Foxp1 and Lhx1 Coordinate Motor Neuron Migration with Axon Trajectory Choice by Gating Reelin Signalling

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Abstract

Topographic neuronal maps arise as a consequence of axon trajectory choice correlated with the localisation of neuronal soma, but the identity of the pathways coordinating these processes is unknown. We addressed this question in the context of the myotopic map formed by limb muscles innervated by spinal lateral motor column (LMC) motor axons where the Eph receptor signals specifying growth cone trajectory are restricted by Foxp1 and Lhx1 transcription factors. We show that the localisation of LMC neuron cell bodies can be dissociated from axon trajectory choice by either the loss or gain of function of the Reelin signalling pathway. The response of LMC motor neurons to Reelin is gated by Foxp1- and Lhx1-mediated regulation of expression of the critical Reelin signalling intermediate Dab1. Together, these observations point to identical transcription factors that control motor axon guidance and soma migration and reveal the molecular hierarchy of myotopic organisation.

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Abbreviations: arb, arbitrary; HH, Hamburger and Hamilton; LMC, lateral motor column; LMCI, lateral LMC; LMCm, medial LMC; LS, lumbo-sacral; PG, preganglionic; PLAP, placental alkaline phosphatase; St, stage; wt, wild type

Introduction

Neural circuits are frequently organised in a topographic manner such that the position of a neuronal cell body is correlated with the location of the post-synaptic target and therefore its axon trajectory. Since the inference of such organisational principles [1], the molecular identity of many neuronal migration and axon guidance cues has been uncovered [2,3]. Recent studies have also begun to identify the transcription factors that control neuronal identity and deploy the repertoire of neuronal migration and axon guidance receptors and signals employed in neural circuit assembly [4,5,6]. These observations raise the possibility that correlated neuronal soma localisation and axon trajectory of topographically ordered neural circuits arise as a consequence of specific transcription factors directing both axon guidance and cell body migration effector expression.

Vertebrate spinal motor neurons are organised myotopically in longitudinal columns such that the location of their soma in the ventral spinal cord corresponds to the position of their muscle targets in the periphery [7]. In mouse and chick, motor neurons innervating axial and body wall muscles are located in medially positioned columns, whereas motor neurons innervating limb muscles are located in the lateral motor column (LMC) present only at spinal cord levels in register with limbs. LMC neurons are further subdivided according to their axon trajectory within the limb: lateral LMC (LMCl) neurons innervate dorsal limb muscles, whereas medial LMC (LMCm) neurons innervate ventral limb muscles [8,9,10]. Motor pools are also organised myotopically such that, in general, the antero-posterior location of a pool within the LMC correlates with the proximo-distal location of its limb muscle target [7,9,11,12]. A motor axon guidance decision point is at the base of the limb where LMC axons interact with mesenchymal cells resulting in the selection of a dorsal or a ventral limb nerve trajectory [10,13]. Concomitant with this process, LMC somata migrate from the progenitor-rich ventricular zone to the ventral horn of the spinal cord [14,15], with the later-born LMCi neurons migrating past the earlier-born LMCm neurons in a manner reminiscent of the inside-out lamination of the developing cerebral cortex [16,17,18]. Recent studies also describe a topographic relationship between motor neuron soma and...
Author Summary

Many areas of our nervous system are organized in a topographic manner, such that the location of a neuron relative to its neighbors is often spatially correlated with its axonal trajectory and therefore target identity. In this study, we focus on the spinal myotopic map, which is characterized by the stereotyped organization of motor neuron cell bodies that is correlated with the trajectory of their axons to limb muscles. An open question for how this map forms is the identity of the molecules that coordinate the expression of effectors of neuronal migration and axonal guidance. Here, we first show that Dab1, a key protein that relays signals directing neuronal migration, is expressed at different concentrations in specific populations of limb-innervating motor neurons and determines the position of their cell bodies in the spinal cord. We then demonstrate that Foxp1 and Lhx1, the same transcription factors that regulate the expression of receptors for motor axon guidance signals, also modulate Dab1 expression. The significance of our findings is that we identify a molecular hierarchy linking effectors of both neuronal migration and axonal projections, and therefore coordinating neuronal soma position with choice of axon trajectory. In general, our findings provide a framework in which to address the general question of how the nervous system is organized.

dendrite localisation in Drosophila and the patterns of motor neuron recruitment during swimming in fish [19,20].

The molecular signals controlling the trajectory of LMC axons are characterised, but those controlling LMC soma position in the spinal cord are poorly understood. The LIM homeodomain proteins Isl1 and Lhx1, expressed by LMCm and LMCl neurons respectively, act in conjunction with the pan-LMC forkhead domain transcription factor Foxp1 to specify the dorsoventral axon trajectory in the limb by regulating the expression of axonal Eph tyrosine kinase receptors that enable LMC growth cones to respond to ephrin ligands in the limb mesenchyme. Genetic evidence argues that ephrin-A ligands in the ventral limb repulse EphA-expressing LMCm axons into the dorsal limb nerve, while ephrin-B ligands in the dorsal limb repulse EphB-expressing LMCm axons into the ventral limb nerve [21,22,23,24,25,26]. The clustering of some motor pools relies on EphA5, type II cadherins, and the ETS transcription factor Pec3 [27,28,29], while migration of LMCl and LMCm neurons into their appropriate columnar location can be biased by Lhx1 and Isl1 and requires Foxp1 [21,22,23]. These observations raise the possibility that Foxp1, Lhx1, and Isl1 control the migration of LMC cell bodies within the ventral horn by restricting the expression of specific effectors of neuronal migration.

The extracellular matrix protein Reelin is a crucial neuronal migration signal that acts through the lipoprotein receptors VLDLR or ApoER2 to induce the phosphorylation of the intracellular adaptor protein Dab1 leading to remodelling of the actin cytoskeleton [30]. Loss of Reelin or its signalling effectors disrupts the layering of the neuronal somata within the cerebral cortex [31,32,33] but the role of Reelin in neuronal migration remains controversial. Reelin has been proposed to act as a neuronal migration stop signal [34]; however, since Reelin expression in the ventricular zone can partially rescue the pre-neuronal migration stop signal [34]; however, since Reelin remains controversial. Reelin has been proposed to act as a neuronal migration stop signal [34]; however, since Reelin remains controversial. Reelin has been proposed to act as a permissive signal enabling neurons to interpret distinct migration cues [35]. Similar to cortical neurons, spinal neuron progenitor clones migrate away from the ventricular zone in radial spoke-like trajectories [14] and the migration of preganglionic (PG) motor neurons and the layering of the dorsal horn laminae is controlled by Reelin [36,37]. These studies raise the possibility that Reelin may also regulate the localisation of LMC neurons and is thus a general migration cue specifying the position of many different classes of spinal neurons including LMC motor neurons.

Using gain and loss of function experiments in chick and mouse, we provide evidence that Reelin directs LMC neuron migration but not the selection of limb axon trajectory. We also show that Foxp1 and Lhx1, the transcription factors specifying LMC axon trajectory choice, gate Reelin signalling through the restriction of Dab1, a key signalling intermediate. Thus, the same transcription factors are directing neuronal soma migration and axon trajectory selection revealing the molecular hierarchy controlling the establishment of a somatotopic map.

Results

Expression of Reelin, VLDLR, ApoER2, and Dab1 in the Ventral Spinal Cord

To explore the possibility that Reelin signalling might control LMC soma migration, we monitored the expression of Reelin, its receptors, and their adaptor protein Dab1 in mouse embryos between embryonic day of development (e) 11.5 and e12.5 and in chick embryos between Hamburger and Hamilton (HH) stages (St) 23 and 30 [38] in limb-level spinal cord. These stages correspond to the times at which LMCl neurons are migrating out of the ventricular zone and reach their final position lateral to LMCm neurons [17,22]. We used the transcription factor Foxp1 as a pan-LMC marker and subdivided the LMC based on the presence of Isl1 and Lhx1 transcription factors [21,23,25].

