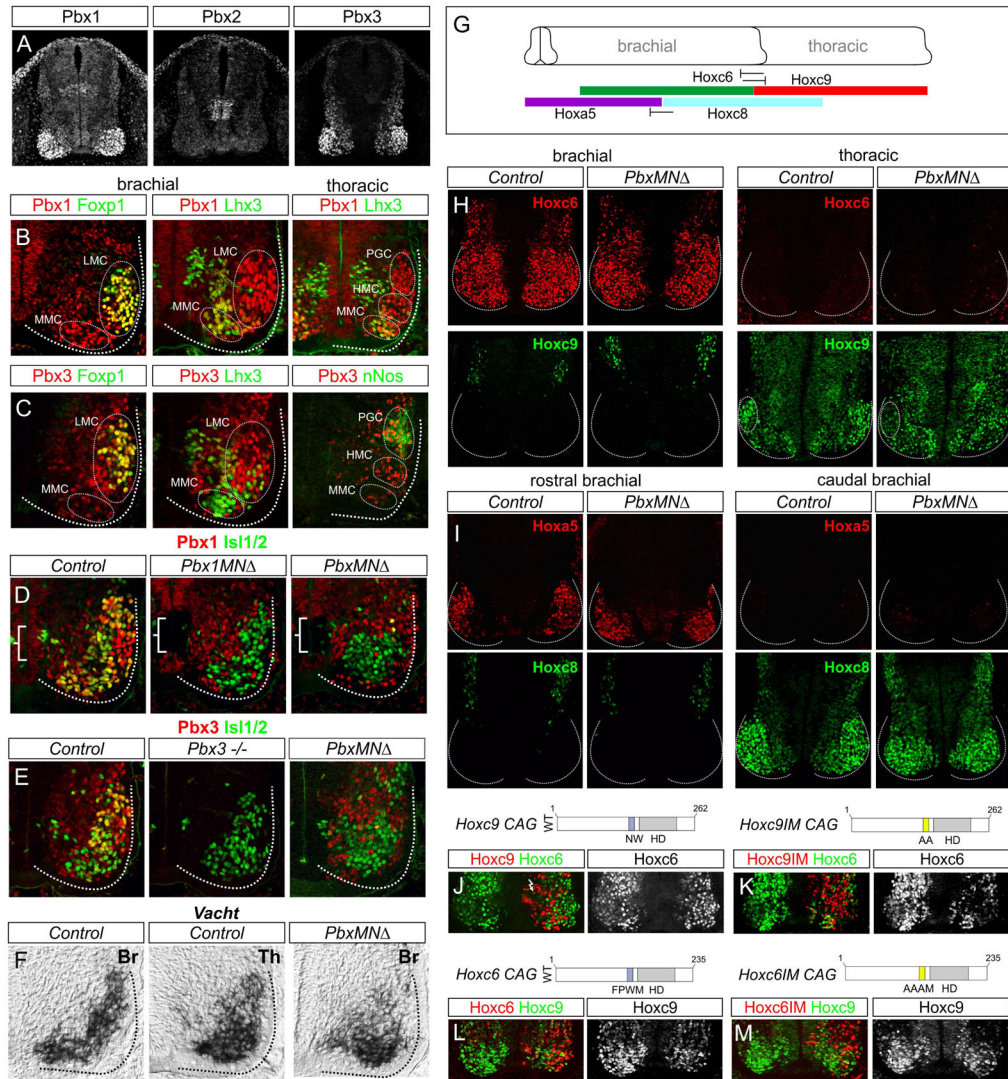


Figure 1. *Pbx* Genes are Not Required for MN Generation or Establishing *Hox* Boundaries

