

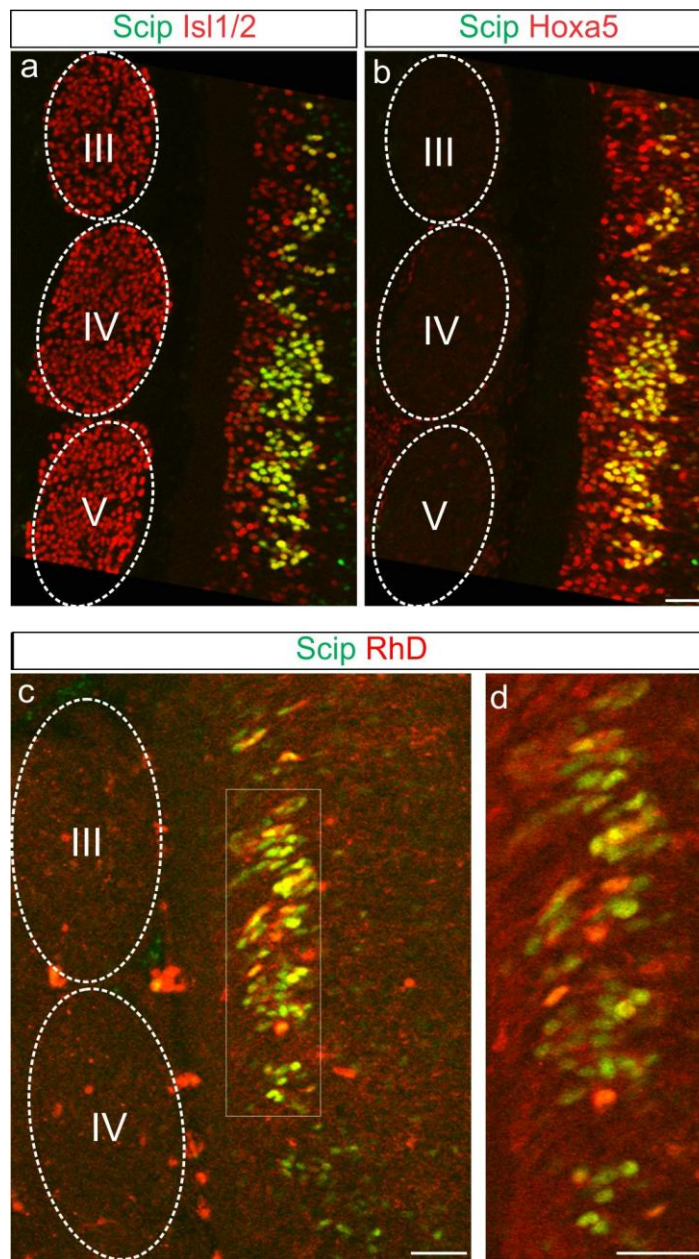
Sustained *Hox5* Gene Activity is Required for Respiratory Motor Neuron Development