Reelin has previously been detected in the thoracic spinal cord adjacent to PG neurons [36]. At limb levels Reelin is expressed from e10.5 (Figure S1) and in e11.5 mouse embryos we observed Reelin expression in cells medio-dorsal to LMC neurons, and by e12.5 this domain expanded ventrally, resulting in a Reelin-rich band intercalated between the ventricular zone and the LMC (Figure 1A–H). We also observed a similar Reelin mRNA and protein distribution in chick embryos (Figure S1).

We next monitored the expression of Reelin receptors VLDLR and ApoER2 and their intracellular adaptor protein Dab1 in mouse and chick spinal cords. In e11.5 mouse embryos at both limb levels, VLDLR protein and mRNA were apparently expressed in all LMC neurons (Figure 1I–L; unpublished data). However, VLDLR protein levels appeared higher in LMCl neurons relative to LMCm neurons (Figure 1K), whereas VLDLR mRNA and protein levels appeared uniform throughout the LMC (Figure 1M–P; unpublished data). In chick embryos, VLDLR mRNA was present in apparently all lumbar LMC neurons at both HH St 24 and HH St 30 (Figure S1). At the stages examined, ApoER2 mRNA was expressed in the ventricular zone adjacent to the floor plate of both mouse and chick embryos; however, its expression in LMC neurons was only apparent in mouse embryos (Figure 1Q–T; Figure S1; unpublished data).

In mouse, Dab1 mRNA and protein were present throughout the LMC from e10.5, at both limb levels; however, at later ages examined, an LMC subpopulation expressed Dab1 mRNA and protein at noticeably higher levels (Figure 1U–AF; Figure S1, Figure S4; unpublished data). At e11.5, this expression domain (Dab1high) was confined to the medio-ventral aspect of the LMC corresponding to Foxp1+Isl1– LMCm neurons while the low-level Dab1 expression domain (Dab1low) was confined to the dorsally positioned Isl1+Foxp1+ LMCm neurons (Figure 1U–X). By e12.5,
Dab1\textsuperscript{high} and Dab1\textsuperscript{low} LMC neurons were found in, respectively, lateral and medial aspect of the LMC, and corresponded to LMCl and LMCm neurons (Figure 1Y–AB). Similar Dab1\textsuperscript{mRNA} distribution was observed in chick embryos (Figure S1). Together, our expression data raise the possibility that Reelin signalling directs LMC soma migration and the disparate Dab1 expression levels in LMCl and LMCm neurons suggest that these neuronal populations may differ in their responsiveness to Reelin.

**LMC Migration Defects in Dab1 and Reln Mutant Mice**

To determine whether Reelin signalling influences LMC neuron migration, we examined the spinal cord of Dab1 and Reln (Reln) mutant mice (Figure 2) [31,32]. Since Reelin signalling is required for the appropriate positioning of PG neurons which share a part of their migration trajectory with LMC neurons [36,39], we focused on the analysis of caudal lumbar-sacral (LS) levels, which contain no PG neurons, as assessed by phospho-Smad1 expression [23]. During LMC migration, the total number of LMCm neurons, LMCl and LMCm subtype specification, and radial glia development was unaffected by Dab1 and Reln loss of function (Figure S2, Figure S3; unpublished data). Additionally, most likely because of its impaired degradation [40], Dab1 protein levels in LMC neurons were increased in Reln mutants, suggesting that all LMC neurons are responsive to Reelin (Figure S4).

We next analysed the localisation of lumbar LMC neurons in Dab1 and Reln mutants at e12.5, the time at which, in control embryos, the majority of wild type LMC neurons have terminated their migration and are positioned lateral to LCMc neurons (Figure 2A–D). In Dab1 mutants, LMCm neurons settled ventral to LMCl neurons, which were abnormally shifted to a lateral position in the ventral horn, and many LMCl and LMCm neurons

**Figure 1. Ventral spinal cord expression of Reelin, VLDLR, ApoER2 and Dab1.** (A–P, U–AB) Protein detection in e11.5 and e12.5 mouse lumbar spinal cord. LMCl (M) and LMCm (L) neurons are identified as Foxp1\textsuperscript{+} Isl1\textsuperscript{+} and Foxp1\textsuperscript{+} Isl1\textsuperscript{2}, respectively. Reelin is expressed in a domain medio-dorsal to the LMC at e11.5 (A–C) and expands more ventrally at e12.5 (G; arrowheads). VLDLR expression is stronger in LMCl neurons (arrowheads) at e11.5 (I–K). Arrow in (O) indicates VLDLR protein in neuronal processes extending towards the ventricular zone. Dab1 expression is stronger in LMCl neurons (arrowheads in W and AA) than in LMCm neurons at both e11.5 (U–W) and e12.5 (Y–AA). (Q–S, AC–AE) Detection of mRNA in consecutive lumbar spinal cord sections of an e11.5 (Q–S) and e12.5 (AC–AE) mouse embryo. Isl1 (Q, AC) and Lhx1 (R, AD) expression highlights LMCm and LMCl motor neurons, respectively. ApoER2 mRNA is detected throughout the ventral spinal cord (S), with higher levels of expression in the ventricular zone (VZ). Inset in (S) shows a lower magnification image of the same section, highlighting the specificity of the probe. Dab1 mRNA expression levels are higher in LMCl neurons than in LMCm neurons (AE). (AF) Quantifications of Dab1 mRNA and protein levels in LMCl (Isl1\textsuperscript{+} Foxp1\textsuperscript{+}) and LMCm (Isl1\textsuperscript{2} Foxp1\textsuperscript{+}) in lumbar spinal cord of e12.5 mouse embryos. Quantifications were gated on Lhx1\textsuperscript{+} LMCl and Isl1\textsuperscript{2} LMCm regions in neighbouring sections and represent a mean intensity value within the gated region with the background (gating on the ventricular zone) subtracted. arb: arbitrary units on a 0 (no signal) to 255 (maximum intensity) scale. Measurements are based on three embryos and six sections. (D, H, L, P, T, X, AB) Schematic representation of LMCm, LMCl, and Reelin signalling components. Blue intensity varies with expression levels. Stippled lines outline the spinal gray matter and LMC neurones. Scale bar: 65 \textmu m (A–AB), 50 \textmu m (AC–AE).

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Figure 2. Impaired LMC position in Dab1 and Reln mutants. (A–H, J–Q) LMCm (Foxp1<sup>+</sup> Isl1<sup>+</sup>) and LMCl (Foxp1<sup>+</sup> Isl1<sup>2</sup>) neurons in the lumbar spinal cord of e12.5 Dab1 mutants (E–H), Reln mutants (N–Q), and wild type littermates (A–D, J–M). In both mutants, LMCl neurons are positioned more ventrally, while LMCm neurons are shifted laterally, relative to control embryos. Some overlap between LMCl and LMCm domain is also evident (M/L in panels E and N). Superimposed ventral spinal cord position (D, H, M, Q) of LMCm (red) and LMCl (green) neurons in several consecutive sections of representative embryos highlights the altered position of LMC neurons. (I, R) Density plots of mediolateral (ML) and dorsoventral (DV) position of LMCm and LMCl neurons as percentage of LMC width and height. See Materials and Methods for details. Mean position (ML, DV) is in
were intermingled (Figure 2E–H). This neuronal displacement was more evident when we superimposed the position of LMC and LMCM neurons in images of adjacent wild type (wt) and Dab1 mutant spinal cord sections (Figure 2D, H). To assess the expressivity of this phenotype and to account for LMC neuron displacement along mediolateral (ML) and dorsoventral (DV) axes simultaneously, we performed a two-dimensional position analysis of LMC neuron position using the bivariate statistical Hotelling’s $T^2$ test. We measured the mean ML and DV coordinates of wild type and Dab1 mutant LMC neurons within the ventral spinal cord. To compensate for sectioning artefacts, we normalised the ML coordinates to the distance from the ventricular zone to the lateral edge of the Foxp1 expression domain and the DV coordinates to the dorsoventral extent of the Foxp1 expression domain, two standard measurements that are not different between Dab1 mutants and wild type littermates (see Experimental procedures for details; unpublished data). Thus, with the lateral-most edge of the LMC defined as ML: 100%, and with the dorsal-most domain of the LMC defined as DV: 100%, in wild type embryos, these neurons were spread over a larger mediolateral zone compared to wild type littermates (Figure 2I; Table S2). In contrast, by visual inspection of at least six spinal cord sections per embryo, we noted that in six out of six embryos analysed, LMCM neurons were positioned aberrantly. Quantification revealed that LMC neuron position was significantly shifted in a medio-ventral direction in Dab1 mutants relative to wild type littermates (ML: 73%; DV: 33%) versus (ML: 79%; DV: 39%); $p<0.0035$, Hotelling’s $T^2$ test; Table S2), which could be observed at least until e15.5 (Figure 2S–U, W–Y; unpublished data). A similar LMC migration phenotype was also observed in the cervical spinal cord as well (unpublished data). We also noted that in four out of four embryos, the position within the ventral spinal cord of a Pea3-expressing motor neuron pool was shifted medio-ventrally at e15.5 (Figure 2V, Z). Together, these results demonstrate that in the limb-level spinal cord, Dab1 is essential for the normal migration of LMC neurons and motor pool position.

