Motor axon exit from the mammalian spinal cord is controlled by the homeodomain protein Nkx2.9 via Robo-Slit signaling

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SUMMARY
Mammalian motor circuits control voluntary movements by transmitting signals from the central nervous system (CNS) to muscle targets. To form these circuits, motor neurons (MNs) must extend their axons out of the CNS. Although exit from the CNS is an indispensable phase of motor axon pathfinding, the underlying molecular mechanisms remain obscure. Here, we present the first identification of a genetic pathway that regulates motor axon exit from the vertebrate spinal cord, utilizing spinal accessory motor neurons (SACMNs) as a model system. SACMNs are a homogeneous population of spinal MNs with axons that leave the CNS through a discrete lateral exit point (LEP) and can be visualized by the expression of the cell surface protein BEN. We show that the homeodomain transcription factor Nkx2.9 is selectively required for SACMN axon exit and identify the Robo2 guidance receptor as a likely downstream effector of Nkx2.9; loss of Nkx2.9 leads to a reduction in Robo2 mRNA and protein within SACMNs and SACMN axons fail to exit the spinal cord in Robo2-deficient mice. Consistent with short-range interactions between Robo2 and Slit ligands regulating SACMN axon exit, Robo2-expressing SACMN axons normally navigate through LEP-associated Slits as they emerge from the spinal cord, and fail to exit in Slit-deficient mice. Our studies support the view that Nkx2.9 controls SACMN axon exit from the mammalian spinal cord by regulating Robo-Slit signaling.

KEY WORDS: Nkx2.9, Robo, SACMN, Slit, Motor axon exit, Mouse

INTRODUCTION
Motor neurons (MNs) project their axons out of the central nervous system (CNS) and make stereotyped connections with peripheral muscle targets to form circuits that control movement (Bonanomi and Pfaff, 2010; Dalla Torre di Sanguinetto et al., 2008; Sharma and Peng, 2001). Motor axons grow in a directed manner to specialized exit points through which they emerge from the CNS (Bravo-Ambrosio and Kaprielian, 2011; Jacob et al., 2001; Lieberam et al., 2005; Schneider and Granato, 2003; Sharma et al., 1998; Shirsaki and Pfaff, 2002). MN subtypes can be distinguished by the positions of their exit points: ventral MNs (vMNs) and dorsal MNs (dMNs) utilize ventral and dorsal exit points, respectively (Chandrasekhar, 2004; Cordes, 2001; Dillon et al., 2005; Guthrie, 2007; Lieberam et al., 2005; Schubert and Kaprielian, 2001; Sharma et al., 1998; Snider and Palavali, 1990). Although Cxcl12-Cxcr4 signaling regulates the growth of vMN axons to their exit points in mice (Lieberam et al., 2005) and myotomal-derived divanka (plod3 – ZFIN) glycosyltransferase is required for motor axon growth into the periphery in zebrafish (Schneider and Granato, 2006), the molecular mechanisms that control motor axon exit from the vertebral spinal cord are poorly understood.

Transcription factors (TFs) control axon pathfinding by regulating the expression of cell surface molecules (Broihier et al., 2004; Garcia-Frigola et al., 2008; Labrador et al., 2005; Landgraf et al., 1999; Lee et al., 2008; Wilson et al., 2008). In Drosophila, Zfh1 (Layden et al., 2006) and Nkx6 (HGTX – FlyBase) (Broihier et al., 2004) are required for the exit of vMN axons, whereas Eve (Landgraf et al., 1999) is necessary for dMN axon exit. Nkx6 and Eve are likely to mediate motor axon exit by regulating the expression of Fas3 (Broihier et al., 2004) and Unc5 (Labrador et al., 2005), respectively. In vertebrates, Lhx3/Lhx4 (Sharma et al., 1998) and Phox2b (Hirsch et al., 2007) are required for the directed growth of vMN and dMN axons, respectively, to their exit points. However, these TFs appear to control the specification of vMNs/dMNs rather than motor axon exit per se.

Spinal accessory motor neurons (SACMNs) are branchiomotor dMNs that reside within the cervical spinal cord and project dorsally directed axons to and through a highly localized lateral exit point (LEP) situated midway along the dorsoventral axis of the spinal cord (Dillon et al., 2005; Hirsch et al., 2007; Lieberam et al., 2005). Upon exiting the CNS, SACMN axons execute a rostral turn and assemble into the longitudinally projecting spinal accessory nerve (SAN), which innervates particular neck and back muscles (Dillon et al., 2005; Dillon et al., 2007; Schubert and Kaprielian, 2001; Snider and Palavali, 1990). We identified the immunoglobulin (Ig) domain-containing protein BEN (Alcam or SC1 – Mouse Genome Informatics) (Dillon et al., 2005; Schubert and Kaprielian, 2001) as a selective marker of SACMN cell bodies/axons (Dillon et al., 2005; Schubert and Kaprielian, 2001). Since SACMNs are a molecularly homogeneous and readily identifiable population of spinal MNs, which leave the CNS through a circumscribed exit point, they represent an ideal model system for elucidating molecular programs that control motor axon exit.

