

# Neuropilin receptors guide distinct phases of sensory and motor neuronal segmentation

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The segmented trunk peripheral nervous system is generated by ventrally migrating neural crest cells that exclusively invade the anterior sclerotome and differentiate into metameric dorsal root and sympathetic ganglia. Meanwhile, ventral spinal motor axons also project through the somites in a segmental fashion. How peripheral nervous system segmentation is generated is unknown. We previously showed that neuropilin 2 (Nrp2)/semaphorin 3F (Sema3F) signaling is required for segmental neural crest migration, but not for metameric dorsal root gangliogenesis. We now expand these results to show that Nrp2 patterns initial motor axon outgrowth as well. Later, Nrp1/Sema3A signaling is essential for segmental dorsal root gangliogenesis and motor axonal fasciculation into ventral roots. Strikingly, Nrp/Sema signaling is not required for sympathetic ganglia segmentation. These data show that Nrp2 and Nrp1 work together to produce segmentation of sensory and motor nerves, and that dorsal peripheral nervous system metamerism is generated in a stepwise, Nrp-dependent process.

**KEY WORDS:** Dorsal root ganglia, Guidance, Motor axon, Neural crest, Neuropilins, Peripheral nervous system

## INTRODUCTION

The vertebrate neural crest is a migratory population of multipotent cells that give rise to many derivatives, including neurons and glia of the peripheral nervous system (PNS). In the trunk, neural crest-derived neurons are arranged in metameric, segmentally iterated groups: the dorsal root ganglia (DRGs) lateral to the spinal cord, and the sympathetic ganglia (SGs) at the level of the dorsal aorta. The first indication of this segmentation is the patterned ventral migration of neural crest cells through the anterior portion of each somitic sclerotome (Bronner-Fraser, 1986; Rickmann et al., 1985; Serbedzija et al., 1990). This is soon followed by segmental motor axon outgrowth from the ventral spinal cord, also through the anterior somite (Keynes and Stern, 1984). Segmentally migrating neural crest cells subsequently condense to form the metameric peripheral ganglia, and sensory axons extending from the DRGs fasciculate with motor axons to form the equally segmental mixed spinal nerves. The end result is metameric sensorimotor complexes within anterior somites that are ultimately in register with the somite-derived vertebrae.

Our work (Gammill et al., 2006) has challenged the classical assumption that segmental neural crest migration creates the metameric organization of the PNS (Bronner-Fraser, 1986; Rickmann et al., 1985; Teillet et al., 1987). We previously showed that neuropilin 2/semaphorin 3F (Nrp2/Sema3F) signaling is required to guide segmental neural crest migration through the sclerotome, but is not essential for metameric DRG formation. In addition to establishing the molecular basis for trunk neural crest guidance, this surprising result indicated that segmental neural crest migration and metameric DRG formation are separately regulated steps in PNS development.

The cue(s) that segments DRGs is unclear. Disruption of somite polarity indicates that signals from the somite restrict neural crest-derived ganglia to the anterior sclerotome (Bronner-Fraser and Stern, 1991; De Bellard et al., 2002; Hrabe de Angelis et al., 1997;

Kalcheim and Teillet, 1989; Stern and Keynes, 1987). Although previous work implicated Eph/ephrin and neuropilin 1/semaphorin 3A (Nrp1/Sema3A) in somite to neural crest signaling (Eickholt et al., 1999; Krull et al., 1997; Wang et al., 1998), mouse knockouts of these molecules failed to exhibit segmentation defects in trunk neural crest migration or subsequent DRG formation (Adams et al., 2001; Davy et al., 2004; Hrabe de Angelis et al., 1997; Kawasaki et al., 2002; Orioli et al., 1996; Wang et al., 1998) unless accompanied by somite patterning defects (Davy and Soriano, 2007). One possibility is that Eph/ephrin and/or Nrp1/Sema3A signaling is required to direct segmental DRG formation downstream of Nrp2. We reasoned that this requirement would not be apparent in mice mutant for these pathways because migrating neural crest cells are already segmentally arranged by Nrp2/Sema3F signaling. However, we predicted that a requirement for DRG patterning would be revealed if neural crest cells were migrating non-segmentally.