(A) *Pbx* protein expression in mouse spinal cord at e11.5. *Pbx1* is expressed in progenitors and postmitotic MNs. *Pbx2* is expressed at low levels in progenitors and postmitotic neurons, and a population of interneuron progenitors. *Pbx3* is expressed by postmitotic spinal neurons. (B) *Pbx1* colocalizes with *Foxp1*⁺ LMC neurons at brachial levels, *Lhx3*⁺ MMC neurons, and thoracic HMC and PGC neurons. (C) *Pbx3* is restricted to rostral brachial *Foxp1*⁺ LMC neurons and excluded from *Lhx3*⁺ MMC neurons. At thoracic levels *Pbx3* is expressed in HMC and *nNos*⁺ PGC neurons. (D) *Pbx1* expression is lost in progenitors (brackets) and postmitotic MNs in *Pbx1*^{MN} and *Pbx*^{MN} mice. (E) *Pbx3* expression is lost in *Pbx3*^{-/-} and *Pbx*^{MN} mice. *Pbx3* staining in *Pbx*^{MN} section is from the *Pbx3* conditional allele. (F) *Vacht* mRNA expression at e12.5 in control brachial (Br) and thoracic (Th) MNs and in Br MNs of *Pbx*^{MN} mice. Loss of Br MNs in *Pbx* mutants does not appear to be due to increased apoptosis (Figure S1H). (G) Summary of *Hox* expression boundaries in MNs at brachial and thoracic levels. (H) *Hoxc6* and *Hoxc9* boundaries are maintained in *Pbx*^{MN} mutants, but *Hoxc9* levels are reduced in PGC neurons. (I) *Hoxa5*

and Hoxc8 boundaries are maintained in *Pbx^{MN}* mutants. (J, K) Misexpression of Hoxc9 or a Hoxc9-Pbx interaction mutant (Hoxc9IM) at brachial levels represses *Hoxc6*. (L, M) Misexpression of Hoxc6 or a Hoxc6IM at thoracic levels represses *Hoxc9*. See also Figure S1.

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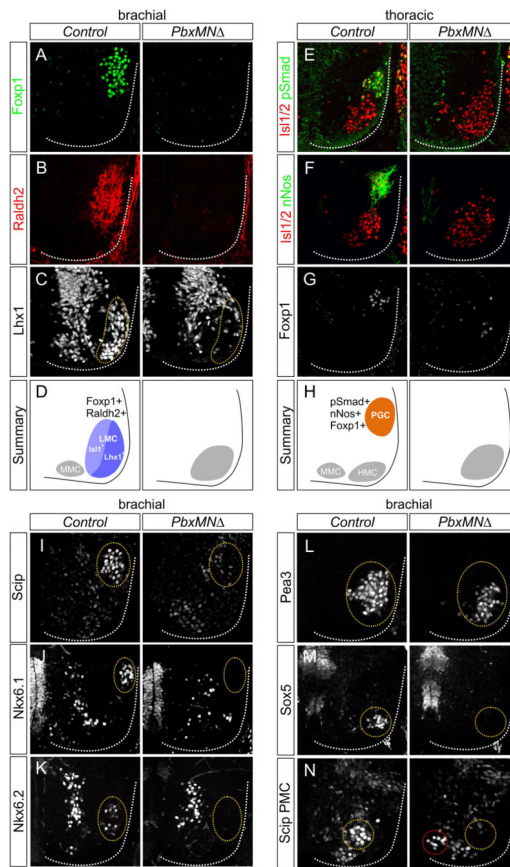


Figure 2. *Pbx* Genes are Essential for MN Columnar, Divisional, and Pool Specification
 (A,B) Expression of Foxp1 and Raldh2 is reduced in *Pbx^{MN}* mutants at e12.5. (C) Expression of Lhx1 within the lateral division of the LMC is depleted at brachial levels in *Pbx^{MN}* mice. (D) Summary of LMC neuron organization at brachial levels in control and *Pbx^{MN}* mice. (E–G) At thoracic levels there is a loss of nNos, Foxp1, and pSmad expression in *Pbx^{MN}* mutants. (H) Summary of MN organization at thoracic levels in control and *Pbx^{MN}* mice. (I) Loss of Scip expression from median and ulnar MN pools in *Pbx^{MN}* mice (J,K) Loss of Nkx6.1 and Nkx6.2 expression from rostral brachial pools in *Pbx^{MN}* mutants. (L) Expression of Pea3 is detected in *Pbx^{MN}* mice, but mispositioned ventromedially. (M) Loss of the non-LMC Sox5⁺ pool in *Pbx^{MN}* mice. (N) Expression of Scip in phrenic MNs is reduced and mislocalized (red circle) in *Pbx^{MN}* mice. In panels I–N circled areas discriminate MNs that express indicated factors from other spinal populations. In *Pbx^{MN}* mice circled areas represent position where these pools would be present normally. See also Figure S2.