Our observation that the homeodomain TF Nkx2.9 is likely to be required for SACMN axons to leave the CNS (Dillon et al., 2005) prompted us to further characterize the role of Nkx2.9 in motor axon exit. Here we show that, in mice lacking Nkx2.9, SACMN axons
appropriately project to the LEP but assemble into an ectopic longitudinally projecting SAN within the spinal cord. We also identify the axon guidance receptor roundabout 2 (Robo2) (Ypsilanti et al., 2010) as a likely downstream effector of Nkx2.9 by showing that Robo2 expression in SACMNs is downregulated in Nkx2.9 null mice and that SACMN axons fail to exit the spinal cord in Robo2-deficient animals. Furthermore, the Robo2 ligands Slit1-3 are present at the LEP, SACMN axons fail to exit the CNS in Slit null mice, and Slit promotes SACMN axon outgrowth in vitro. Collectively, our findings are consistent with Nkx2.9 controlling SACMN axon exit from the CNS by regulating Robo2-Slit interactions at the LEP.

MATERIALS AND METHODS

Mice

CD-1 wild-type (WT) embryos were used for expression studies (Charles River Laboratories). Nkx2.9 mutant embryos were generated by mating Nkx2.9<sup>–/–</sup> mice and genotypes determined by PCR (Tian et al., 2006). Nkx2.9 breeding pairs were obtained from J. Locker (Albert Einstein College of Medicine). Pregnant dams were sacrificed as described (Dillon et al., 2005). The morning on which a vaginal plug was detected was considered embryonic day (E) 0.5. Robo and Slit mutant embryos were generated and genotyped as described (Andrews et al., 2008; Farmer et al., 2008; Grieshammer et al., 2004; Long et al., 2004; Lopez-Bendito et al., 2007; Lu et al., 2007; Plump et al., 2002).

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Fig. 1. In Nkx2.9<sup>–/–</sup> embryos, SACMN axons fail to exit the CNS and assemble into an ectopic SAN within the spinal cord. (A-H) Wild-type (WT) (A,B, E10.5; E,F, E11.5) and Nkx2.9<sup>–/–</sup> (C,D, E10.5; G,H, E11.5) embryos were labeled with either anti-BEN and anti-laminin (A-D) or anti-BEN and anti-L1 (E-H). (A,B) In E10.5 WT embryos, BEN-labeled spinal accessory motor neuron (SACMN) axons extend through the lateral exit point (LEP) and assemble into a spinal accessory nerve (SAN) located outside the CNS. (C,D) SACMN axons appropriately extend to the LEP in Nkx2.9<sup>–/–</sup> mice but fail to exit and remain within the anti-laminin-labeled margin of the spinal cord (arrow), where they appear to assemble into an ectopic SAN (arrowhead) (n>5). B and D are magnified views of the boxed areas in A and C, respectively. (E-H) A BEN/L1-expressing SAN (arrowhead) forms outside of the spinal cord and adjacent to the LEP (arrow) in WT embryos, whereas in Nkx2.9<sup>–/–</sup> mice the SAN inappropriately forms within the spinal cord adjacent to the LEP and BEN-expressing SACMNs (H, asterisks). F and H are magnified views of the boxed areas in E and G, respectively. (I) The distance from the base of the floor plate (FP) to the SAN (see upper schematic) was not statistically different in WT (dark gray, SAN) as compared with Nkx2.9<sup>–/–</sup> (light gray, ectopic SAN) embryos (n=4). Error bars indicate s.d. (J) Schematized open-book (OB) preparation from cervical levels (C1-C4) of the spinal cord. RP, roof plate. (K-N) OB preparations derived from an E11.5 Nkx2.9 null embryo and a WT littermate labeled with anti-BEN. In contrast to WT embryos (K,L), an ectopic SAN is located within the spinal cord of Nkx2.9<sup>–/–</sup> embryos (M,N; n=3). L and N are magnified views of the boxed areas in K and M, respectively. (O) The trajectory of SACMN axons in WT and Nkx2.9<sup>–/–</sup> embryos. Scale bars: 50 µm in C for A,C; 25 µm in D for B,D; 100 µm in G for E,G, in K for K,M, in L for L,N; 5 µm in H for F,H.
RNA isolation, cDNA microarray hybridization, data acquisition and analysis

The Qiagen RNeasy Micro Kit was used to isolate total RNA from E11.5 cervical spinal cords (excluding dorsal spinal cord and floor plate; WT, \(n=3\); \(Nkx2.9^{+/–}\), \(n=3\)). RNA was amplified using the WT-Ovation Pico RNA Amplification Kit (NuGEN Technologies), followed by hybridization to GeneChip Mouse Genome 430 2.0 Array (Affymetrix) by the Albert Einstein College of Medicine Affymetrix facility. Results from three independent arrays were averaged. The raw data (CEL files) from each probe set were normalized by RMA methods using GeneSpring GX 10.0 software (Agilent). The log2 transformed signal intensities were averaged for biological replicates and the mean value was used to compute fold change. Differentially expressed genes were identified as those exhibiting a fold change exceeding 1.5, as several previous studies have used this cut-off (Hughes et al., 2000; Schachter et al., 2002; Wang et al., 2003; Yuan et al., 2005) and a fold change of less than 2 can be biologically significant (Hughes et al., 2000).