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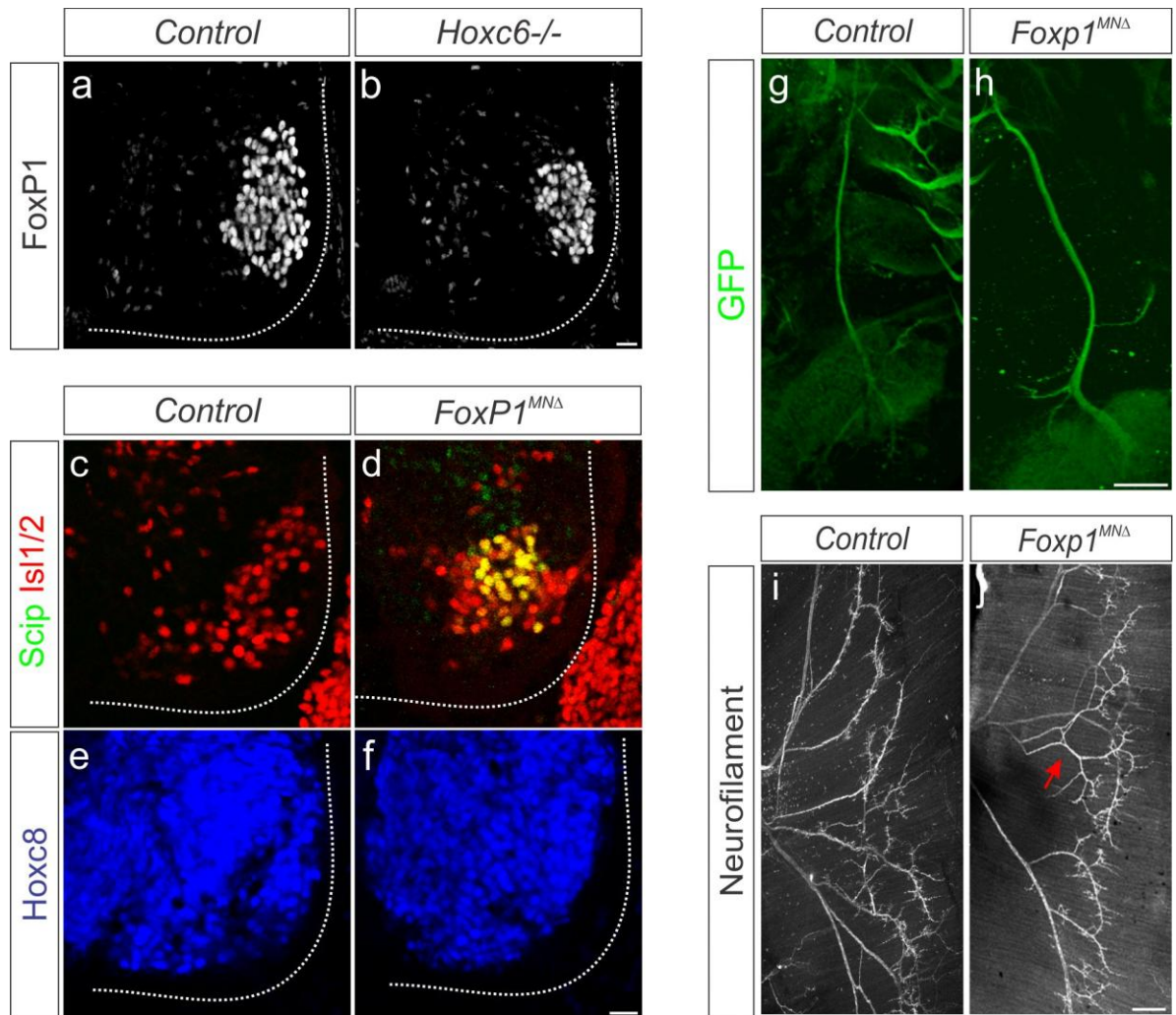
Supplementary Information



Supplementary Figure 1. Rostrocaudal extent of PMC position.

(a-b) Scip expression in longitudinal spinal cord sections at e12.5. Scip+ motor neurons are located in segments III-V, within the domain of Hox5 expression. Scale bar=50 μ m.

(c-d) Retrograde labeling of Scip+ motor neurons after RhD injection into the phrenic nerve at e12.5. Scale bar=50 μ m.



Supplementary Figure 2. Hox and FoxP1 activities define PMC size and position.