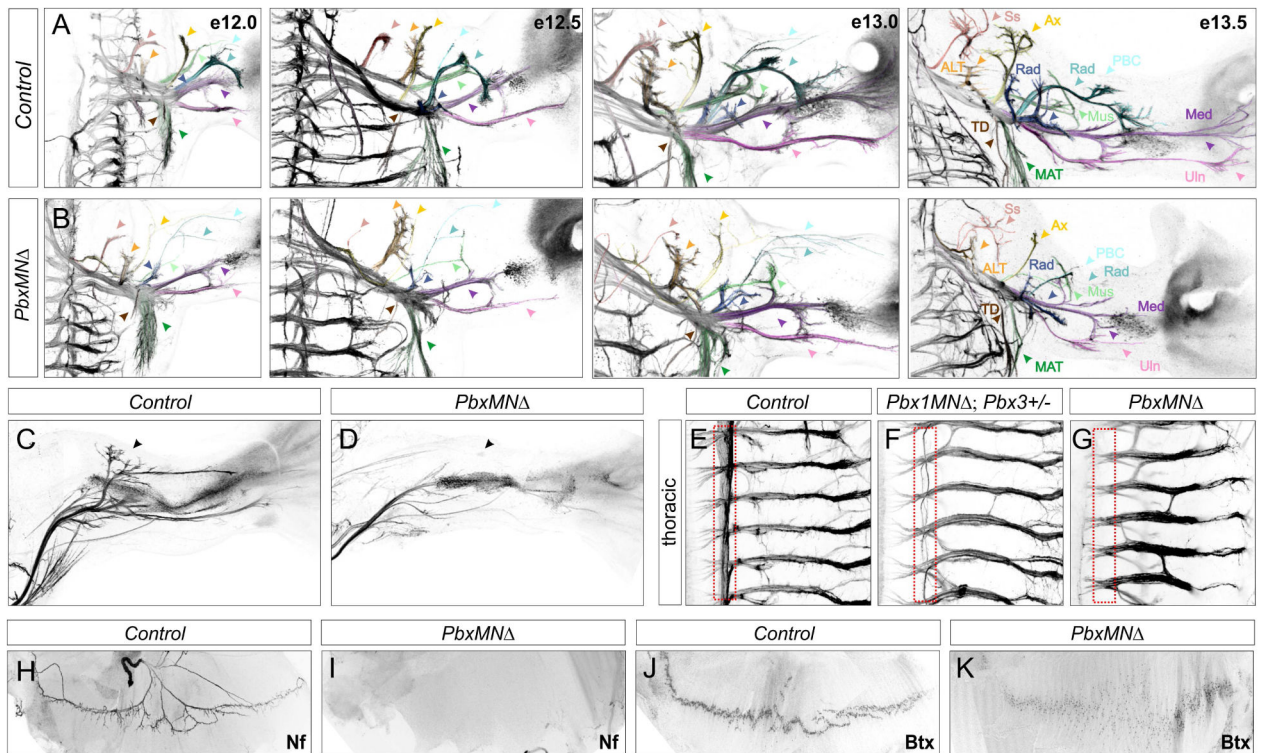


Figure 3. Motor Axon Projections in *Pbx^{MN}* Mice

(A,B) Dorsal view of forelimb innervation in control and *Pbx^{MN}* mice between e12.0 and e13.5. *Pbx^{MN}* mice have thinner axons and display defects in axonal branching and nerve trajectories. Color coding: Suprascapular Nerve (N.) (Ss, red), Anterior Lateral Thoracic N. (ALT, orange), Axillary N. (Ax, yellow), Musculocutaneous N. (Mus, neon green), Radial N. (Rad, blue), Posterior Brachial Cutaneous N. (PBC, aqua), Radial/Musculospiral N. (Rad, dark blue), Median N. (Med, dark purple) Ulnar N. (Uln, light purple), Thoracodorsal nerve to Lattismus Dorsi (LD) (TD, brown) and Medial Anterior Thoracic N. to the Cutaneous Maximus (CM) (MAT, dark green). (C,D) In *Pbx^{MN}*; *Hb9::GFP* mice the dorsal tibialis anterior nerve fails to form in the hindlimb. (E–G) Motor axon projections at thoracic levels showing loss of sympathetic chain ganglia innervation (outlined in red). Mice retaining one allele of *Pbx3* also display projection defects. Projections along the intercostal nerves are retained in *Pbx* mutants, although some aberrant branching is observed. (H,I) Diaphragm innervation defects in *Pbx^{MN}* mutants at e16.5. Motor axons are labeled using Neurofilament (Nf) staining. In *Pbx^{MN}* mice phrenic axons fail to innervate the diaphragm. (J,K) Staining with α-bungarotoxin (Bgt) showing acetylcholine receptor (AChR) clustering in control animals and absence of concentrated clusters in *Pbx^{MN}* mice. See also Figure S3.