Immunohistochemistry and in situ hybridization

Antibodies used were: anti-BEN (RIKEN BioResource Center), anti-β-galactosidase (Abcam), anti-NF (DSHB), anti-HB9 (S. Pfaff, Salk Institute), anti-Islet1 (DSHB), anti-laminin (Sigma), anti-L1 (Chemicon), anti-myc (Millipore), anti-Phox2b (G. Goridis, INSERM) and anti-Robo2 (R&D Systems). Standard immunohistochemistry, digoxigenin-labeled riboprobe generation and in situ hybridization were carried out as described (Imondi et al., 2000). The mouse \(Slit1\), \(Slit2\), \(Slit3\) (Yuan et al., 1999), \(Nkx2.9\) (Pabst et al., 1998), chick \(Robo2\) (Reeber et al., 2008) and \(BEN\) (Dillon et al., 2005) cDNAs were generated as described. Detection of β-galactosidase activity with X-Gal was as described (Corrales et al., 2004). Open-book (OB) spinal cord preparations were generated from E11.5 mouse embryos and subject to whole-mount immunohistochemistry as described (Imondi et al., 2000).

Fluorescent in situ hybridization followed by immunohistochemistry

Cervical spinal cord-containing cryosections derived from E9.5 mouse embryos were hybridized with \(Slit1\), \(Slit2\) or \(Slit3\) riboprobe for 12 hours at 72°C, and then incubated with anti-digoxigenin POD (Roche) and anti-β-galactosidase (Abcam) overnight at 4°C. Labeled mRNA and protein were visualized using the Tyramide Signal Amplification Plus Cyanine 3 System (Perkin Elmer) and an Alexa Fluor 488-conjugated secondary antibody (Invitrogen), respectively (see Vosshall et al., 2000).

In ovo electroporation

Full-length mouse \(Nkx2.9\) cDNA was subcloned into the \(pMES\) expression vector [which contains the chicken β-actin promoter/CMV-IE enhancer and \(IRES\)-EGFP (Swartz et al., 2001)]. Purified \(pMES\) (2–4 μg/μl), \(pMSENkx2.9\) (2–4 μg/μl) or cytopcig-Slit1-LRR (0.5 μg/μl) vectors (Shiau...
and Bronner-Fraser, 2009) were microinjected into the central canal of E2 chick embryos, which were then subjected to unilateral electropropraxation as described (Reeber et al., 2008).

**In vitro axon outgrowth assay**

Floor plate-containing ventral spinal cord explants were generated from E11.5 WT mouse embryos as described (Imondi and Kaprielian, 2001), placed in 1:1 rat-tail collagen:Matrigel (BD Biosciences) pads and co-cultured with aggregates of mock- or Slit2-transfected (1-2 µg/ml) (Kadison et al., 2006) HEK293 cells for 15-24 hours in 69% OptiMEM with GLUTAMAX (Gibco), 23% F12 (Gibco), 5% FBS (Gemini BioProducts), 2% 2 M glucose and 1% penicillin-streptomycin-glutamine (Gibco). Explants were then fixed in 4% paraformaldehyde for 16 hours at 4°C and subjected to whole-mount immunohistochemistry (Dillon et al., 2005) (data not shown).

**RESULTS**

**In Nkx2.9+/− mice, SACMN axons fail to exit the spinal cord and inappropriately assemble into an ectopic SAN**

We previously reported that SACMN axons apparently fail to exit the CNS in Nkx2.9 null mice (Dillon et al., 2005). To unambiguously determine whether SACMN axons remain confined to the spinal cord in these mice, we labeled transverse cryosections from the cervical spinal cord of E10.5 Nkx2.9+/− embryos and their WT littermates with anti-BEN, as a selective marker of SACMNs, and anti-laminin, which demarcates the margin of the spinal cord (Fig. 1A-D). BEN-expressing SACMN axons failed to project across the laminin border in Nkx2.9+/− mice (Fig. 1C,D). This does not appear to reflect a developmental delay as SACMN axons do not exit the spinal cord in Nkx2.9+/− mice analyzed as late as E13.5, well after SACMN axons normally assemble into the SAN (Dillon et al., 2005) (data not shown).