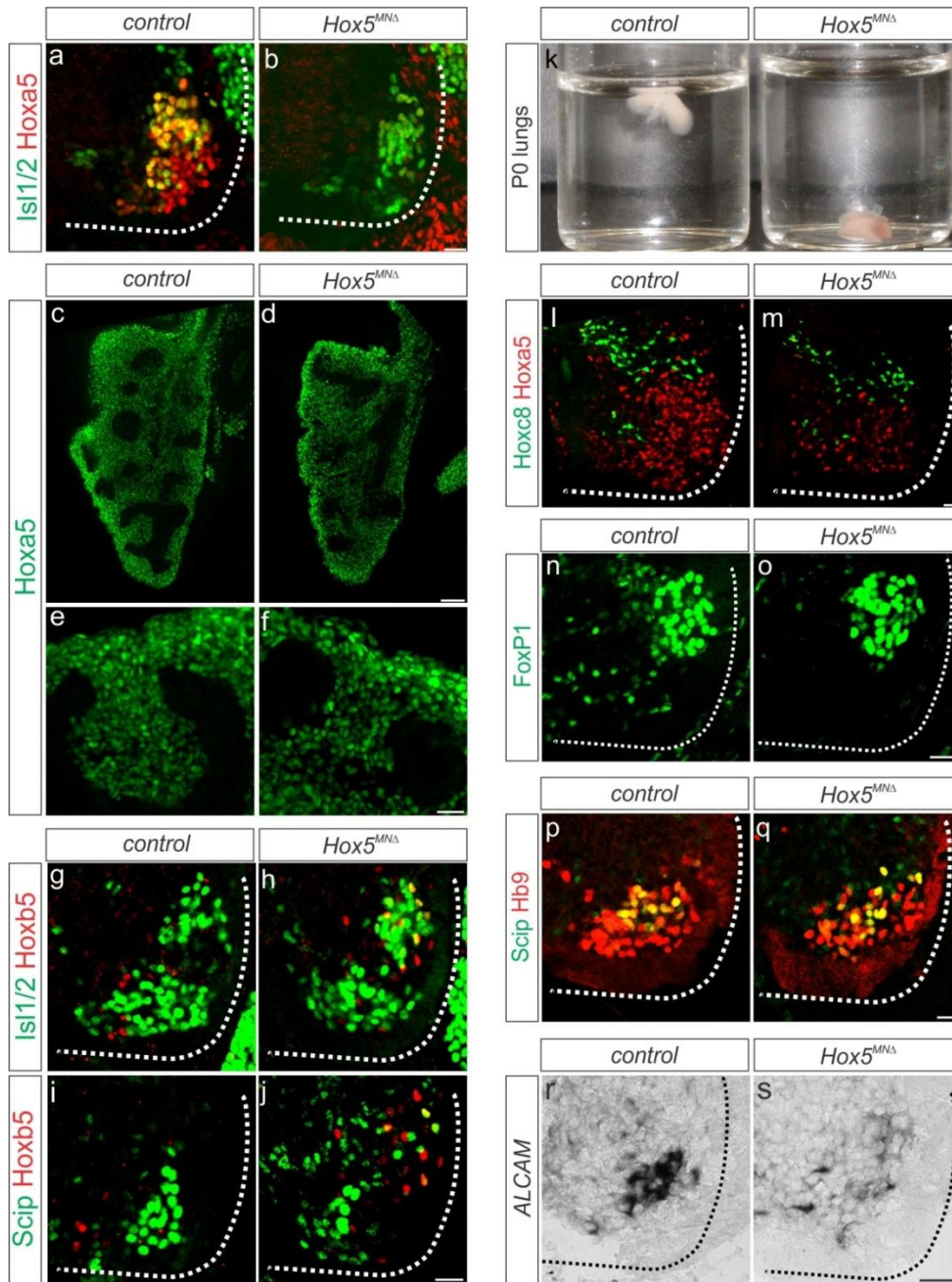
(a-b) Reduction of FoxP1 expression at cervical levels in *Hoxc6*^{-/-} mice.

(c-f) Ectopic Scip expression in the Hoxc8+ domain in *Foxp1*^{MNA} mice.

(g-h) The thickness of the phrenic nerve is increased in *Foxp1*^{MNA} mice. Phrenic nerves were visualized by wholemount GFP staining in an *Hb9::GFP* background.