We next examined the position of lumbar LMC neurons in Rehn mutant embryos at e12.5. Rehn mutation did not alter the mean position of LMCM neurons (Figure 2J–Q; Table S2), although as in Dab1–/– embryos, these neurons were spread over a larger area of the LMC when compared to controls (Figure 2R). In contrast, in three out of four embryos, we observed that LMCM neurons were positioned abnormally, with quantification revealing that the mean LMC neuron position in Rehn mutants was significantly shifted in the medio-ventral direction relative to wild type, with many LMCM neurons found intermingled with LMCM neurons (ML: 75%; DV: 33%) versus (ML: 80%; DV: 41%); $p<0.0473$, Hotelling’s $T^2$ test; Figure 2J–R; Table S2). Migration defects observed in Rehn mutants mirrored those observed in Dab1 mutants, thus implicating Reelin signalling in the specification of LMC soma position in the ventral spinal cord.

**Dab1 Expression Determines LMC Soma Position**

Based on the differential expression and the requirement for its function in LMCM and LMCM neurons, we reasoned that the levels of Dab1 expression, rather than simply its presence or absence, might influence the migration of LMC neurons. We therefore asked whether increasing Dab1 expression would shift the position of LMC soma laterally. To do this, we used in ovo electroporation to introduce a Dab1::GFP fusion protein or GFP expression plasmids into the lumbar spinal cord of HH St 17/19 embryos and monitored the position of GFP+ LMC neurons at HH St 29 [22]. Dab1::GFP was expressed with equal efficiency in LMCM and LMCM neurons and did not change their identity nor affect their axon trajectory in the limb (Figure S6; unpublished data). The mean position of LMCM neurons with elevated Dab1 levels was the same as that of LMCM neurons expressing GFP (Figure 3A–G, I; Table S3). However, in four out of five embryos, we observed that LMCM neurons with elevated Dab1 expression were observed in a more ventro-lateral position (Figure 3E–I; (ML: 70%; DV: 49%)) compared to LMCM neurons expressing GFP (Figure 3A–D, I; (ML: 67%; DV: 59%); $p=0.0165$, Hotelling’s $T^2$ test; Table S3), demonstrating that increasing Dab1 expression levels in LMC neurons is sufficient to shift their position laterally.

**Dissociation of Axon Trajectory from Soma Position in Rehn and Dab1 Mutants**

The myotopic relationship between LMC soma position and axon trajectory within the limb raises the possibility that changes in LMC soma position in Dab1 or Rehn mutants could result in the selection of inappropriate limb trajectory by LMC axons. To examine the LMC axon limb trajectory in Dab1 mutants, we used the Isl1-LacZ reporter marker [42] and quantified the proportion of LacZ+ LMC axons projecting into e11.5 forelimb dorsal and ventral limb nerves in Dab1–/–; Isl1+/- Lhx1LacZ, and Lhx1LacZ littermate embryos [24]. In Lhx1LacZ embryos we observed ~99% of LacZ+ axons within the dorsal limb nerves and ~1% of LacZ+ axons within the ventral limb nerves (Figure 4A, B, E). The proportions of LacZ+ in dorsal and ventral limb nerves of littermate Dab1–/–; Lhx1LacZ embryos were not significantly different (Figure 4C–E; 98% and 2%, respectively, $p>0.5$, Student’s t test). Additionally, in whole mount e12.5 Dab1–/–; Lhx1LacZ embryos, we did not detect any aberrantly projecting LMC axons at either limb level (unpublished data).

To trace LMC axons we used the herest/Isl1-PLAP reporter line in which the Isl1 enhancer-promoter drives the expression of placental alkaline phosphatase (PLAP) in LMC neurons at forelimb levels [43]. PLAP enzymatic reaction was used to detect LMCM axons in Dab1–/–; herest/Isl1-PLAP and control herest/Isl1-PLAP e11.5 forelimbs, followed by axonal signal quantification. In herest/Isl1-PLAP embryos, ~99% of PLAP+ axons were found in the ventral limb nerve, while ~1% of PLAP+ axons were found in the dorsal limb nerve (Figure 4F, G, J), proportions not significantly different from Dab1–/–; herest/Isl1-PLAP embryos (Figure 4H–J; 99% and 1%, respectively; $p=0.335$, Student’s t-test).
test). LMCm limb trajectory in Reelin mutants was also apparently normal (unpublished data), indicating that neither Dab1 nor Reelin are required for the selection of limb trajectory by LMC axons and demonstrating that the LMC soma position can be dissociated from axon trajectory selection.

**Foxp1 Controls Dab1 Expression in LMC Neurons**

Since our results indicated that the Dab1 protein level determines the position of LMC neuron somata but not their axon trajectory, we next evaluated whether the deployment of effector pathways governing these processes might be coordinated by a common set of transcriptional inputs. To determine whether Foxp1, a transcription factor specifying LMC cell fate, participates in the control of Dab1 expression in LMC neurons, we analyzed the embryonic spinal cords in which Foxp1 is expressed in all motor neurons (Hb9::Foxp1 transgenic) as well as in those lacking Foxp1 function [21,23]. We first focused our analysis on upper cervical levels, where Foxp1 and Dab1 expression levels are normally low or undetectable (Figure 5A–C; Figure S7; unpublished data). In e12.5 Hb9::Foxp1 transgenic spinal cords, compared to control embryos, we observed a significant increase in Dab1 mRNA levels (30 arbitrary units) versus 16 in controls; t-test; 0.0002, Student’s t test; Note that Foxp1 and Dab1 expression levels associated with ectopic Foxp1 transgenic embryos, without any obvious changes in Reelin expression (Figure 5A, B, D, E, F, M) as well as protein expression levels associated with ectopic Foxp1 transgenic embryos, without any obvious changes in Reelin expression (Figure 5A, B, D, E, F, M). We then examined the expression of Dab1 in the lower cervical spinal cord of Foxp1 mutant mice at e12.5. When compared to controls, Foxp1 mutant spinal cords exhibited a significant decrease in Dab1 mRNA levels (15 arbitrary units versus 33 in control littermates; p<0.001, Student’s t test; Figure 5G, I, J, L, M) as well as Dab1 protein levels (Figure 5G, H, J, K, M; Figure S7; 12 arbitrary units versus 37 in control littermates; p<0.001, Student’s t test). These results suggest that Foxp1 is both sufficient and required for Dab1 expression in migrating LMC neurons.