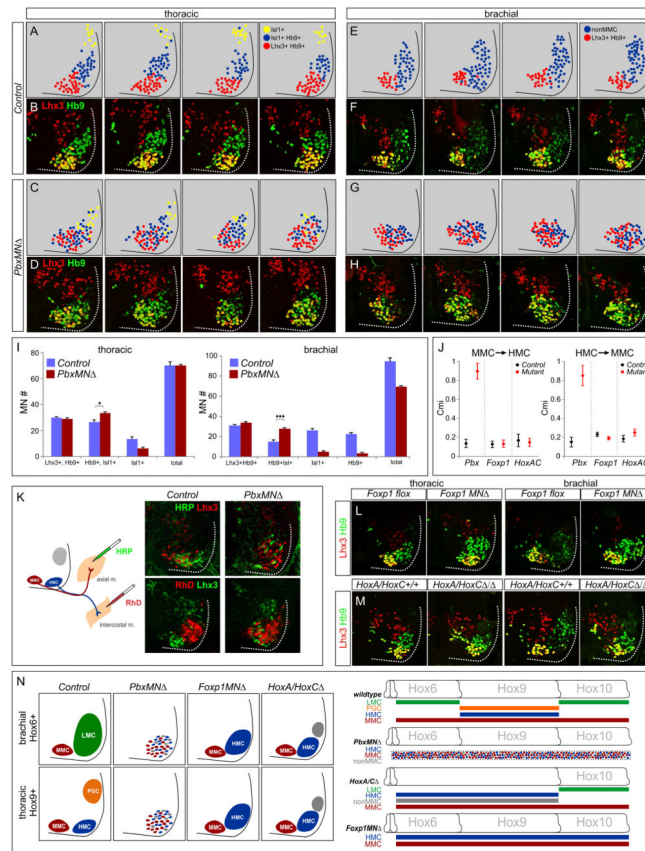


Figure 4. Motor Neuron Columnar Disorganization in *Pbx*^{MN} Mice

(A–D) Position of molecularly defined thoracic MN subtypes in control and *Pbx*^{MN} mice at e12.5. Schematics represent serial thoracic sections of control and *Pbx* mutants indicating the position of MN subtypes defined by *Lhx3*, *Hb9*, and *Isl1* expression. MMC neurons are *Lhx3*⁺, *Hb9*⁺; HMC neurons are *Hb9*⁺, *Isl1*⁺, *Lhx3*⁻; and PGC neurons are *Isl1*⁺. (E–H) Position of MN subtypes at brachial levels. Non-MMC neurons are defined as MNs that are *Lhx3*⁻, and express *Hb9* and/or *Isl1*. Brachial sections shown are from segments C6–C8. (I) Quantification of MN subtypes in control and *Pbx* mutants at brachial and thoracic levels. Molecular codes for columnar subtypes are indicated. MN counts show average of indicated subtype on one side of spinal cord \pm SEM. * $p < 0.05$, *** $p < 0.001$. (J) Quantification of columnar mixing indices (Cmi) for the indicated mutant at thoracic levels. Data are shown as mean Cmi \pm SEM, averaged from $n > 4$ sections, from $n > 3$ animals of indicated genotype. (K) Retrograde labeling of HMC and MMC neurons in control and *Pbx*^{MN} mice at e12.5. MMC neurons were labeled by injection of horseradish peroxidase (HRP) into axial muscles, HMC neurons by injection of intercostal nerves with rhodamine dextran (RhD). (L–M) Comparison of MN organization at thoracic levels in control, *Pbx*^{MN}, *Foxp1* and *HoxA/C* mutants. In *Foxp1* and *HoxA/C* mutants MMC neurons are organized and segregated from non-MMC neurons. (N) Summary of defects in MN specification and positioning in *Pbx*, *Foxp1*, and *Hox* cluster mutants. See also Figure S4