We next asked whether SACMN axons assemble into an ectopic SAN within the spinal cord of Nkx2.9 null mice by labeling transverse cryosections from E11.5 embryos with anti-BEN and anti-L1 (L1cam – Mouse Genome Informatics), which labels longitudinally projecting spinal axons (Imondi et al., 2000). A BEN/L1-labeled nerve was located adjacent to the LEP but within the spinal cord in Nkx2.9+/− mice (Fig. 1G arrow, 1H arrowhead). To determine whether this ectopic nerve projects along the anterior-posterior (A-P) extent of the cervical spinal cord, we analyzed OB preparations derived from E11.5 Nkx2.9+/− and Nkx2.9+/− embryos. Consistent with OB preparations lacking an external SAN (Fig. 1J), a BEN-positive SAN was only observed within the spinal cord of Nkx2.9+/− mice (Fig. 1K-O). Together, these findings indicate that Nkx2.9 is selectively required for SACMN axon exit. By contrast, vMN axons appropriately emerge from the spinal cord in Nkx2.9+/− mice (supplementary material Fig. S1).

**Appropriate numbers of SACMNs, which likely arise from Nkx2.9+ progenitors, form in Nkx2.9 null mice**

To rule out the possibility that the lack of SACMN axon exit in Nkx2.9+/− embryos reflects a reduction in SACMN number, we first identified a panel of SACMN markers by labeling

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**Fig. 3. Robo2 expression is reduced in Nkx2.9+/− mice.** (A-F) E10.5 cryosections derived from WT (A,C,E) or Nkx2.9+/− (B,D,F) mice were subjected to in vitro hybridization for BEN (A1cam) (A,B) or Robo2 (C,D) mRNA, or labeled with anti-BEN and anti-Robo2 (E,F). Similar levels of BEN mRNA are expressed by SACMNs (black arrowheads) in WT (A) and Nkx2.9+/− (B) embryos (n=3). SACMN-associated Robo2 mRNA is reduced in Nkx2.9+/− embryos (D; n=3) as compared with WT littermates (C). In Nkx2.9+/− embryos, SACMN axons (arrow) and the ectopic SAN (white arrowhead) display reduced levels of Robo2 protein (F; n=3) compared with WT littermates (E). The dotted line indicates the margin of the spinal cord. Scale bars: 100 µm in D for A-D; 25 µm in F for E,F.
cryosections from the cervical spinal cord of WT embryos with anti-BEN and antibodies specific for the MN-associated TFs Islet1 (Ericson et al., 1992; Pfaff et al., 1996), HB9 (Mnx1 – Mouse Genome Informatics) (Arber et al., 1999) or Phox2b (Pattyn et al., 2000). At E10.5-11, BEN-positive SACMNs express the generic MN marker Islet1 (Fig. 2A-C) and the branchiomotor neuron marker Phox2b (Hirsch et al., 2007) (Fig. 2F,G). Therefore, we analyzed the numbers of BEN+ Phox2b+ and BEN+ Islet1+ SACMNs in Nkx2.9 null and WT littermates and found that appropriate numbers of properly specified SACMNs are generated in the absence of Nkx2.9 (Fig. 2H-M).

To determine whether Nkx2.9 functions cell-autonomously to facilitate SACMN axon exit we compared the distribution of lacZ-expressing cells and SACMNs within the spinal cord of a Nkx2.9-lacZ knock-in mouse line (Tian et al., 2006). In E9.5 embryos, anti-β-galactosidase staining overlapped with Nkx2.9 mRNA above the floor plate (FP), suggesting that Nkx2.9+ progenitors give rise to lacZ-positive cells (supplementary material Fig. S2A-C). At E10.5, X-Gal staining of sections from Nkx2.9-lacZ+/– embryos identified strong labeling above and adjacent to the FP and within cells (presumably SACMNs) that migrate dorsolaterally towards the LEP (supplementary material Fig. S2D). To investigate the possibility that Nkx2.9 is required to maintain the identity of these lacZ-positive cells, we labeled cryosections derived from E10.5 Nkx2.9-lacZ+/– and Nkx2.9-lacZ–/– embryos with anti-β-galactosidase and antibodies to the SACMN markers BEN, Islet1 or Phox2b. β-galactosidase-expressing neurons were labeled by each of these markers (supplementary material Fig. S2E-X), and a subset of β-galactosidase-positive cells within the ventricular zone, which were likely to be Nkx2.9+ progenitors, expressed Phox2b.