**Control of Differential Expression of Dab1 in LMC Neurons by Is1 and Lhx1**

Although Foxp1 controls Dab1 expression, because of its uniform expression throughout the LMC, it appears to us an unlikely determinant of the differential level of Dab1 expression in LMCm and LMCl neurons. LIM homeodomain proteins Is1 and Lhx1 are determinants of, respectively, LMCm and LMCl neuronal fate, can influence their migration, and can control their axon trajectory by modulating Eph receptor expression (Figure S8 and Text S1; [22,24,25,42]). We thus hypothesized that while Foxp1 activates Dab1 expression in all LMC neurons, Is1 and Lhx1 have opposing effects on Dab1: (1) Is1 lowers Dab1 expression in LMCl neurons while (2) Lhx1 elevates Dab1 expression in LMCl neurons. We tested the first of these hypotheses by electroporating Is1 and LacZ expression plasmids, or a control LacZ expression plasmid alone into HH St 17/19 chick lumbar spinal cords and measuring changes in Dab1 mRNA levels relative to the unelectroporated control side at HH St 29 [22]. Expression of LacZ did not affect Is1 or Dab1 mRNA expression while overexpression of Is1 significantly reduced Dab1 mRNA expression levels in LMC neurons (Figure S9; e/a values: 1.4 for LacZ versus 0.7 for Is1, p<0.001, Student’s t test) indicating that Is1 can suppress Dab1 mRNA expression. To test whether Is1 is required to control Dab1 expression, we examined the effects of siRNAs directed against Is1 in LMC neurons but observed no significant difference in Dab1 expression when compared to controls (Figure S9 and Text S1). Together, these data suggest that Is1 is sufficient but might be dispensable for the modulation of Dab1 expression in LMC neurons.

We next tested whether Lhx1 is required to specify the position of LMCi neurons by examining embryos with a conditional loss of Lhx1 function in LMC neurons, obtained by crossing Lhx1<sup>floxed</sup> homozygotes with Isl1<sup>IRES-LacZ</sup> or Isl1<sup>IRES-LacZ</sup> mice, in which Isl1<sup>IRES-LacZ</sup> drives Cre recombinase expression in all LMC neurons. We focused our analysis on e12.5 lumbarosacral levels in two groups of embryos obtained from these crosses: Lhx<sup>1/2</sup> and Isl1<sup>IRES-LacZ</sup> mice, designated as...
Figure 4. Loss of Reelin signalling disrupts LMC myotopy. (A–D) Axonal neurofilament (NF) and LacZ protein in a representative forelimb section of e11.5 control Lhx1flo/flo litterate (A–B) and Dab1−/−; Lhx1flo/flo embryos (C–D). (E) Quantification of LacZ+ axons within dorsal (d) and ventral (v) limb nerves expressed as a percentage of total LacZ+ signal within both limb nerves. Student’s t test p value: Dab1+/+; Lhx1×/× versus Dab1−/−; Lhx1×/×, p≥0.5. n = 3 embryos per genotype; N = 8 sections quantified per embryo. (F–I) Neurofilament (NF) immunodetection and alkaline phosphatase (PLAP) enzymatic detection in axons of a representative forelimb section of e11.5 control hCrest/Isl1-PLAP litterate (F–G) and Dab1−/−; hCrest/Isl1-PLAP embryos (H–I). PLAP enzymatic reaction signal images (G, I) were colour-inverted and overlaid with same section NF signal images in (F, H). (J) Quantification of PLAP+ axons within dorsal (d) and ventral (v) limb nerves expressed as percentage of total PLAP+ signal within both limb nerves. Student’s t test p value: Dab1+/+; hCrest/Isl1-PLAP versus Dab1−/−; hCrest/Isl1-PLAP, p = 0.335. n = 3 embryos per genotype; N = 6 sections quantified per embryo. Scale bar: 90 μm.

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Lhx1COND, and control Lhx1×/×, designated as Lhx1+/−. Lhx1 loss of function did not affect the total number of LMC or LMCm neurons but resulted in ∼60% of LMCm neurons (Foxp1+Isl1−) losing their Lhx1 expression (Isl1−Lhx1+/−Foxp1+: 37.3% versus 95.2% in controls; p<0.001, Student’s t test, Figure 6I, unpublished data). We determined the soma position of three LMC neuronal populations: LMCm, LMCi, and LMCi neurons lacking Lhx1 expression, which were defined as Isl1−Foxp1+Lhx1+/− (LMCi). As in control embryos, in which the majority of LMCi neurons resided in the most lateral part of the LMC, in Lhx1COND embryos, a significant proportion of LMCi* neurons settled laterally and the mean position of LMCi, LMCi, or LMCi* neurons was not changed when compared to controls (Figure 6A–J; Table S4). However, in Lhx1COND embryos, many LMCl* neurons were found in medial locations, intermingled with LMCm neurons (Figure 6A–H), and these neuronal displacements were more evident when we superimposed the positions of LMCi*, LMCi, and LMCm neurons in images of adjacent control and Lhx1COND spinal cords sections (Figure S10). To further characterise the medially displaced population of LMCi* neurons, we counted the number of LMC neurons in four equal quadrants of the LMC (Figure 6J, K, unpublished data). In both Lhx1 mutant and control embryos the majority of LMCi neurons were in the medial half of the LMC (unpublished data). In control embryos, 60% of LMCl neurons were in the lateral half of the LMC, compared to 42% of LMCi* neurons in Lhx1 mutants, representing a significant change (p = 0.003, Student’s t test, Figure 6K), indicating that Lhx1 is required for LMCl position specification.

To determine whether Lhx1 directs LMCl migration by controlling Dab1 expression, we compared Dab1 protein levels in the lumbar spinal cord of e12.5 Lhx1 mutants in which at least 50% of LMCi neurons lost their Lhx1 expression and littermate controls [22]. Our analysis revealed that in Lhx1 mutants, Dab1 protein expression in LMCl neurons was decreased by ∼20% when compared to control embryos (Figure 7A–H, O; p = 0.038, Student’s t test). We also quantified Dab1 mRNA and protein levels in the LMCm, defined as containing >90% of Isl1/Foxp1* neurons and LMCl defined as Isl1−Foxp1+. Within the LMCm, Dab1 mRNA and protein levels were not significantly different from controls, while in LMCl of Lhx1 mutants, relative to controls, Dab1 mRNA was decreased significantly by approximately 40% (p = 0.01, Student’s t test) and Dab1 protein was decreased significantly by ∼14% (p = 0.017, Student’s t test, Figure 7O), indicating that Lhx1 is required for the differential expression of Dab1 in LMCl neurons. Together, our results reveal that Foxp1 and Lhx1 coordinate LMC myotopy through their modulation of expression of neuronal migration and axon guidance effectors.

Discussion

Our observation that Reelin is an essential signal specifying the location of LMC neurons in the ventral spinal cord allowed us to address how neuronal migration and axon guidance are coordinated to achieve topographical organisation. Our experiments demonstrate that the transcription factors specifying the axon trajectory of LMC neurons occupy a privileged position in the molecular hierarchy controlling myotopy as they also control LMC soma migration by gating Reelin signalling. Here we discuss Reelin as a motor neuron migration signal, coordination of axon trajectory selection and soma placement, and the possible functional consequences of myotopic organisation of motor neurons.

Reelin as a Migration Signal for Motor Neurons

Following their birth near the ventricular zone, spinal neurons first migrate radially by perikaryal translocation, then tangentially, either in dorsal or ventral direction [14]. Reelin has been proposed as a radial migration signal; however, our observations argue that the initial, apparently radial trajectory of LMC motor neurons is Reelin signalling independent as is the case of PG and hindbrain motor neurons [36,39]. Thus, in general, the radial migration trajectory of motor neurons might not require Reelin signalling, but once it is terminated, Reelin becomes an important guidance signal, suggesting that unlike cortical neurons that rely on Reelin for their localisation in the radial plane, motor neurons at different rostrocaudal levels of the spinal cord depend on Reelin for the tangential aspect of their migration.