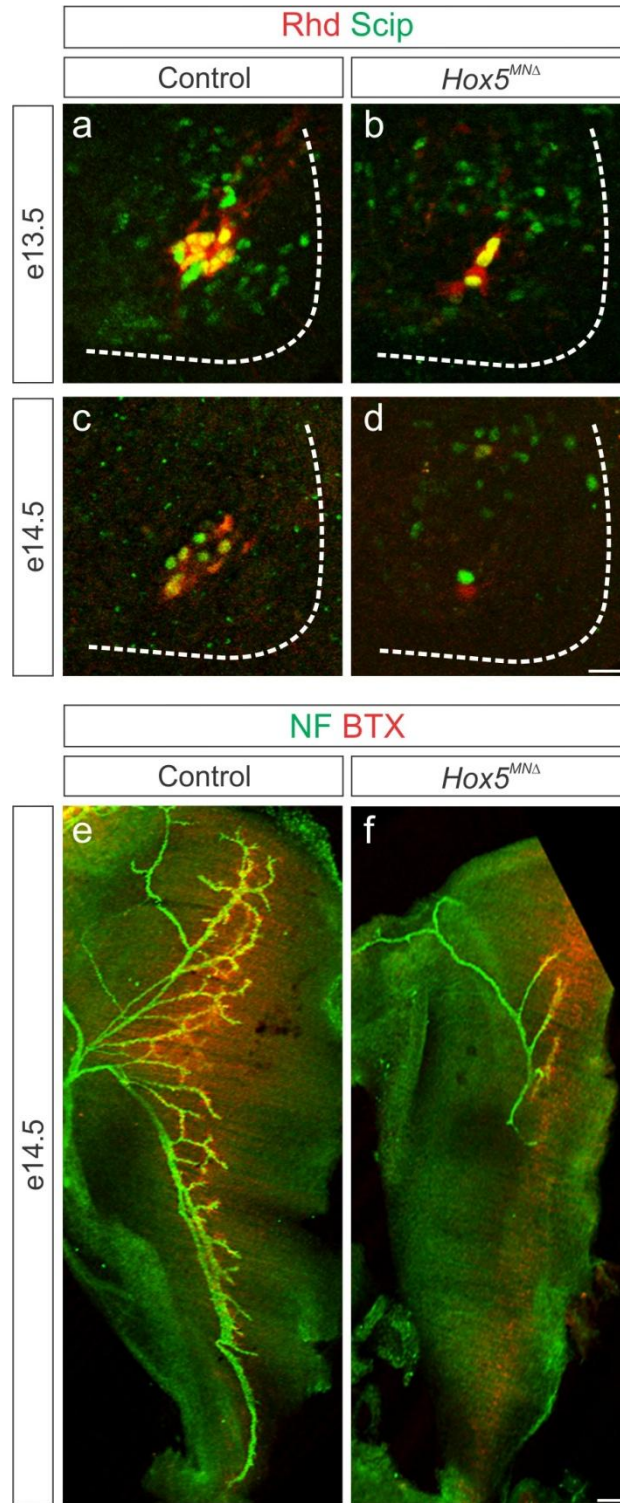
(i-j) Diaphragm innervation is grossly normal in *Foxp1*^{MNA} mice, with the exception of some overlapping branches (arrow in j).

Scale bars=25μm (a-f), 100μm (g-j).



Supplementary Figure 3. Analysis of *Hox5*^{MNA} mice.

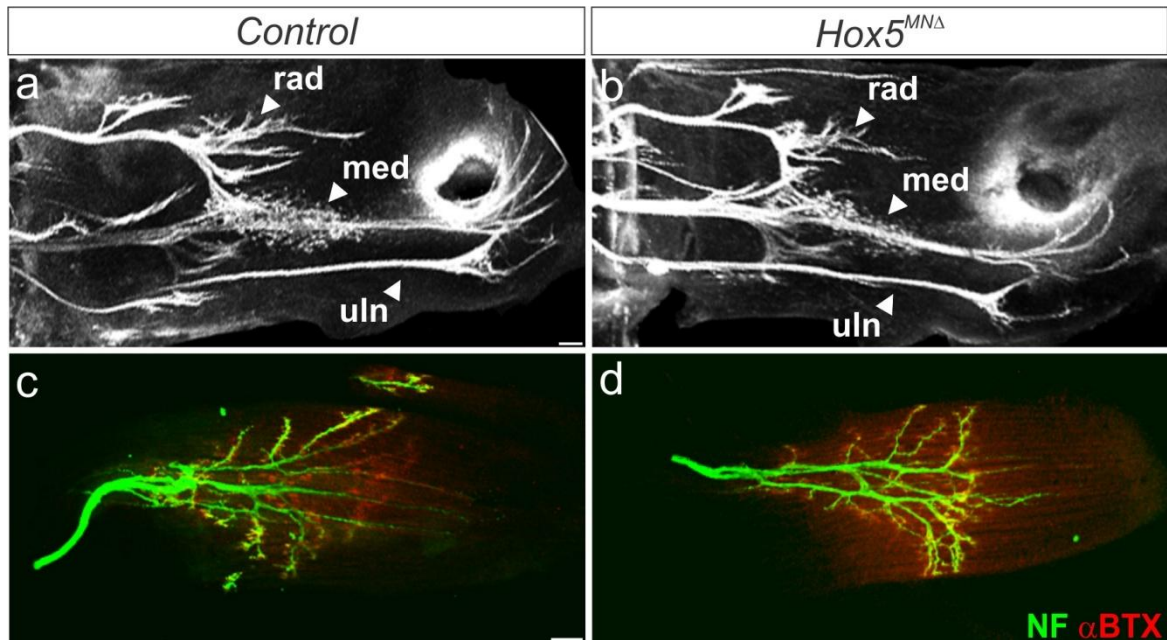
(a-b) *Hoxa5* expression is lost in motor neurons at e11.5 in *Hox5*^{MNA} mice. (c-f) *Hoxa5* expression is maintained in cells of the lung at e12.5 in *Hox5*^{MNA} mice. (g-j) *Hoxb5* is upregulated in a few *Isl1/2*+ cells in *Hox5*^{MNA} mice, however they are *Scip*-. (k) Lungs of *Hox5*^{MNA} newborn pups sink in water, indicating they are not inflated with air. (l-m) *Hoxc8* expression does not expand rostrally in *Hox5*^{MNA} mice at e12.5. (n-o) *FoxP1* expression is not affected in *Hox5*^{MNA} mice at e13.5. (p-q) *Hb9* expression is similar between control and *Hox5*^{MNA} mice at e12.5. (r-s) *ALCAM* expression is reduced in *Hox5*^{MNA} mice at e13.5. Scale bars=25µm, except in c,d=100µm and k=500µm.