Fig. 4. SACMN axons fail to exit the spinal cord in Robo2-deficient mice. (A-T) Cryosections derived from E10.5 Robo2+/+ (A,B), Robo2–/– (F,G), Robo1+/+ (K,L) and Robo1–/– (P,Q) embryos were labeled with anti-laminin and anti-BEN (first column) or anti-NF alone (second column). E11.5 Robo2+/+ (C-E), Robo2–/– (H-J), Robo1+/+ (M-O) and Robo1–/– (R-T) embryos were colabeled with anti-BEN (third column) and anti-NF (fourth column). BEN/NF-expressing SACMN axons fail to exit the spinal cord and form an ectopic SAN (asterisk) within the CNS in Robo2–/– mice (H-J), whereas in Robo1–/– mice (R-T), just as in Robo2+/+ (C-E) and Robo1+/+ (M-O) embryos, a SAN (A) formed outside the CNS. As in E10.5 Robo2–/– embryos, the majority of BEN/NF-expressing SACMN axons fail to exit the spinal cord at E11.5. At E11.5, several small ectopic SANs (asterisks) form within the spinal cord (H-J), as compared with WT mice (C-E). A small number of SACMN axons emerge from the CNS and form an external SAN at E11.5 (H-J, arrowhead). The bar charts show that the average number of external SANs per section is reduced in E10.5 Robo2–/– mice (top; n=4), but unaltered in Robo1–/– mice (bottom; n=3), as compared with WT mice. Error bars indicate s.d. SAN formation is unperturbed in E11.5 Robo1–/– mice (R-T; n=2), as compared with WT littermates (M-O; n=2). Scale bar: 50 μm.
(supplementary material Fig. S2S,U,V,X, asterisk). These observations are consistent with SACMNs arising from a subset of Nkx2.9<sup>−/−</sup> progenitors and indicate that the specification/identity of SACMNs is unaltered in Nkx2.9<sup>−/−</sup> mice.

**Identifying putative downstream effectors of Nkx2.9**

To identify novel downstream effectors of Nkx2.9 that might mediate SACMN axon exit, we used in situ hybridization and immunohistochemistry to examine whether the following known cell surface molecules/axon guidance receptors are expressed by SACMNs: Robo1, Robo2 (Brose et al., 1999; Kidd et al., 1999; Yuan et al., 1999), neuropilin 1 (Npn1) (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), Npn2 (Chen et al., 1997), NCAM (Ncam1 – Mouse Genome Informatics) (Maness and Schachner, 2007), polysialylated-NCAM (PSA-NCAM) (Boisseau et al., 1991), EphB1-3 (Birgbauer et al., 2001; Holland et al., 1997; Imondi et al., 2000; Jevince et al., 2006; Palmer and Klein, 2003) and VEGFR2 (Kdr – Mouse Genome Informatics) (Ruiz de Almodovar et al., 2009) (data not shown). Robo2 mRNA expression most closely overlapped with the distribution of SACMNs, and Robo2 protein was present on SACMNs axons (see below).

We also carried out an unbiased microarray screen to identify genes dysregulated in Nkx2.9<sup>−/−</sup> embryos (supplementary material Table S1) by comparing total RNA isolated from the ventral half of the cervical spinal cord of E11.5 WT and Nkx2.9<sup>−/−</sup> embryos. This revealed that Robo2 mRNA levels were ~2-fold lower in Nkx2.9<sup>−/−</sup> mice than in WT mice (supplementary material Table S1).

Given these findings, we focused our subsequent studies on investigating the regulation of Robo2 expression in Nkx2.9<sup>−/−</sup> embryos and the role of Robo2 in SACMN axon exit.

**Robo2 is expressed by SACMNs and is downregulated in Nkx2.9<sup>−/−</sup> null mice**

To validate the results of our screens, we labeled cryosections derived from E10.5 Nkx2.9<sup>−/−</sup> embryos and their WT littermates with a Robo2 riboprobe or anti-Robo2. In WT embryos, BEN-expressing SACMNs express Robo2 mRNA (Fig. 3A,C, black arrowheads) and Robo2 protein is expressed on SACMNs axons (Fig. 3E, arrows) and on the SAN (Fig. 3E, white arrowheads). In Nkx2.9<sup>−/−</sup> embryos, SACMNs-associated Robo2 mRNA levels are reduced (Fig. 3B,D, black arrowheads) and Robo2 protein is downregulated on SACMNs axons (Fig. 3F, arrows) that fail to exit the spinal cord, as well as on the ectopic SAN (Fig. 3F, white arrowheads). These observations are consistent with Robo2 operating as a downstream effector of Nkx2.9.

**SACMN axons do not exit the spinal cord in Robo2-deficient mice**

To determine whether Robo2 is required for SACMN axon exit, we labeled E10.5 cryosections derived from Robo2<sup>−/−</sup> mice and their WT littermates with anti-BEN and anti-laminin or anti-neurofilament (NF) (Fig. 4A-J). Phenocopying Nkx2.9 null mice, the majority of SACMN axons appropriately projected to the LEP but failed to exit the CNS in E10.5 Robo2<sup>−/−</sup> animals (Fig. 4F,G, asterisks), and most SACMN axons remained confined to the spinal cord at E11.5 (Fig. 4H-J). Since a small subset of SACMN axons exited the spinal cord in Robo2<sup>−/−</sup> mice (Fig. 4H-J, arrowheads), we quantified the presence or absence of an external SAN (SACMN axon-containing nerve bundle) on both sides of the spinal cord in WT and Robo2<sup>−/−</sup> embryos. Consistent with the failure of most SACMN axons to exit the CNS, there was a significant reduction in the mean number of external SANs in Robo2<sup>−/−</sup> mice (Fig. 4, top bar chart). Although Robo1 mRNA is expressed by SACMNs and Robo1 and Robo3 protein is present on the SAN (data not shown), SACMN axon pathfinding/exit is not perturbed in Robo1<sup>−/−</sup> mice (Fig. 4K-T, bottom bar chart) and Robo3 (data not shown) null embryos. Accordingly, Robo2 is the sole Robo receptor required for SACMN axon exit from the spinal cord.