How does Reelin act in motor neuron migration? The initial model where Reelin is a migration stop signal has been challenged by observations that Reelin overexpression in the cortical ventricular zone can rescue, at least in part, pre-plate splitting defects associated with Reelin loss of function [34,35]. Likewise, overexpression of Reelin in the ventricular zone of the spinal cord rescues Reln mutant PG neuron migration defects but does not
cause an overt phenotype in a wild type background [44]. In the context of LMC neurons, the Reelin expression domain is intercalated between the emerging postmitotic neurons and their final lateral position, thus precluding a function as a migration stop signal, unless at the time of their early migration LMC motor neurons are insensitive to Reelin. Our functional Reelin fragment overexpression in the ventral spinal cord resulted in LMC motor neurons moving beyond their normal lateral position (E.P., T.-J.K., and A.K., unpublished observations); thus, in the context of motor neurons, Reelin is unlikely to function as a migration stop signal, rather, it likely promotes migration or enables LMC neurons to respond to a cue that provides spatial information.

What is the relationship of the Reelin-mediated LMC position specification to that mediated by cadherins, Eph receptors, and the transcription factor Pea3 [27,28,29]? Because of their restricted expression patterns and functional analysis phenotypes, these are thought to operate at the level of motor pools, in contrast to Reelin signalling which appears to specify the position of the entire LMC division. Cadherins have been shown to be involved in the clustering of specific motor pools via their combinatorial expression imparting different adhesion properties on specific motor pools. Similarly, although the early migration of LMC motor neurons in EphA4 mutants appears to be normal, eventually the position of the tibialis motor pool is shifted. Because of these observations, it is likely that Cadherins, EphA4, and Pea3 act at a step following Reelin-mediated migration of LMC neurons.

Unfortunately, since ETS genes, arguably the earliest molecular markers of motor pools, begin to be expressed at the time when LMCI somata attain their lateral position [45], it is technically difficult to ascertain experimentally whether motor pool clustering precedes or coincides with LMCI lateral migration. The differences between the LMC position phenotypes in Dab1 and Lhx1COND mutants might shed some light on this hierarchy. In Dab1 mutants, although shifted medio-ventrally, LMCI neurons remain clustered, in contrast to Lhx1 mutant LMCI motor neurons that can be found intermingled with LMCm neurons. These observations suggest that while the Dab1 mutation probably only leads to the absence of sensitivity to Reelin, the loss of the transcription factor Lhx1 might have consequences beyond the loss of Dab1, resulting, for example, in a change in expression of cell surface adhesion molecules allowing LMCI and LMCm neurons to intermingle.

Dab1 as a Neuronal Position Determinant

Our findings demonstrate that migration of LMC neurons within the ventral spinal cord requires Reelin signalling through the intracellular adaptor protein Dab1. This requirement is principally evident in LMCI neurons and corresponds to the high level of Dab1 protein and mRNA expressed in this population when compared to LMCm neurons. Other studies have also implicated Dab1 protein levels controlled by Cullin5 and Notch signalling as a determinant of neuronal migration [46,47], raising

Figure 5. Foxp1 regulates Dab1 expression. (A–L) Dab1 mRNA and protein expression in the cervical spinal cord of e12.5 Hb9::Foxp1 transgenic (D–F), Foxp1 mutant (J–L), and control embryos (A–C, G–I). (M) Dab1 mRNA and protein mean pixel intensity measurement within Foxp1+ Lhx3− LMC regions (fine stippled outline; D–F, G–I) or the corresponding Foxp1− Lhx3+ region (arrowheads; B–C, K–L). arb: arbitrary units on a 0 (no signal) to 255 (maximum intensity) scale. Student’s t test p values for mRNA quantifications: Hb9::Foxp1 versus wt, p = 0.002, n = 3 embryos per genotype analysed; Foxp1−/− versus Foxp1+/+, p < 0.001, n = 3 embryos per genotype analysed; Student’s t test p values for protein quantification: Hb9::Foxp1 versus wt, p < 0.001, n = 4 embryos per genotype analysed; Foxp1−/− versus Foxp1+/+, p < 0.001, n = 3 embryos per genotype analysed. All sections of the same genotypes are consecutive. Arrows: Foxp1+ interneurons (INs); LMC*: ectopic LMC; bracket: expanded MMC/HMC*. Thicker stippled lines outline the spinal gray matter. Scale bar: 50 μm.

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the question of how might differential Dab1 expression specify LMC soma position in the ventral spinal cord. Upon activation of the Reelin pathway, Dab1 is phosphorylated and rapidly degraded [30,34]. Therefore, in the presence of Reelin, the low Dab1 protein levels in LMCm neurons might be depleted faster than the higher Dab1 protein levels in LMCl neurons, resulting in the termination of Reelin signalling and thus a migration stop occurring sooner in LMCm neurons than in LMCl neurons. This mode of Dab1 function assumes that Reelin promotes migration of LMC neurons, or is a factor enabling their reception of a migration cue and is consistent with our observation that both LMCl and LMCm neurons can respond to Reelin. Thus similar to the Toll-like receptor (TLR) [48] and chemokine [49] signalling pathways regulated by the level of expression of a signalling intermediate, Reelin signal is differentially gated in two neuronal populations through opposing levels of Dab1 expression. In such a model, we would favour the idea that Dab1 concentration, in the presence of Reelin, is an instructive determinant of LMC neuron position in the spinal cord.

Figure 6. Impaired LMC migration in Lhx1 mutants. (A–H) Foxp1, Isl1, and Lhx1/5 expression identifies LMCm (Foxp1⁺ Isl1⁻, LMCl (Foxp1⁺ Isl1⁻ Lhx1/5⁻) and LMCl* (Foxp1⁺ Isl1⁻ Lhx1/5⁻) neurons in the lumbar spinal cord of e12.5 Lhx1 mutants (E–H), and heterozygous littermates (A–D). In Lhx1 mutants, LMCl* cells are found more medially and intermingled with LMCm neurons (arrowheads). (I) Proportion of LMCI motor neuron in Lhx1 mutants and control embryos expressed as percentage of Isl1⁺ Foxp1⁺ motor neurons (37.3%±9% versus 95.2%±2%; p<0.001, Student’s t test). (J) Density plots of mediolateral (ML) and dorsoventral (DV) position of LMCm and LMCI neurons as percentage of LMC width and height. Mean position (ML, DV) is indicated in brackets. Hotelling’s T² test p values: LMCm(Lhx1+/–) versus LMCl(Lhx1COND), p = 0.1613; LMCl*(Lhx1COND) versus LMCI(Lhx1COND), p = 0.2825; LMCl*(Lhx1COND) versus LMCI(Lhx1+/–), p = 0.9886. (K) Proportion of LMCI and LMCl* neurons in quadrants 1–3 and 2–4 of Lhx1+/– and Lhx1COND embryos expressed as percentage of all neurons analysed. Student’s t test p value: LMCI(Lhx1+/–) versus LMCl*(Lhx1COND), p = 0.008. Dashed lines in (J) divide the plots into four equal quadrants. Stippled lines outline the spinal gray matter. n=7 (Lhx1COND) and 5 (Lhx1+/–) embryos; N>1,400 neurons per genotype. Scale bar: 50 μm.

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Figure 7. Lhx1 controls the differential expression of Dab1 in LMC neurons. (A–N) e12.5 lumbar spinal cord Dab1 protein (A–H) and mRNA (I–N) expression in LMC (Isl1+ Foxp1+) is decreased in Lhx1COND mutants (E–H, L–N) compared to control embryos (A–D, I–K). (I–N) Detection of Lhx1, Isl1, and Dab1 mRNA in consecutive sections of e12.5 Lhx1COND and control lumbar spinal cord. (O) Quantification of Dab1 protein and mRNA levels in
position, although the formal demonstration of this through, for example, the change of LMCl Dab1 levels to match exactly those in LMCl neurons is technically challenging. Following its phosphorylation, Dab1 is targeted for polyubiquitination and degradation by Cul5 [47], raising the possibility that in LMCl neurons, Dab1 protein stability might contribute to the differences in Dab1 protein in LMCl neurons. However, since in LMCl neurons Cul5 is apparently expressed at equal levels by LMCl and LMClm neurons (E.P. and A.K., unpublished observations), and because of the selective enrichment of Dab1 mRNA in LMCl neurons, compared to LMClm neurons, we favour the hypothesis that differential transcriptional regulation of the Dab1 gene or its mRNA stability is an important factor contributing to Dab1 protein levels in LMCl neurons.