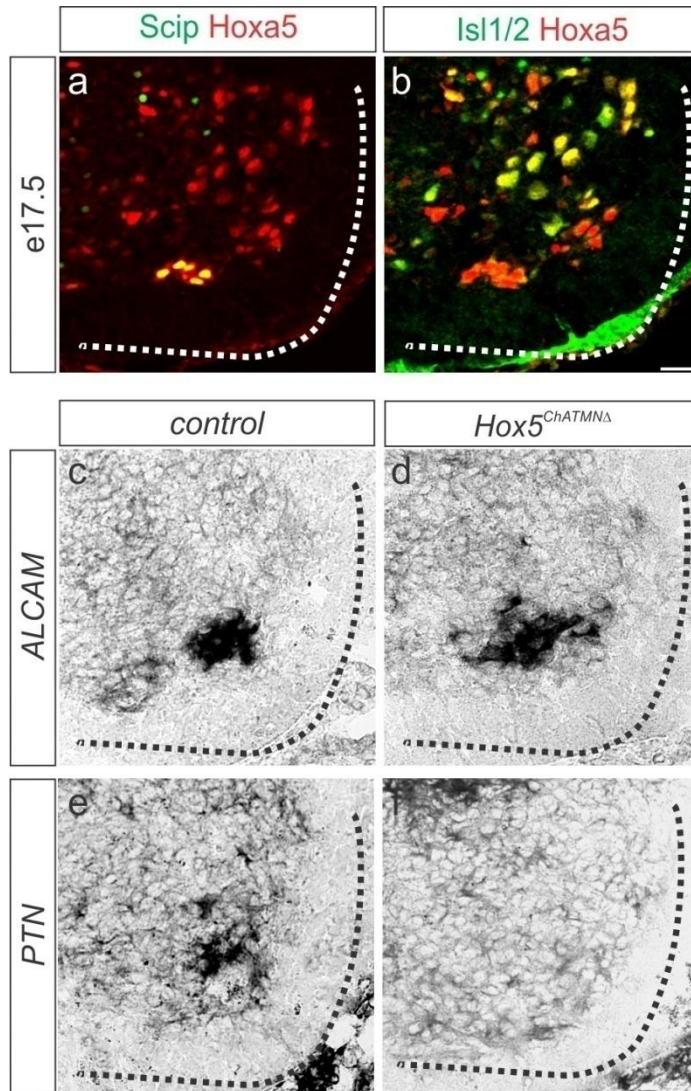
Supplementary Figure 4. Innervation defects in *Hox5^{MNΔ}* mice.

(a-d) Reduction in RhD⁺ cells after retrograde tracing of the phrenic nerve at e13.5 (a-b) and e14.5 (c-d) in *Hox5^{MNΔ}* mice. Scale bar=25μm.