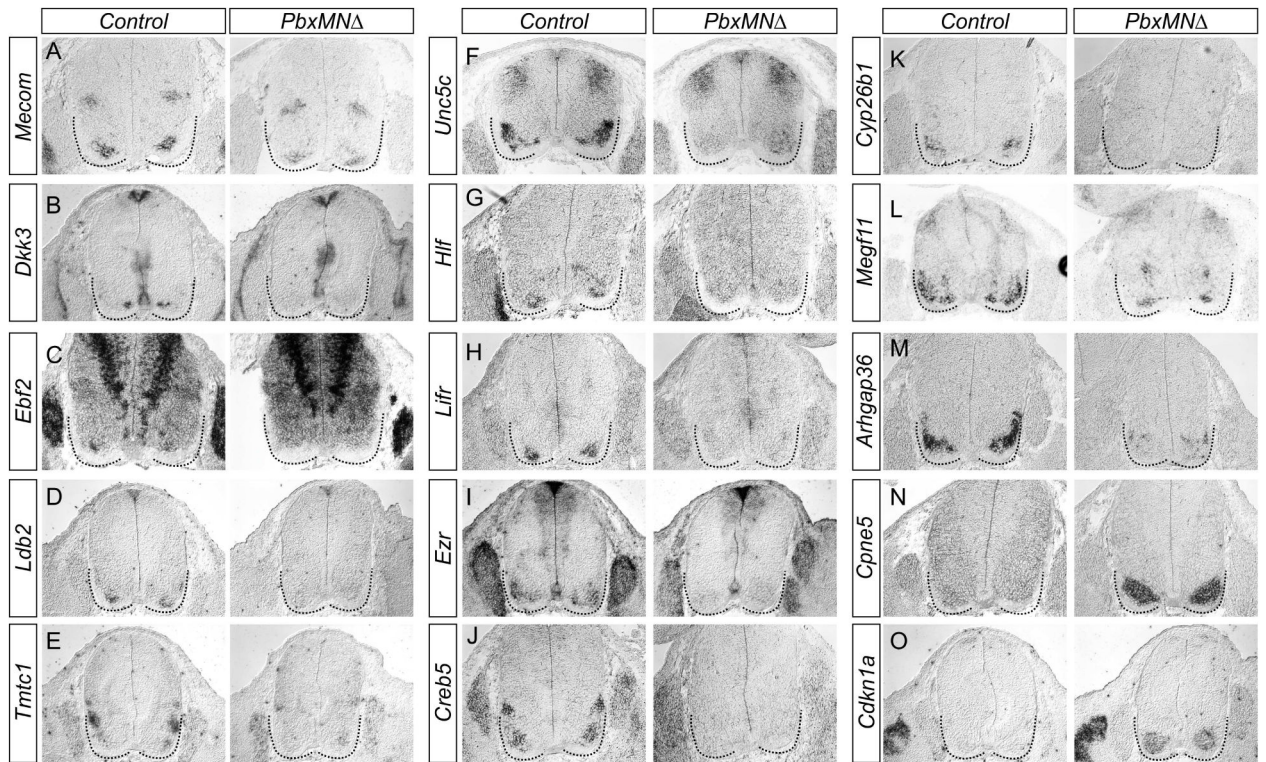


Figure 6. Characterization of Pbx Target Gene Expression in Motor Neurons

(A–O) Analysis of genes common to brachial and thoracic levels that are differentially expressed between control and *Pbx^{MN}* mice. Panels show *in situ* hybridization of indicated genes in spinal cord sections of e12.5 mice. All sections are brachial except J and K, which are thoracic. Sections are derived from embryos which are *Pbx1 flox/flox*; *Pbx3-/flox*; *Olig2::Cre⁺*. (A) *Mecom* is downregulated in ventromedial neurons but preserved in dorsal interneurons. (B–K) Expression of *Dkk3*, *Ebf2*, *Ldb2*, *Tmtc1*, *Unc5c*, *Hlf*, *Lifr*, *Ezr*, *Creb5*, and *Cyp26b1* is markedly decreased in MNs of *Pbx* mutants. (L) *Megf11* is downregulated in LMC neurons but maintained in a subset of ventromedial MNs. (M) *Arhgap36* expression is markedly reduced in *Pbx^{MN}* mice. (N,O) *Cpne5* and *Cdkn1a* are upregulated in *Pbx* mutants. See also Figure S6.

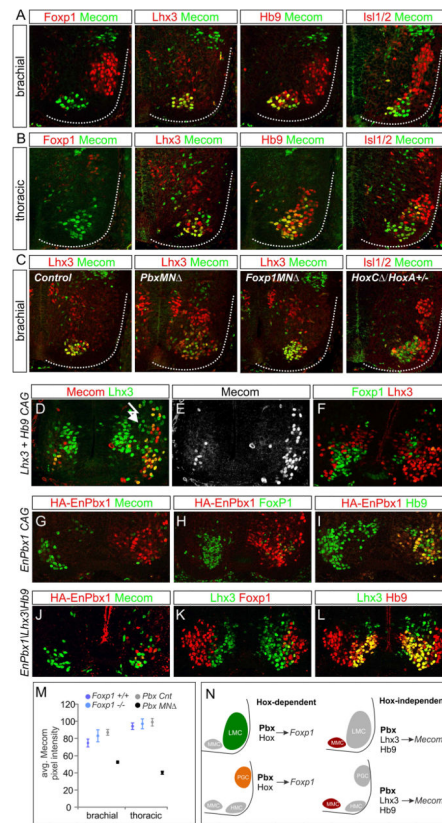


Figure 7. Hox-Independent Regulation of *Mecom* by Pbx Proteins and *Lhx3*