Gating of Reelin Signalling by Transcription Factor Restriction of Dab1 Expression

Our results demonstrate that Dab1 expression levels in LMCl neurons are set by Foxp1 and Lhx1, two transcription factors that are essential for the specification of LMCl soma position [21,22,23]. Our data suggest the following model of Dab1 expression control in LMCl neurons: a basal level of Dab1 expression in LMCl neurons is induced or maintained by Foxp1, while Lhx1, a transcription factor selectively expressed in LMCl neurons, could act to elevate Dab1 expression in LMCl neurons. Additionally, based on its ability to suppress Lhx1 [22] and Dab1 mRNA expression in LMCl neurons, Isl1 might function to diminish Dab1 expression in LMClm neurons. Thus, although we cannot exclude the influence of other transcription factors or distinguish whether the control of Dab1 expression by Foxp1 and Lhx1 occurs at the level of the Dab1 promoter, through intermediary transcription factors or regulation of Dab1 mRNA stability, we propose that the concerted action of Foxp1 and Lhx1 leads to differential Dab1 expression levels in LMCl neurons.

Could transcription factor control of Dab1 expression be a general mechanism gating Reelin signalling in the CNS? In the cortex, examples of control of migration effectors by transcription factors include the coupling of neurogenesis to migration by bHLH control of doublecortin and p35, Tbr2 control of the planar cell-polarity pathway, and Nks2.1 control of Neurogin2 expression [6], but to our knowledge, a general link between a specific transcription factor and Dab1 expression has so far only been established for CREB/CREM [50]. Intriguingly, in the spinal cord, like LMCl neurons, PG neurons migrate in response to Reelin and also require Foxp1 for their specification [21,23,36], yet although their initial lateral migration path is shared, they eventually occupy two distinct locations in the spinal cord, raising the question of the identity of the divergent migration cues that act on these two motor neuron populations.

Coordination of Myotopy by Transcription Factors

The myotopic organisation of spinal motor neurons is the consequence of the selection of a specific axon trajectory in the limb mesenchyme and of a particular soma location within the spinal cord. The two processes can be uncoupled by loss of Reelin, Eph signalling, or mutation of Lmx1b, a LIM homeodomain transcription factor that controls ephrin ligand expression in the limb [24,26,42], raising the question of the molecular hierarchy controlling myotopy. Foxp1 and Lhx1 determine the selection of a dorsal or ventral LMCl axon trajectory through restriction of Eph receptor expression [21,22,23], and our data suggest that they gate LMCl neuron sensitivity to Reelin signals, thereby specifying the position of LMCl soma in the ventral spinal cord. These observations imply that the selection of an LMCl axon trajectory in the limb and soma position within the ventral horn are normally controlled coordinately by Foxp1 and LIM homeodomain transcription factors. Based on these observations, a probable simple hierarchy for motor axon trajectory and soma position selection coordination (Figure 8) Foxp1 together with Lhx1 and Isl1 transcription factors are required for the expression of Eph receptors in LMCl axons, and thus their repulsion from ephrin ligands in the limb mesenchyme, leading to their selection of a dorsal or a ventral limb trajectory. Foxp1, Lhx1, and possibly Isl1 also establish disparate Dab1 protein levels in LMCl neurons, thus enabling their cell bodies to segregate into distinct mediolateral positions. A number of transcription factors regulating reception of specific axon guidance receptors has already been described [4,5], implying that some of them may also direct neuronal migration, thus coordinating topographic organisation of neuronal circuits. Moreover, topographical organisation also extends to dendrite arborisation and synaptic activity [19,51], and since Foxp1 regulates the position of motor neuron dendrites [21], it remains plausible that the transcription factors controlling migration and axon projections may be used to control other facets of topographic organisation.

Why should neuronal migrations and axon trajectories be controlled coordinately? LMCl neurons within a specific motor pool, i.e. those innervating a particular muscle, are electrically coupled through gap junctions, possibly to consolidate their electrical activity patterns during the time of spinal motor circuit assembly [52]. Aberrant soma position could result in the inability of LMCl neurons to form electrically coupled motor pools even though neuromuscular junctions with appropriate muscle targets in the limb might be maintained. Thus, a motor neuron might receive appropriate signals from its muscle target but is unable to synchronize its electrophysiological maturation, such as calcium transient waves [53], with other motor neurons in its pool because of their dispersed position. The emergence of functional motor circuitry also depends on the formation of specific sensory-motor contacts achieved by sensory axons synapsing on the dendrites of homonymous motor neurons within the ventral spinal cord [54]. Motor neurons in distinct pools have stereotypic dendritic arbor shapes which in principle could be dictated by the position of the motor neuron soma [28], although it remains to be determined whether motor neuron soma displacement, without any effects on molecular markers of cell fate, results in dendritic arborisation defects and whether such defects alter the sensory-motor connectivity. Reelin signalling has also been implicated in cortical dendrite formation, raising the possibility that Reln mutation might...
lead to LMC dendritic arbour defects independently of its effect on soma localisation. Moreover, in *Reln* mutant mice, although retrograde and electrophysiological analysis reveals relatively normal cortico-thalamic connectivity, retinal circuit connectivity is perturbed possibly due to defects in neuronal layer formation [55,56]. Because of the involvement of Reelin in synapse function [57], it is difficult to dissociate the functional consequences of altered topography in Reelin signalling loss of function from altered synaptic function. However, examples of severe functional deficits caused by neural circuit topography disruption apparently independent of Reelin signalling [58] highlight the importance of topographic organisation of the nervous system.

**Materials and Methods**

**Animals**

All mice were maintained and genotyped by PCR as previously described [21,31,43,59,60,61,62]; *Reln* allele was *Reln* [Relnrl/J (Jackson Laboratory, USA). Fertilised chick eggs (Couvoir Simentin, Canada) were staged according to Hamburger and Hamilton [38].

**Expression Plasmid Generation and Chick In Ovo Electroporation**

Chicken Dab1L isoform (NM_204238) [63] was cloned by RT-PCR (Invitrogen, USA) and fused in frame to GFP at the C-terminus in pN2-eGFP (Invitrogen, USA).

Chick spinal cord electroporation was performed using an Ovodyne TSS20 square pulse generator (Intracell, UK) as described [24,64].

**Immunostaining and In Situ mRNA Detection**

Immunofluorescence stainings were carried out on 12 μm cryosections as described [22,24]. For antisera used and dilutions, see Table S1.

In situ mRNA detection was performed as previously described [65,66]. Probe sequence details are available upon request.

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**Figure 8. Model of transcriptional control of LMC myotopy.** (A) Foxp1, Isl1, and Lhx1 control Dab1 and Eph receptor expression in LMC neurons and thus coordinate LMC neuronal migration and axon projections. (B) Molecular hierarchy of transcription factor control of LMC myotopy. See discussion for details.

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Image Quantification

Images were acquired using a Zeiss LSM confocal microscope or a Leica DM6000 microscope with Improvision Volocity software. Quantification of protein and mRNA expression, GFP-, and β-gal-labelled axon projections was as described [24,65]. To quantify axon projections in hCrgt/Isl1-PLAP embryos, 12 μm cryosections were immunostained (see Table S1), post-fixed, washed, and incubated at 65°C. Phosphatase activity was revealed simultaneously in sections containing mutant and control tissue. The signal was quantified in sections sampled at 30–50 μm rostrocaudal intervals at the cervical level with at least six sections analysed per embryo.

Motor Neuron Position Quantification

All quantifications were done between lumbosacral (LS)4 and LS6 levels as assessed by vertebra counts and absence of pSmad1/5/8 PG neurons [23]. Neurons were imaged in 12 μm cryosections sampled at 100 μm intervals using a Zeiss LSM confocal or Leica DM6000 fluorescent light microscope; ML and DV values were calculated. [i.e., ImageJ] software measurements of distance (D) and angle (α) of motor neuron soma from the ventral edge of the ventricular zone (see Text S1 for details) and then plotted using Matlab software running the “scatter” function, which creates a scatter plot with contour lines linking data points with similar frequency and colour intensities that increase with data point frequency.