The signals that pattern motor axon outgrowth are also incompletely understood. Motor axons only extend from the spinal cord adjacent to anterior sclerotome, and this segmentation is imposed by the somites (Keynes and Stern, 1984; Tosney, 1988). Nrp1 in conjunction with Nrp2 guides motor axon pathfinding in the limbs and intercostal bundles (Huber et al., 2005), but the role of neuropilin signaling in patterning initial growth and restricting motor axons to the anterior somite has not been assessed. Although Eph-ephrin expression and activity are consistent with a role in motor axon outgrowth (Wang and Anderson, 1997), Eph-ephrin signaling is not required for this process, leading to the suggestion that neural crest and motor axon segmentation are regulated by different signals (Koblar et al., 2000; Vermeren et al., 2000).

Here, we demonstrate that Nrp1/Sema3A signaling acts downstream of Nrp2/Sema3F to segment DRG formation and motor axon outgrowth. We focused on Nrp1/Sema3A because Nrp1 and Nrp2 signaling often cooperate; for example, in peripheral motor axon guidance (Huber et al., 2005) and in cranial neural crest migration (Schwarz et al., 2008). As previously described, we find that migrating neural crest cells and forming DRGs express Nrp1 (Kawasaki et al., 2002), while Sema3A is expressed in the dermomyotome and, transiently, in the posterior sclerotome as DRGs condense (Adams et al., 1996; Giger et al., 1996; Wright et al., 1995). Nrp1/Sema3A signaling is required to sequester

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migratory neural crest cells to the sclerotome, but is not essential for segmental neural crest migration (Kawasaki et al., 2002). In single mutants, *Nrp1*/*Sema3A* signaling is dispensable for DRG formation; however, in the absence of segmental neural crest migration in an *Nrp2* mutant background, *Nrp1*/*Sema3A* signaling is required for metameric DRG formation. The absence of a neuropilin gene-dosage effect and the expression patterns of *Sema3f* and *Sema3a* suggest that these receptors act sequentially during PNS segmentation. Additionally, segmental motor axon outgrowth proximal to the spinal cord requires first *Nrp2* and later *Nrp1*. These data suggest that sequential neuropilin signaling is required for segmental patterning of both sensory and motor components of the PNS.

## MATERIALS AND METHODS

### Embryos

Mouse embryos were surgically isolated into phosphate buffered saline (PBS) on ice and fixed in 4% paraformaldehyde for 2 hours at room temperature or overnight at 4°C. *Nrp2* and *Nrp1*<sup>Sema</sup> mutant mouse lines were genotyped as previously described (Giger et al., 2000; Gu et al., 2003).

### In situ hybridization

In situ hybridization and histology were performed as previously described (Gammill et al., 2006). Antisense probe templates were: *Nrp1* (Stalmans et al., 2002), *Sema3a* (Puschel et al., 1995), *Sema3f* (Giger et al., 2000), *Sox10* (Kuhlbrodt et al., 1998) and *Uncx4.1* (Mansouri et al., 2000).

### Immunohistochemistry

Whole embryos were incubated in Dent's fixative (80% methanol, 20% dimethylsulfoxide) plus 6% hydrogen peroxide, followed by anti-neurofilament (1:100; Developmental Studies Hybridoma Bank) and anti-mouse-Biotin (1:100; Jackson ImmunoResearch) antibodies. Staining was developed using ABC-horseradish peroxidase (Vector Laboratories) and 0.1 mg/ml 3,3'-diaminobenzidine (DAB; Sigma) with 0.01% hydrogen peroxide.

Unstained embryos were infiltrated with 5% sucrose, 15% sucrose and 7.5% gelatin in 15% sucrose, frozen in liquid nitrogen and sectioned at 20 µm. Sections were degelatinized for 20 minutes in PBS at 37°C and then stained. Neural crest cells were stained with 1:500 anti-p75 (Weskamp and Reichardt, 1991) followed by 1:250 anti-rabbit-AlexaFluor 568 (Invitrogen). Anti-neuropilin1 (R&D Systems) was used at 1:100, followed by 1:250 anti-goat-Cy2 (Jackson ImmunoResearch). DRGs and motor axons were stained with 1:500 anti-TuJ1 (neuron specific class III β-tubulin; Babco) followed by 1:250 anti-mouse-AlexaFluor 568 (Invitrogen).