(A) *Mecom* protein expression at brachial levels at e12.5. *Mecom* is detected in *Lhx3*⁺, *Hb9*⁺ MMC neurons but excluded from *Foxp1*⁺ LMC neurons. (B) *Mecom* expression at thoracic levels. *Mecom* is detected in MMC neurons, a subset of *Lhx3*⁻, *Hb9*⁺ MNs, but is excluded from *Foxp1*⁺ PGC neurons. (C) Analysis of *Mecom* expression in *Pbx*, *Foxp1*, and *HoxC* mutants. *Mecom* is reduced in MMC neurons of *Pbx*^{MN} mice, but is unaffected in *Foxp1*^{MN} and *HoxC* mutants. (D,E) Chick electroporations at thoracic levels showing *Mecom* expression is induced after *Lhx3* and *Hb9* misexpression. (F) *Lhx3*/*Hb9* also represses *Foxp1* expression. (G–I) Expression of *EnPbx1* represses *Mecom* and *Foxp1* in MNs. (J–L) Coexpression of *EnPbx1*, *Lhx3*, and *Hb9* fails to generate ectopic *Mecom*⁺ MNs. (M) Quantification of *Mecom* levels in *Pbx* and *Foxp1* mutants. Data are shown as average pixel intensities of *Mecom* immunofluorescence \pm SEM. (N) Summary MN columnar specification by Pbx proteins. Pbx proteins act in Hox-dependent pathways to induce expression of columnar determinants such as *Foxp1*. Pbx proteins also act in a Hox-independent manner to regulate expression of MMC-restricted genes including *Mecom*. See also Figure S7.