(e-f) Diaphragm innervation is reduced at e14.5 in *Hox5^{MNΔ}* mice. Scale bar=100μm.



Supplementary Figure 5. Normal forelimb and biceps muscle innervation in *Hox5*^{MNΔ} mice.
 (a-b) Motor axons project to limb muscles normally in *Hox5*^{MNΔ} mice. Images show pattern of e13.5 forelimb innervation in an *Hb9::GFP* background. Scale bar=100μm.
 (c-d) The pattern of biceps muscle innervation is not altered in *Hox5*^{MNΔ} mice at e18.5. Scale bar=100μm.



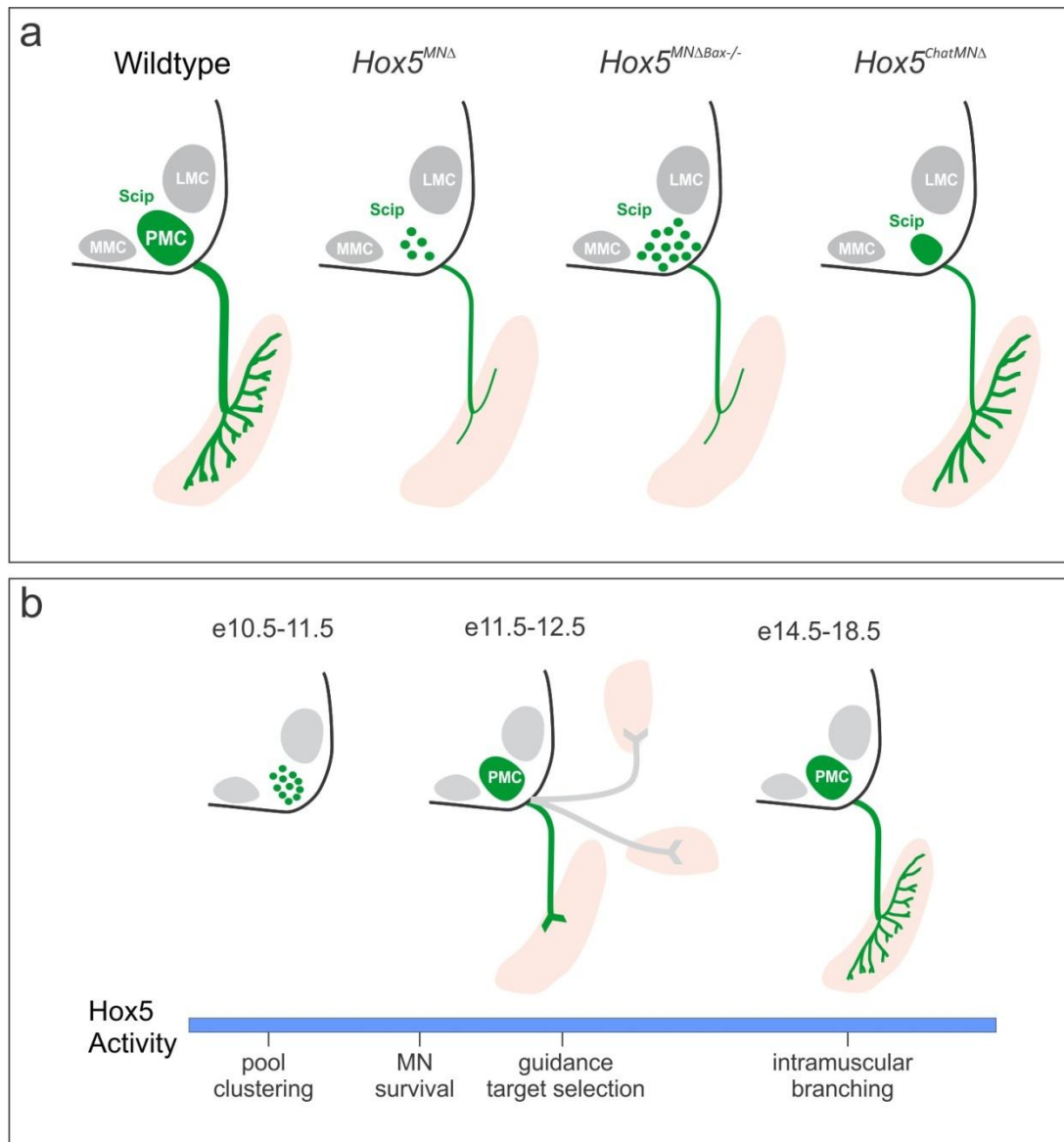
Supplementary Figure 6. Hoxa5 expression persists at late embryonic stages.