In all cases, to compare the vectors of means between experimental and control groups, we used a two-sample Hotelling’s T², which is a two-dimensional generalization of the Student’s t test, combined with a randomization test under the stringent assumptions of the parametric Hotelling’s T², to circumvent the difficulty of having moderately sized samples. The analysis was implemented using the NCSS software package (Hitz J. [2007]; Kaysville, Utah, www.ncss.com).

Supporting Information

Figure S1 Expression of Dab1 and Reelin in e10.5 mouse and Dab1, Reelin, VLDDL, and ApoER2 in the chick spinal cord. (A–L) Detection of Isl1, Lhx1/5, Foxp1, and Dab1 in cervical (A–D) and lumbar spinal cord (E–H) and Reelin in cervical spinal cord (I–L). Dab1 is expressed in Foxp1+ LMC neurons, while Reelin expression is restricted to a small domain dorsal to the LMC. (M–P, R–U) Detection of mRNA in consecutive lumbar spinal cord sections of HH St 24 and HH St 30 chick embryos. Isl1 (M, R) and Lhx1 (N, S) expression highlights LMCm and LMCl neurons, respectively. Reelin mRNA is expressed medio-dorsally to the LMC at HH St 24 (P), but at HH St 30 this expression domain is expanded medio-ventrally (U). Detection of Dab1 mRNA expression is stronger in LMCl neurons compared to LMCm neurons at both HH St 24 (O) and HH St 30 (T). (W–Z) Detection of Isl1 (W), Lhx1 (X), ApoER2 (Y), and VLDDL (Z) mRNA in HH St 25/26 chick embryos. ApoER2 mRNA is present in the ventricular zone (VZ) but not in LMC neurons (Y). VLDDL expression is uniform throughout the ventral spinal cord (Z). (Q, V) Schematic view of LMCl and LMCm position within ventral spinal cord. VZ: ventricular zone; yellow stippled lines outline the spinal gray. Scale bar: 62 μm (A–L), 52 μm (M–U), and 59 μm (W–Z).

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Figure S2 Expression of RADLH2, RC2, and Cadherins in Dab1 mutants. (A–L) Protein expression in lumbar spinal cord of e12.5 embryos. Foxp1 expression highlights LMC neurons; LMCm (M) neurons are Foxp1+ Isl1+, LMCl (L) neurons are Foxp1+ Isl1+. In Dab1 mutants, RADLH2 (A–F) and RC2 (G–L) distribution is unchanged when compared to control embryos. (M–X) Detection of cadherin mRNAs in consecutive sections of e12.5 lumbar spinal cord of Dab1 mutants (S–X) and control littermate (M–R). Isl1 (M, S) and Lhx1 (N, T) expression highlights LMCm (M) and LMCl (L) motor neurons, respectively. Scale bar: 50 μm (A–L) and 94 μm (M–X).

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Figure S3 Quantification of motor neurons in Dab1 and Reln mutant embryos. (A, C) Quantification of LMC motor neuron numbers in e12.5 Dab1, Reln, and wild type (WT) littermate embryos expressed as the average number of total Foxp1+ LMC neurons per 12 μm section (# Foxp1+/section). LMC neuron numbers are not significantly different in mutants when compared to controls (p > 0.17, Student’s t test). (B, D) Quantification of LMCm (Isl1+ Foxp1+) and LMCl (Isl1– Foxp1+) motor neuron numbers in lumbar spinal cord of e12.5 Dab1 (B) and Reln (D) mouse embryos expressed as the percentage of all LMC motor neurons in 12 μm sections (Foxp1+ MN %). LMCm and LMCl neuron numbers are not significantly different in mutants when compared to controls (p > 0.25 Student’s unpaired t test).

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Figure S4 Characterisation of Dab1 and Reelin mutants. (A–F) Loss of Dab1 immunoreactivity (arrowheads) in the lumbar spinal cord of an e12.5 Dab1 mutant embryo (D–F) compared to a control littermate (A–C). LMCm (L) neurons are Foxp1+ Isl1+ and LMCl (M) neurons are Foxp1+ Isl1+. (G–L) Loss of Reln protein immunoreactivity (arrows) in the lumbar spinal cord of a e12.5 Reln mutant embryo (J–L) compared to a control littermate (G, I). Dab1 protein levels are increased in the Reln mutant (J, K) relative to the control littermate (G, H) (arrowheads). Isl1 expression highlights LMCm neurons. Asterisks indicate blood vessels. Stippled lines outline the spinal gray or LMCl (L) neurons. Scale bar: 50 μm in all panels.

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Figure S5 Cell autonomous requirement for Dab1 signaling in LMCl migration. (A) Quantification of LMC motor neurons electroporated with LacZ or Dab1V5F and LacZ expression plasmids in chick HH St 29 embryos, expressed as the average number of total LacZ+ LMC neurons per 12 μm section (# LacZ+ MN %). The difference in numbers between control and experimental embryos is not significant (n.s.; p = 0.440, Student’s unpaired t test; n = 7 embryos; N = 100 neurons per embryo per experimental condition analysed). (B) Quantification of LMCm (Isl1+ Foxp1+) and LMCl (Isl1– Foxp1+) motor neurons in lumbar spinal cord of chick HH St29 embryos electroporated with LacZ or Dab1V5F and LacZ expression plasmids, expressed as the percentage of total electroporated motor neurons [LacZ+ MN %]. The difference in numbers between control and experimental embryos is not significant (n.s.; p = 0.393, Student’s unpaired t test; n = 7 embryos; N = 100 neurons per embryo per experimental condition analysed). (C–J) Detection of LacZ, Foxp1, and Isl1 protein in LMCm (Foxp1+ Isl1+) and LMCl (Foxp1– Isl1+) motor neurons in lumbar spinal cord of chick HH St 29 embryos electroporated with LacZ, (C–F) or Dab1V5F and LacZ expression plasmids (G–J). LMCm neurons expressing Dab1V5F are frequently found in a zone ventromedial to LMCm neurons (arrowheads in J). Examples of electroporated LMCm neurons are indicated by arrowheads (G–J). LMCm neurons expressing Dab1V5F are shown at higher magnification (insets of G–J). (K) Density plots of medio-lateral (ML) and dorsoventral (DV) position of electroporated LMCm and LMCl neurons expressed as percentage of LMC.
width and height. The position of LacZ-expressing LMCm neurons was not significantly different from Dab1+/- expressing LMCm embryos (ML: 58.6% ± 2.9%; DV: 50.8% ± 5.5%) versus (ML: 58.1% ± 3.3%; DV: 50.2% ± 7.9%); p = 0.9293, Hotelling’s T² test). Dab1+/- expressing LMCm neurons were shifted significantly in the mediolateral direction with respect to the LacZ-expressing LMCm neurons (ML: 72.1% ± 3.6%; DV: 42.2% ± 3.8%) versus (ML: 78.3% ± 3.3%; DV: 45% ± 3.4%); p = 0.0233, Hotelling’s T² test). Number of embryos analysed per experimental condition: 7; number of electroporated neurons analysed per experimental condition: >100 per embryo. Dashed lines divide the LMCm into four equal quadrants (I). All values are expressed as mean ± s.d. Yellow stippled lines outline the spinal gray matter. Scale bar: 23 μm in all panels.