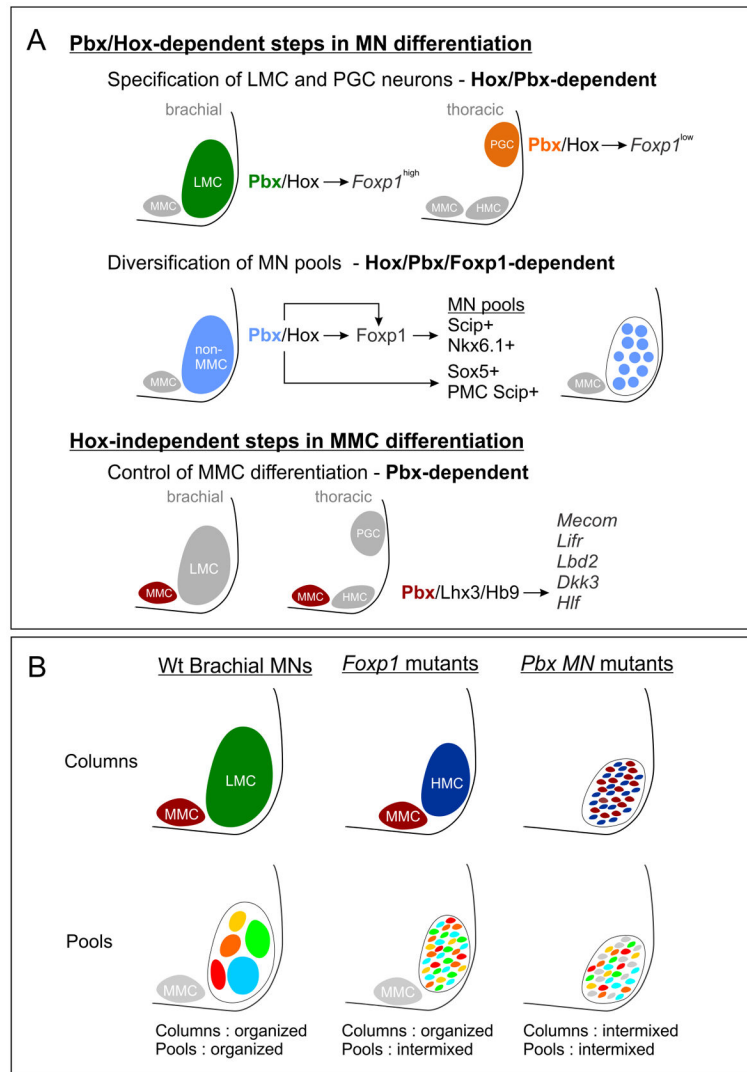


Figure 8. *Pbx* Genes Function in Parallel MN Differentiation Pathways

(A) *Pbx* genes are essential for Hox-dependent and -independent steps in MN differentiation. *Pbx* genes are required for two critical Hox-dependent steps: the specification of LMC and PGC columnar subtypes, and the diversification of LMC motor pools. *Pbx* genes are also necessary for the maturation of MMC neurons and govern expression of a subset of MMC-restricted genes, including *Mecom*. (B) Summary of defects in MN organization in *Foxp1* and *Pbx* mutants at brachial levels. MN organization in wildtype (Wt) mice at brachial levels is shown. In *Foxp1* mutants LMC neurons revert to an HMC-like identity but are clustered and segregated from MMC neurons. However, the position of MN pools projecting to individual muscles is disordered. In *Pbx* mutants the remaining HMC and MMC neurons are intermixed, leading to severe defects in MN clustering.