### Quantification of phenotype

The extent of staining and the anteroposterior length of each segment were measured in micrographs (Image J). At least three segments were independently measured from each embryo. Calculating the ratio of the segment occupied by staining allowed us to compensate for differences in embryo size or warping (stretching or compaction) during sectioning. The average proportion was calculated for each embryo and each genotype. *P*-values were determined using Student's *t*-tests (Excel).

## RESULTS

### Migrating neural crest cells express *Nrp1* whereas somites express *Sema3a*

To determine when and where *Nrp1* and *Sema3A* act during PNS development, we visualized *Nrp1* and *Sema3a* expression in E9.5 to E11.5 mouse embryos. Although elements of these expression patterns have been published (Adams et al., 1996; Kawasaki et al., 2002), we wanted to compare expression levels and localization directly over a defined time period. Trunk neural crest cells migrate segmentally through the somites at E9.5, begin condensing into DRGs at E10.5, and form discrete ganglia by E11.5. Migrating neural crest cells, identified by p75 expression (Rao and Anderson,

1997), exhibited *Nrp1* immunofluorescence at E9.5 (Fig. 1A). *Nrp1* expression was maintained in sensory precursors throughout DRG formation (Fig. 1B,C). *Nrp1* was also expressed by intersomitic vessels (Fig. 1A) but, unlike *Nrp2* (Gammill et al., 2006), was not expressed in the somite (Fig. 1A, all sclerotomal *Nrp1* immunofluorescence overlaps with p75 expression). Although *Sema3a* was expressed in the dermomyotome throughout trunk neural crest migration and DRG formation (Fig. 1D-F), it was not expressed in the sclerotome at E9.0 (Fig. 1D) or E9.5 (data not shown). *Sema3a* was transcribed in the posterior sclerotome beginning at E10.0, just prior to the stage when uniform *Nrp2*<sup>-/-</sup> neural crest migration first exhibits segmentation (Gammill et al., 2006), and corresponding to the stage at which DRG condensation and motor axon outgrowth begins (Fig. 1E) (Marmigere and Ernfor, 2007). *Sema3a* transcript was maintained in the sclerotome through E11.5, when DRG formation was essentially complete (Fig. 1F). In contrast to *Sema3a*, *Sema3f* expression in the posterior sclerotome is at its highest at E9.5 (Gammill et al., 2006), with decreased levels being observed at E10.5 (Fig. 1G) (Giger et al., 2000), and no expression at E11.5 (Fig. 1H), indicating that *Nrp2*/*Sema3F* signaling cannot pattern DRG formation at these stages. The complementary expression patterns of *Nrp1* and *Sema3A*, along with the timing of *Sema3a* expression in the posterior sclerotome, made *Nrp1*/*Sema3A* signaling an ideal candidate for regulating metameric DRG formation.

### *Nrp2* but not *Nrp1* is essential for segmental neural crest migration

As a starting point for our analysis, we wanted to characterize neural crest migration in the absence of *Nrp1*/*Sema3A* signaling, with or without *Nrp2* function. Because *Nrp1*/*Nrp2* double mutants die from vascular defects at E8.5, prior to trunk neural crest migration and DRG formation (Takashima et al., 2002), we took advantage of a line of *Nrp1* mutant mice that are deficient in semaphorin signaling but are still able to transmit vascular endothelial growth factor (VEGF) signaling (*Nrp1*<sup>Sema</sup>-null, referred to here as *Nrp1*<sup>Sema-/-</sup>) (Gu et al., 2003). *Nrp1*<sup>Sema-/-</sup>;*Nrp2*<sup>-/-</sup> double mutant embryos survive until late gestation (e.g. Huber et al., 2005). This allele also ensures that any phenotype is specifically due to the loss of *Nrp1*/semaphorin signaling and not disrupted VEGF signaling. Because *Sema3c* is not expressed in the sclerotome (Adams et al., 1996), we could specifically assess *Nrp1*/semaphorin signaling.