**Slits are expressed at the LEP and SACMN axon exit is perturbed in Slit null mice**

Since SACMN axons appropriately project away from the FP and towards the LEP but fail to exit the spinal cord in Robo2<sup>−/−</sup> mice, short-range interactions between Robo2 and its Slit ligands at the LEP might normally facilitate SACMN axon exit. To test this hypothesis, we labeled serial cryosections derived from E10.5 WT embryos with Slit1, Slit2 or Slit3 riboprobes (Fig. 5A-D). High levels of Slit2 and lower levels of Slit1 and Slit3 are present at the LEP, and each Slit gene is expressed in vMNs and the FP. Based on these observations, we analyzed SACMN axon pathfinding in mice lacking one or more Slits by labeling cryosections from E10.5 Slit<sub>1−/−</sub>, Slit<sub>2−/−</sub> or Slit<sub>1−/−</sub>Slit<sub>2−/−</sub> embryos with anti-BEN and anti-NF. Whereas SACMN axon pathfinding/exit was not perturbed in the absence of Slit1 (Fig. 6D–F) or Slit2 (Fig. 6G–I), SACMNs axons failed to exit the spinal cord in mice lacking both Slit1 and Slit2 (Fig. 6J–L). In the double-mutant mice, SACMNs axons formed more than one ectopic SAN within the spinal cord, just as they do in Robo2<sup>−/−</sup> mice (Fig. 6L–I, asterisks). The number of ectopic nerve bundles was significantly increased, and there was a corresponding reduction in the number of normally positioned external SANs, in Slit<sub>1−/−</sub>Slit<sub>2−/−</sub> embryos as compared with WT mice and mutant mice lacking other combinations of Slits (Fig. 6M,N). These findings identify Slit1 and Slit2 as the LEP-associated Robo2 ligands required for SACMN axon exit from the spinal cord.

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*Fig. 5. Distribution of Slit mRNA in the cervical spinal cord. (A–D) Serial cryosections derived from E10.5 WT embryos were labeled with Slit1 (A), Slit2 (B) or Slit3 (C) riboprobes. (D) Pseudo-color image representing the merge of A–C. High levels of Slit2 (B,D, arrowheads) and low levels of Slit1 and Slit3 are expressed at the LEP (A,C,D). High-magnification micrographs depicting LEP-associated expression are included as insets (A–C, arrowheads). Dotted lines demarcate spinal cord margin. Scale bar: 50 μm.*
for SACMN axon exit, and suggest that the increase in the number of ectopic nerves within Slit1−/− Slit2−/− mice might arise from defasciculation of longitudinally projecting SACMN axons.

Our expression and genetic data raise the possibility that short-range interactions between Robo2-expressing SACMN axons and LEP-associated Slits normally facilitate SACMN axon exit. Since SACMN cell bodies eventually settle near the LEP (Dillon et al., 2005), it is possible that they themselves might represent the source of Slit mRNA expression. We tested this hypothesis by colabeling cryosections derived from E9.5 Nkx2.9-lacZ−/− embryos with Slit1, Slit2 or Slit3 riboprobes and anti-β-galactosidase to visualize Nkx2.9+ progenitor-derived SACMNs (supplementary material Fig. S2). At E9.5, when the majority of SACMN axons have reached the LEP but have yet to exit the spinal cord, LEP-associated Nkx2.9-lacZ-expressing SACMNs do not express Slit mRNA (Fig. 7A-I, arrowheads). By contrast, SACMNs are likely to express Slit as they begin to migrate away from the FP (Fig. 7A-I). Together, our data suggest that short-range interactions between Slits expressed by LEP-associated (non-SACMN) cells and Robo2-expressing SACMN axons facilitate SACMN axon exit. To determine whether SACMN axons are attracted or repelled by Slits in vitro, we co-cultured SACMN-containing embryonic mouse spinal cord explants and aggregates of Slit2-expressing HEK293 cells. Anti-BEN-labeled SACMN axons exhibited more robust outgrowth towards Slit2-expressing HEK293 cells than towards mock-transfected HEK293 cells (Fig. 8). Therefore, short-range attractive Robo2-Slit interactions at the LEP are likely to promote SACMN axon exit (Fig. 10).
of these mice with anti-BEN (Fig. 9A-D). These analyses revealed that SACMNs axons form disorganized nerve fascicles along the A-P axis of the spinal cord in mice lacking either Robo2 or Slit1/2 (Fig. 9C,D, arrows), supporting a role for Robo2-Slit interactions in the proper growth of longitudinally projecting SACMNs axons.