(a-b) PMC neurons continue to express Hoxa5 at least until e17.5, the latest embryonic stage examined.

(c-d) *ALCAM* expression is not affected in *Hox^{ChATMNA}* mice at e14.5.

(e-f) *PTN* expression is reduced in *Hox^{ChATMNA}* mice at e14.5.

Scale bar=25μm.



Supplementary Figure 7. *Hox5* genes are essential for multiple facets of PMC differentiation.

(a) Summary of the phenotypes observed with the different *Hox5* mutant strains. Removal of *Hox5* genes from motor neuron progenitors leads to cell death, PMC disorganization and severe diaphragm innervation defects. Rescue of cell death restores cell number but not cell body organization or innervation. Removal of *Hoxa5* from postmitotic motor neurons leads to PMC loss and branching defects, but preserves columnar organization. (b) *Hox5* genes define PMC identity by controlling facets of PMC development at specific temporal phases.

Gene	Ratio c/m	Accession number	Gene name
COX7A2L	3.384665	XM_123188.1	cytochrome c oxidase subunit VIIa polypeptide 2-like
LYPD1/Lynx2	1.527575	NM_145100.3	Ly6/Plaur domain containing 1
MAML3	1.498015	NM_001004176.1	mastermind like 3
MEGF9	1.48558	NM_172694.2	multiple EGF-like-domains 9
IMPACT	1.458044	NM_008378.2	imprinted and ancient
HOXC6	1.427784	NM_010465.2	homeobox C6
UNCX	1.416359	NM_013702.3	UNC homeobox
TAGAP1	1.413392	NM_147155.2	T cell activation GTPase activating protein 1
HMG2	1.402983	NM_016957.3	high mobility group nucleosomal binding domain 2
GADD45A	1.394091	NM_007836.1	growth arrest and DNA-damage-inducible 45 alpha
TBC1D20	1.370696	NM_024196.3	TBC1 domain family, member 20
MRPS35	1.369718	NM_145573.2	mitochondrial ribosomal protein S35
PAQR9	1.369276	NM_198414.2	progesterin and adipoQ receptor family member IX
RABL4	1.361176	NM_025931.2	intraflagellar transport 27
CXX1A	1.351944	NM_024170.1	CAAX box 1 homolog A
CITED2	1.335656	NM_010828.2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
HOXA5	1.335396	NM_010453.2	homeobox A5
EXOSC2	1.333737	NM_144886.1	exosome component 2
RCCD1	1.332452	NM_173445.1	RCC1 domain containing 1
TMEM90A	1.33012	NM_001033334.1	transmembrane protein 90a
LLPH	1.319654	NM_025431.2	long-term synaptic facilitation homolog
RTN4	1.312443	NM_194054.1	reticulon 4
NAP1L5	1.307726	NM_021432.2	nucleosome assembly protein 1-like 5
ZFP131	1.301916	NM_028245.1	zinc finger protein 131
TNFSF13B	1.293779	NM_033622.1	tumor necrosis factor (ligand) superfamily, member 13b
DDX20	1.292543	NM_017397.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20
MBTD1	1.285682	NM_134012.2	mbt domain containing 1
TLE3	1.284471	NM_001083928.1	transducin-like enhancer of split 3
TAF1A	1.27814	NM_021466.1	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, A
CDK5RAP1	1.275018	NM_025876.2	CDK5 regulatory subunit associated protein 1
KLC1	1.274117	NM_001025360.2	kinesin light chain 1
CEP78	1.262065	NM_198019.2	centrosomal protein 78

Supplementary Table 1. List of genes found to be downregulated in cervical motor neurons of *Hox5^{MNA}* embryos at e12.5. Genes were included on this list if the change in expression was >1.25 and showed a p-value of <0.1. The ratio of expression between control/mutant embryos (c/m) is indicated in Column 2. Expression of these genes was subsequently analyzed by quantitative PCR and in situ hybridization.