At E9.5, in situ hybridization for the neural crest marker *Sox10* (Kuhlbrodt et al., 1998) revealed anteriorly restricted segmental neural crest migration through each wild-type sclerotome (Fig. 2A,E; *n*=10, *n*=9). *Nrp2*/*Sema3F* signaling is required for this restriction, and loss of *Nrp2* resulted in neural crest migration throughout the sclerotome (Fig. 2B,F; *n*=8) (Gammill et al., 2006). The ratio of sclerotome covered by neural crest cells in *Nrp2* mutant embryos was significantly different from that of controls (Fig. 2J; *P*=4.29×10<sup>-10</sup>). By contrast, *Nrp1*<sup>Sema-/-</sup> neural crest cells were appropriately restricted within the sclerotome (Kawasaki et al., 2002), but also migrated ectopically along somite borders (Fig. 2C,G; *n*=4). Interestingly, *Nrp1*<sup>Sema-/-</sup> neural crest cells migrated through a smaller proportion of the sclerotome than did wild-type neural crest cells (Fig. 2J; *P*=0.016). This increased spacing might reflect lower neural crest cell numbers within the sclerotome owing to ectopic migration along somite borders. *Nrp1*<sup>Sema-/-</sup>;*Nrp2*<sup>-/-</sup> double mutant embryos exhibited a combination of these phenotypes, with neural crest cells migrating non-segmentally through the sclerotome and along somite borders (Fig. 2D,H; *n*=5) in a manner significantly different from control embryos (Fig. 2J;



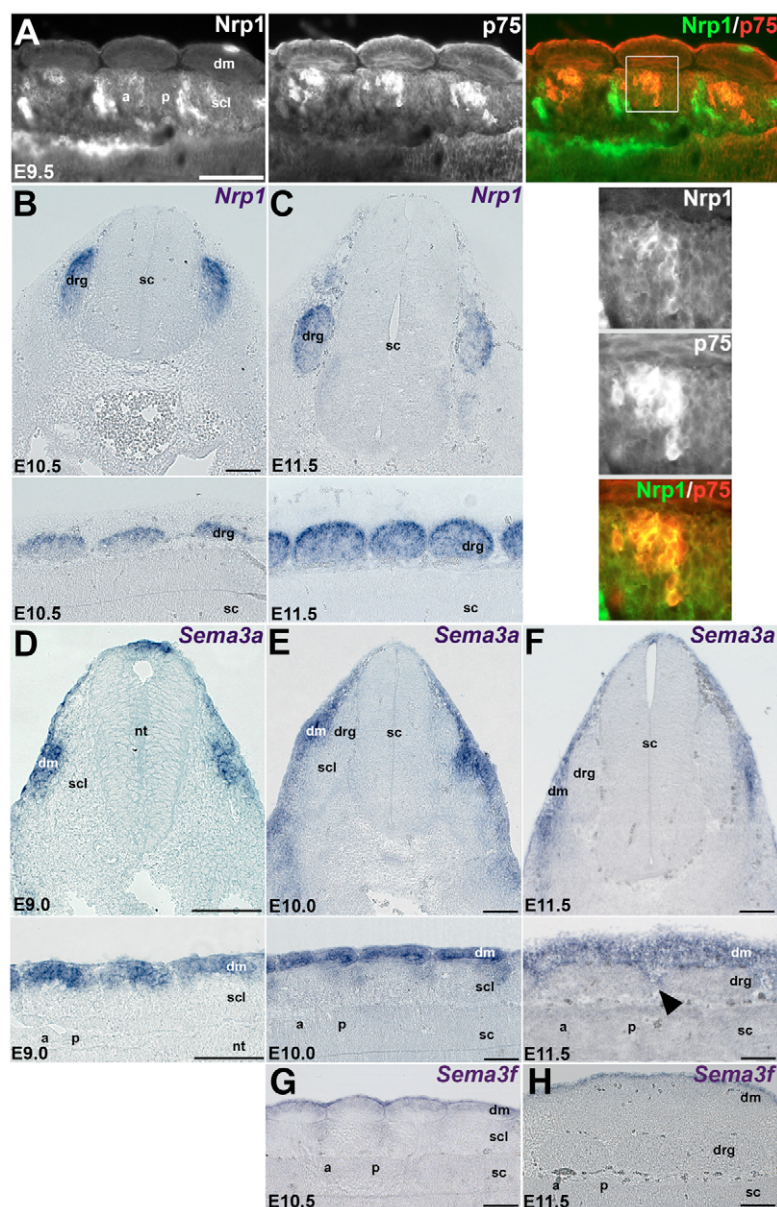
$P=5.10 \times 10^{-10}$ ). These data suggest that *Nrp2* is required to pattern segmental neural crest migration, while *Nrp1/Sema3A* signaling restricts neural crest migration to the sclerotome. Additionally, the strictly additive double mutant phenotype indicates that *Nrp1* and *Nrp2* have defined roles, and do not act redundantly to guide neural crest migration.