**DISCUSSION**

The projection of motor axons out of the CNS is a key, but poorly understood, phase of MN development (Bonanomi and Pfaff, 2010; Hirsch et al., 2007; Lieberam et al., 2005; Schneider and Granato, 2003; Shirasaki and Pfaff, 2002; Vermeren et al., 2003). Here we describe the first identification of a genetic program that regulates spinal motor axon exit in vertebrates.

**Nkx2.9 controls SACMN axon exit**

We show that SACMNs axons appropriately project to the LEP but fail to exit the spinal cord in Nkx2.9–/– mice. This indicates that Nkx2.9 is not required for the pathfinding of SACMNs axons within the spinal cord and suggests that the FP-derived chemorepellent netrin 1 directs Unc5-expressing SACMNs axons away from the FP (Dillon et al., 2005; Dillon et al., 2007) and that LEP-associated chemoattractants (Caton et al., 2000; Guthrie and Lumsden, 1992), possibly secreted by boundary cap cells at motor exit points (Bron et al., 2007; Maro et al., 2004; Maui et al., 2007; Vermeren et al., 2003), are likely to guide SACMNs axons to the LEP. Since normal numbers of properly specified SACMNs are present in Nkx2.9–/– mice (Fig. 2 and supplementary material Fig. S2), and Nkx2.9–/– mice display WT-like ventral spinal cord patterning (Pabst et al., 2003), Nkx2.2 is likely to operate redundantly with Nkx2.9 to control the specification/differentiation of SACMNs (Dillon et al., 2005; Pabst et al., 2003). Although Nkx2.9–/– progenitors are capable of differentiating into interneurons (Briscoe et al., 1999; Holz et al., 2010; Pabst et al., 2003), our data indicate that a subset express SACMN markers, suggesting that Nkx2.9 regulates SACMN axon exit in a cell-autonomous manner. Given that Nkx6 is required for vMN axon exit from the Drosophila ventral nerve cord (Broshier et al., 2004), Nkx TFs might have evolutionarily conserved roles in motor axon exit.

**Nkx2.9 regulates Robo2 expression**

Nkx genes encode homeodomain-containing TFs that regulate cell type specification and organogenesis (Briscoe et al., 2000; Briscoe et al., 1999; Harvey, 1996; Stanfel et al., 2005), and Nkx2.9 is required for FP development and commissural axon guidance (Holz et al., 2010). We show that SACMN-associated Robo2 mRNA and protein are reduced in Nkx2.9–/– embryos, identifying Robo2 as the first candidate CNS-associated downstream effector of any vertebrate Nkx gene. By contrast, we observed no changes in Slit mRNA expression in these animals (data not shown). Although the presence of putative Nkx2 binding sites within the Robo2 promoter (data not shown) is consistent with Robo2 being a direct target of Nkx2.9, forced expression of Nkx2.9 does not modulate Robo2 expression (supplementary material Fig. S3).

**Robo2 and Slit1/2 are required for SACMN axon exit**

Our findings are the first to show that Robo2-Slit signaling is required to facilitate motor axon exit from the spinal cord. Since Slit2 appears to be the most abundant LEP-associated Slit, it was...
surprising to find that SACMN axons appropriately exit the spinal cord in mice lacking Slit2. Possible explanations for these observations are that low levels of Slit1 are capable of facilitating SACMN axon exit and/or that Slit1 and Slit2 operate redundantly to regulate exit. The finding that SACMN axons do not exit the spinal cord in Slit1–/– Slit2–/– mice further indicates that LEP-associated Slit3 does not promote SACMN axon exit on its own. Nevertheless, in the absence of published data indicating that Robo2 binds each of the vertebrate Slits with different affinities, it is difficult to reconcile why Slit1, but not Slit3, appears to compensate for the loss of Slit2. In contrast to our loss-of-function data, misexpression of Slits in the chick spinal cord does not perturb SACMN axon pathfinding (supplementary material Fig. S4).

Robo-Slit signaling regulates the guidance of longitudinally projecting axons in the developing mouse brain stem (Dugan et al., 2011; Farmer et al., 2008; Mastick et al., 2010), forebrain (Devine and Key, 2008; Ricano-Cornejo et al., 2011) and spinal cord (Reeber et al., 2008). Consistent with these observations, we show that Robo2 and both Slit1 and Slit2 are required for the proper assembly of SACMN axons into the SAN. Whereas SACCMN axons form an organized ectopic nerve bundle in Nkx2.9−/− embryos, disorganized/tangled nerve fascicles are present within the spinal cord of Robo2−/− and Slit1−/− Slit2−/− mice. Ventral spinal cord (including vMN)-associated Slits (Brose et al., 1999; Kadison et al., 2006; Yuan et al., 1999) might play a key role in constraining the orientation of longitudinally projecting SACMN axons as Slit1 and Slit2 position retinal ganglion cell axons at the optic chiasm (Plump et al., 2002).