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**Figure S6** Quantification of Dab1 and LMC neuronal identity in embryos over-expressing Dab1. (A) Quantification of LMCm ([Isl1]siRNA1 and LMCc ([Foxp1] Foxp1) neuron numbers in the lumbar spinal cord of chick HH St 29 embryos expressing GFP or Dab1::GFP, expressed as the percentage of total motor neurons ([Foxp1]+ MN (%)). The difference between experimental and control embryos is not significant (p = 0.4324, Student’s unpaired t test; n = 4 (GFP) and 5 (Dab1::GFP) embryos; N = 60 neurons per embryo by experimental condition analysed). (B) Proportions of electroporated LMCm ([Isl1] Foxp1) and LMCc ([Foxp1] Foxp1) motor neurons in lumbar spinal cord of chick HH St29 embryos expressing GFP or Dab1::GFP (GFP+ MN (%)). The difference between experimental and control embryos is not significant (n.s.; p = 0.0540, Student’s unpaired t test; n = 4 (GFP) and 5 (Dab1::GFP) embryos; N = 90 neurons per embryo by experimental condition analysed). (C) In embryos electroporated with Dab1::GFP, Dab1 mRNA is upregulated on the electroporated side with respect to the unelectroporated side of the spinal cord. Quantification of Dab1 mRNA expression in GFP (white bar) or Dab1::GFP electroporated embryos (grey bar), normalized to the expression in the unelectroporated side of the spinal cord (electroporated/unelectroporated ratio; e/u). E/u ratio for Dab1 mRNA expression in GFP electroporated embryo is 1.2 ± 0.04, while in Dab1::GFP electroporated embryos it is 1.3 ± 0.03 (p = 0.001; Student’s t test; n = 3 embryos per experimental condition analysed).

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**Figure S7** Normal Hoxa5, Hoxc6, Reelin, and Chx10 expression in Hb9::Foxp1 transgenics and Foxp1 mutants. (A, D, G, J) Hoxa5 and Hoxc6 protein detection in cervical spinal cord of Hb9::Foxp1 (D), Foxp1 mutant (J), and control littermate (A, G) e12.5 embryos. (B, E, H, K) Foxp1 and Hb9 detection in cervical spinal cord of Hb9::Foxp1 (E), Foxp1 mutant (K), and control littermate (B, H) e12.5 embryos. (C, F, I, L) Reelin and Chx10 detection in forelimbs of Hb9::Foxp1 (F), Foxp1 mutant (L), and control littermates (C, F) e12.5 embryos. LMCc indicates ektomic LMC in upper cervical sections of Hb9::Foxp1 embryos. Found at: doi:10.1371/journal.pbio.1000446.s007 (1.96 MB TIF)

**Figure S8** Isl1 is required for the specification of LMCm position. (A–D, F–I) Detection of LacZ, Foxp1, and Lhx1/5 protein in LMCm (Foxp1+ Lhx1/5+) and LMCc (Foxp1+ Lhx1/5+) neurons in lumbar spinal cord of chick HH St 29 embryos electroporated with LacZ (A–E) or [Isl1]siRNA and LacZ expression plasmids (F–J). Arrowheads point to electroporated LMCm neurons. Examples indicated by arrowheads are shown at higher magnification in insets of (A–D, F–I). Superimposed ventral spinal cord location of electroporated LMCm (red) neurons in consecutive sections of representative embryos highlights the 

lateralized shifted position of [Isl1]siRNA and LacZ expression plasmids electroporated LMCm neurons. The blue dashed line indicates the 50% ML value. (K) Density plots of mediolateral (ML) and dorsoventral (DV) positions of electroporated LMCm and LMCc neurons expressed as percentage of LMCc width and height. The mean position of [Isl1]siRNA-electroporated LMCm neurons (ML: 67.9% ± 1.5%; DV: 53% ± 6%) was significantly shifted laterally with respect to the mean position of LacZ-expressing LMCm neurons (ML: 63.5% ± 1.8%; DV: 56.9% ± 3.8%); p = 0.0473, Hotelling’s T² test). Number of embryos analysed per experimental condition: 4 (LacZ) and 5 (LacZ+ [Isl1]siRNA); number of neurons included in the analysis: >60 per embryo by experimental condition. Dashed lines divide LMCm in four equal quadrants. All values are expressed as mean ± s.d. Yellow stippled lines outline the spinal gray matter. Scale bar: 23 μm in all panels.

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**Figure S9** Isl1 is sufficient to regulate Dab1 mRNA expression. (A–I, N–Y) LacZ Isl1, Lhx1, and Dab1 mRNA detection in chick HH St 28–30 embryos electroporated with LacZ (A–F), Isl1 and LacZ expression plasmids (G–I), GFP (N–S), or [Isl1]siRNA and GFP expression plasmid (T–Y). In embryos electroporated with Isl1, Dab1 is downregulated on the electroporated side (L) with respect to the unelectroporated side of the spinal cord (K). No significant effect on Dab1 expression was observed in LacZ electroporated embryos (E, F); (M) Quantification of Isl1 and Dab1 mRNA expression in LacZ (white bars) or Isl1 electroporated embryos (grey bars), with values normalized to expression in the unelectroporated side of the spinal cord (electroporated/unelectroporated ratio; e/u). E/u ratio for Isl1 mRNA expression in LacZ electroporated embryo is 1.2 ± 0.1, while in Isl1 electroporated embryos it is 3 ± 0.5 (p = 0.002; Student’s t test). Dab1 mRNA expression e/u ratio in Isl1 electroporated embryos (0.7 ± 0.1) is significantly different from that in LacZ electroporated embryos (1.4 ± 0.1; p < 0.001, Student’s t test). (Z) Quantification of numbers of Isl1 or Foxp1 expressing neurons in GFP, (white bars) or [Isl1]siRNA-electroporated embryos (grey bars), expressed as the ratio between electroporated/unelectroporated spinal cord sides (e/u). E/u value for Isl1 Foxp1+ neurons in GFP electroporated embryos is 1.0 ± 0.03, and in [Isl1]siRNA electroporated embryos it is 0.8 ± 0.05 (p < 0.001; Student’s t test). Foxp1+ neurons in [Isl1]siRNA electroporated embryos (0.95 ± 0.03) is not significantly different from GFP electroporated embryos (1.0 ± 0.2; p > 0.075, Student’s t test). All values are expressed as mean ± s.e.m. Fine stippled lines highlight Dab1high area, heavy stippled lines outline the spinal gray. Number of embryos analysed per experimental condition: 3. Scale bar: 59 μm (A–L) and 46 μm (N–Y).

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**Figure S10** Variability of LMCc neuron location in Lhx1 mutants. Superimposed spinal cord position of LMCm (red), LMCc (green), and LMCp (blue) neurons in consecutive sections of analyzed embryos highlights altered position of LMCc neurons. Blue numbers indicate the fractions of LMCc neurons expressed as percentage of Isl1 Foxp1+ motor neurons and the dashed line represents the 50% ML value.

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**Table S1** Antibodies used in this study. (A) Primary antibodies, (B) fluorochrome-conjugated secondary antibodies. DSHB: Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.
Table S2 | Position of LMC neurons in Dab1 and Reln mutants. n: number of embryos analyzed; N: total numbers of neurons counted; p values for position of experimental versus control neurons are from randomized Hotelling’s T^2 test under unequal variances. a Values are ± standard deviation of the mean.

Table S3 | Position of LMC neurons in electrooporated chick embryos. n: number of embryos analyzed; N: total numbers of neurons counted; p values for position of experimental versus control neurons are from randomized Hotelling’s T^2 test under unequal variances. a Values are ± standard deviation of the mean.

Table S4 | Position of LMC neurons in Lhx1 mutants. LMCm (A), LMC1 and LMC1* (B) position analysis. n: number of embryos analyzed; N: total numbers of neurons counted; p values for position versus littermate Lhx1^-/-, Randomization Hotellings T^2 test under unequal variances; p’ values for position LMC1p versus LMC1 littermate Lhx1^-/-; Randomization Hotellings T^2 test under unequal variances. a Values are ± standard deviation of the mean.

Text S1 | Supplemental data and supplemental materials and methods.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: E. Palmesino, A. Kania. Performed the experiments: E. Palmesino, DL. Roussou, TJ. Kao, A. Kania. Analyzed the data: E. Palmesino, DL. Roussou, TJ. Kao, BG. Novitch, A. Kania. Contributed reagents/materials/analysis tools: E. Palmesino, A. Klar, E. Laufer, O. Uemura, H. Okamoto, BG. Novitch, A. Kania. Wrote the paper: E. Palmesino, BG. Novitch, A. Kania.

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