### ***Nrp1* is required for segmental DRG formation**

We next wondered whether *Nrp1/Sema3A* signaling was required during DRG formation. Neural crest cells have begun to coalesce into DRGs by E10.5 (Marmigere and Ernfors, 2007). Whole-mount neurofilament immunostaining of double heterozygous control embryos revealed discrete, metameric ganglia (Fig. 3A; drg,  $n=3$ ) and segmentally arranged dorsal roots (DRs; Fig. 3A), which are sensory projections back to the spinal cord. Sections immunostained with neural specific  $\beta$ -tubulin (TuJ1) clearly showed segmental, compact collections of TuJ1-expressing neurons in these embryos (Fig. 3E;  $n=3$ ). *Nrp1<sup>Sema</sup>* single mutant embryos also exhibited segmental DRGs and DRs in both whole-mount and section views

(Fig. 3B,F;  $n=3$ ). Interestingly, *Nrp1<sup>Sema</sup>* DRGs exhibited a slight but statistically significant increase in spacing compared with control embryos (Fig. 3K,L;  $P=0.024$ ). Despite non-segmental migration at E9.5 (Fig. 2B,F), *Nrp2* mutant neural crest cells formed discrete DRGs and DRs at E10.5 (Fig. 3C,  $n=3$ ). In sections, *Nrp2<sup>-/-</sup>* ganglia were more elongated and therefore more closely spaced than controls, as has been previously described (Fig. 3L;  $P=1.05 \times 10^{-4}$ ) (Gammill et al., 2006), but were nevertheless segmentally arranged (Fig. 3G;  $n=3$ ). Embryos mutant for either neuropilin and heterozygous for the other exhibited identical phenotypes to mutant/wild type combinations, demonstrating that neuropilins do not exhibit gene-dosage effects in trunk neural crest at this stage (see Fig. S1A,B in the supplementary material). SGs formed in their normal, metameric pattern in all genotypes (Fig. 3A-C).

In stark contrast to the single mutants, the DRGs of E10.5 *Nrp1<sup>Sema</sup>*;*Nrp2<sup>-/-</sup>* embryos were not discrete structures (Fig. 3D;  $n=3$ ). DR projections were also completely non-segmental (Fig. 3D). Sections revealed collections of TuJ1-positive cells without tight associations between them that were present continuously



**Fig. 1. Neural crest *Nrp1* expression is complementary to *Sema3a* in the somite.** (A) *Nrp1* (green) immunoreactivity localizes to intersegmental blood vessels (v) and migrating neural crest cells immunostained with p75 (red) in longitudinal sections at E9.5. Anterior is to the left. (B,C) DRGs express *Nrp1* by in situ hybridization at E10.5 (B) and E11.5 (C). (D-F) Dermomyotome expresses *Sema3a* at E9.0 (D), E10.0 (E) and E11.5 (F). Posterior sclerotome expresses *Sema3a* beginning at E10.0 (E) and persisting at least through E11.5 (F, arrowhead). (B-F) Top, transverse sections, dorsal up; bottom, longitudinal sections, anterior to the left. (G) *Sema3f* is expressed in the dermomyotome and at low levels in the posterior sclerotome at E10.5. (H) *Sema3f* is not expressed in posterior sclerotome at E11.5. (G,H) Transverse sections, anterior to the left. a, anterior; dm, dermomyotome; drg, dorsal root ganglion; nt, neural tube; p, posterior; sc, spinal cord; scl, sclerotome. Scale bars: 100  $\mu$ m.