The dynamic role of Robo-Slit interactions in SACMN axon pathfinding

Given that the Slit-rich FP and ventral spinal cord would be expected to represent repulsive territories for Robo2-expressing SACMN axons, how are these axons capable of growing away from the FP and through the ventral spinal cord (Fig. 10A)? As is the case for vMNs (Bai et al., 2011; Brose et al., 1999), SACMNs are likely to co-express Robo2 and Slits as their axons navigate these initial segments of their trajectory (Figs 7, 10). Thus, Slits produced by SACMN cell bodies might occupy SACMN axon-associated Robo receptors, rendering them insensitive to FP- and vMN-derived Slits (Brose et al., 1999). SACMNs express Robo2 and Slits as they pathfind away from the FP and traverse vMNs, and through potential cis attenuation of Robo2-Slit signaling their axons become insensitive to FP- and vMN-derived Slits. SACCMN axons are normally repelled by the FP-derived chemorepellent Netrin (–) and could be guided to the LEP by exit point-derived chemoattractants (+). Once Robo2-positive SACMN axons reach the LEP, they no longer express Slits, become responsive to LEP-associated Slits (3), and attractive Robo2-Slit interactions facilitate their exit from the spinal cord. By contrast, SACMN axons in Nkx2.9−/− (dark gray, Robo2−/−, A right), Robo2−/− (light gray, Robo2, B left) and Slit1−/− Slit2−/− (green, Robo2, B right) mice appropriately reach the LEP but fail to exit the spinal cord. (C) Model for Nkx2.9 control of SACMN axon exit via Robo-Slit signaling. (i) Nkx2.9 regulates Robo2 expression in SACMNs. (ii) Attractive short-range Robo2-Slit interactions at the LEP facilitate SACMN axon exit from the CNS.
axons to Netrin (Bai et al., 2011). Similarly, co-expression of Sema3A and Npn1 on vMNes prevents their axons from responding to exogenous Sema3A (Moret et al., 2007), and the attenuation of Eph receptor signaling via ephrins in cis alters the responsiveness of vMN axons to trans ephrins (Kao and Kania, 2011).

Our observations suggest that short-range, presumably attractive/positive (Fig. 8) interactions between Robo2-expressing SACMN axons and LEP-associated Slits promote SACMN axon exit from the spinal cord. Consistent with this possibility, mesodermal cells (Kramer et al., 2001) and tracheal branches (Englund et al., 2002) are attracted to Slit in Drosophila embryos, and human leukocytes are attracted to a Slit source in vitro (Ye et al., 2010). Drosophila mesodermal cells are also capable of responding to Slit as both an attractant and repellent during sequential phases of their migration (Kramer et al., 2001). By analogy, SACMN axons might alter their responsiveness to LEP-associated Slits, first perceiving Slits as attractants and then as repellents, and this would ultimately push SACMN axons out of the spinal cord. We favor the possibility that Slits produced by LEP-associated cells facilitate SACMN axon exit; however, Robo2-expressing SACMN axons could transport ventral midline-associated Slits to the LEP, just as the Frazzled receptor captures and redistributes its Netrin ligand to modulate axon pathfinding within the Drosophila CNS (Hiramoto et al., 2000). Selectively eliminating Slits from LEP-associated cells would clarify which sources of Slits are required for SACMN axon exit.

To ultimately emerge from the spinal cord, motor axons must break through the basement membrane surrounding the neural tube. Although our studies do not directly address this aspect of SACMN axon pathfinding, it seems plausible that Robo2-Slit interactions promote cytoskeletal remodeling, which might alter the shape of SACMN growth cones and facilitate exit. Consistent with this possibility, the complexity of SACMN axon-associated growth cones is dramatically reduced as they exit the LEP (Snider and Palavalli, 1990), and this might facilitate their passage through small ‘gaps’ formed by glial end-feet at motor exit points (Fraher et al., 2007).

Alternatively, Slit activation of Robo2 at the LEP might trigger the release of either soluble or membrane-bound proteolytic enzymes such as matrix metalloproteinases (MMPs) (McFarlane, 2003), which are capable of breaking down the basal lamina and potentially promoting SACMN axon exit. Our microarray results indicate that the expression of the ADAM metalloproteinase 3 is reduced in Nkx2.9–/- mice, raising the possibility that MMPs might regulate SACMN axon exit (supplementary material Table S1). In one scenario, SACMN axons may extend small, localized protrusions termed invadopodia that selectively secrete MMPs, which degrade the basement membrane (Bravo-Ambrosio and Kaprielian, 2011). No matter how SACMN axons break through the border between the CNS and the peripheral nervous system, we have provided evidence that short-range Robo2-Slit interactions are likely to regulate this crucial phase of motor axon development.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.072256/-/DC1

References


Table S1. Genes that are differentially expressed in Nkx2.9-deficient mouse embryos

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Genes exhibiting a fold change greater than 1.5 in Nkx2.9 null mice as compared with WT littermates.