along the axis (Fig. 3H,L;  $n=4$ ;  $P=2.13 \times 10^{-14}$ ). There were also fewer TuJ1-positive cells, which suggested that neurogenesis was impaired. Normally during DRG development, neural crest cells in the interior of the ganglion differentiate and downregulate *Sox10*, whereas cells at the exterior continue to proliferate and maintain *Sox10* expression (Fig. 3I;  $n=4$ ) (Marmigere and Ernfrors, 2007; Nelson et al., 2002; Wakamatsu et al., 2000). In E10.5 *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos, *Sox10* expression was maintained in all cells of the fused ganglion (Fig. 3J;  $n=3$ ), not just in perimeter cells as in wild types (Fig. 3I). *Sox10* in situ hybridization also revealed completely non-segmental collections of *Sox10*-positive neural crest cells attempting to form DRGs (Fig. 3J), which were strikingly different from the individualized ganglia in wild-type

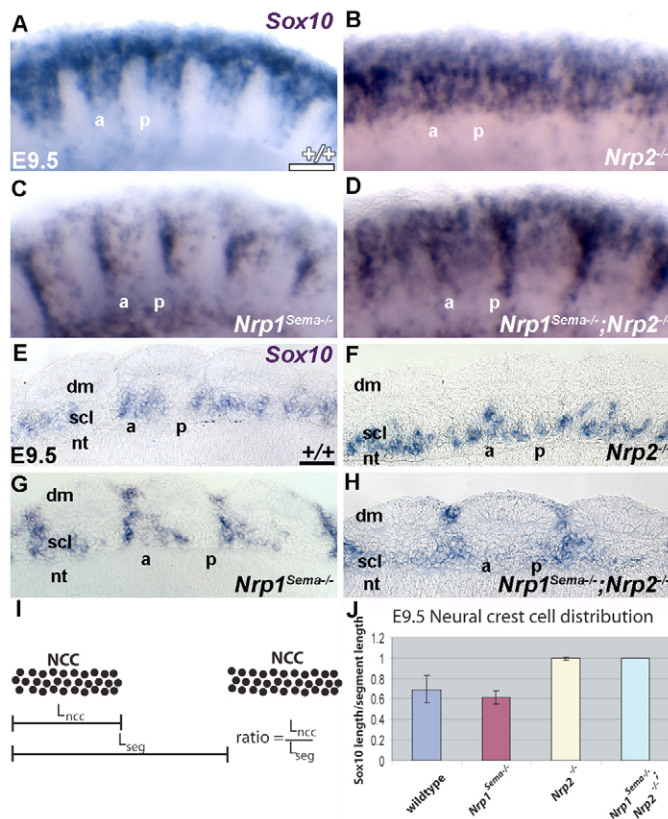
embryos (Fig. 3I). Surprisingly, SGs formed normally in the absence of all Nrp/Sema signaling (Fig. 3D;  $n=5$ ). Thus, although *Nrp1/Sema3A* mutant neural crest cells do form segmental DRGs, this is probably because they are already segmentally arranged by *Nrp2/Sema3F* signaling. In the absence of segmental neural crest migration in an *Nrp2* mutant background, *Nrp1/Sema3A* signaling is required for DRG segmentation. Furthermore, sensory neurogenesis is delayed in *Nrp1/Nrp2* double mutants.

Because neural crest cells migrate and differentiate within the anterior sclerotome, loss of somite polarity indirectly produces unsegmented DRGs (Bronner-Fraser and Stern, 1991; De Bellard et al., 2002; Kalcheim and Teillet, 1989). Because the anterior sclerotome expresses *Nrp2* (Gammill et al., 2006), it is possible that neuropilin double mutant embryos exhibit DRG fusions because of somite patterning defects. We visualized anteroposterior somite polarity in *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos by in situ hybridization for *Uncx4.1*, a transcription factor that marks and maintains posterior somite identity (Leitges et al., 2000; Mansouri et al., 2000). *Uncx4.1* was posteriorly restricted in both wild-type and *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos at E9.5 and E10.5 (see Fig. S2 in the supplementary material; E9.5,  $n=2$ ; E10.5,  $n=1$ ), as in *Nrp2<sup>-/-</sup>* embryos (Gammill et al., 2006). Thus, anteroposterior somite identity is intact in neuropilin double mutant embryos, and neural crest migration defects and DRG fusions are not due to abnormal somite patterning.

### DRG fusions persist in *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos

Because neuropilin double mutant DRGs were initially non-segmental, we wondered whether the final ganglia would also be fused. Lateral views of neurofilament-stained control embryos showed that E11.5 DRGs contained more cells and were more densely packed than those in E10.5 embryos [compare Fig. 4A,E ( $n=6$ ) with Fig. 3A,E]. Additionally, DRs entered the spinal cord and projected directly to the dorsal funiculus in a segmental pattern (Fig. 4A). E11.5 *Nrp1<sup>Sema-/-</sup>* embryos were nearly unaffected (Fig. 4B,F;  $n=3$ ; Fig. 4J). *Nrp1<sup>Sema-/-</sup>* DRs had a more splayed appearance (Fig. 4B), and DRGs were more widely spaced than in controls, although this effect was less pronounced at E11.5 than it was at earlier stages of development (Fig. 4J;  $P=0.0498$ ). Previous studies described *Nrp1<sup>-/-</sup>* DRGs as being less closely packed (Kitsukawa et al., 1997), although this difference was not apparent in *Nrp1<sup>Sema-/-</sup>* embryos. E11.5 *Nrp2<sup>-/-</sup>* DRGs were more closely spaced than those in controls, but were segmental and individualized as previously reported (Gammill et al., 2006) (Fig. 4C,G;  $n=4$ ; Fig. 4J;  $P=1.56 \times 10^{-5}$ ). Occasionally, adjacent ganglia were fused in *Nrp2<sup>-/-</sup>* embryos, although these fusions involved no more than two ganglia and were not observed along the entire dorsoventral length of the ganglia. Loss of one *Nrp2* allele did not exacerbate the phenotype of *Nrp1<sup>Sema-/-</sup>* embryos (compare Fig. 4F with Fig. S1C in the supplementary material). Likewise, loss of one *Nrp1<sup>Sema</sup>* allele did not affect E11.5 *Nrp2<sup>-/-</sup>* DRGs (compare Fig. 4G with Fig. S1D in the supplementary material), although we did observe fusion between multiple DRGs in one embryo, indicating that embryos were at a critical threshold of neuropilin signaling.

By contrast, double mutant *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* DRGs were difficult to visualize in whole-mount views (Fig. 4D;  $n=5$ ). Longitudinal sections revealed that E11.5 *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos contained a single DRG fused along the entire length of the trunk (Fig. 4H,  $n=5$ ; Fig. 4J;  $P=1.78 \times 10^{-13}$ ). Thus, neuropilin signaling is required for DRG segmentation, and loss of segmentation does not recover over the course of DRG morphogenesis. Interestingly, despite their unsegmented appearance



**Fig. 2. *Nrp2* but not *Nrp1* patterns segmental neural crest migration within the sclerotome.** (A–D) Lateral views of *Sox10* in situ hybridization on E9.5 embryos. (E–H) Longitudinal sections of *Sox10* in situ hybridization on E9.5 embryos. Neural crest cells migrate through the anterior sclerotome in wild-type embryos (A,E), whereas neural crest cells migrate non-segmentally in *Nrp2<sup>-/-</sup>* embryos (B,F). Neural crest cells migrate through the anterior sclerotome and ectopically along somite borders in *Nrp1<sup>Sema-/-</sup>* embryos (C,G). *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* neural crest cells migrate nonsegmentally within the sclerotome and along somite borders (D,H). (I) Calculating the segment proportion occupied by neural crest cells. *Sox10* staining length ( $L_{ncc}$ ) and segment length ( $L_{seg}$ ) were measured in ImageJ. Staining length was divided by segment length. The average was calculated for each genotype and statistical significance determined using Student's *t*-test. (J) Neural crest cells in *Nrp2<sup>-/-</sup>* and *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* E9.5 embryos migrate non-segmentally compared with wild-type control embryos (*Nrp2<sup>-/-</sup>*,  $P=4.30 \times 10^{-10}$ ; *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>*,  $P=5.10 \times 10^{-10}$ ). Data are reported as mean  $\pm$  s.d. a, anterior; dm, dermomyotome; nt, neural tube; p, posterior; scl, sclerotome. Anterior is to the left. Scale bars: in A, 100  $\mu$ m for A–D; in E, 50  $\mu$ m for E–H.



at E10.5 (Fig. 3D), E11.5 *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* DR projections avoided segment boundaries in double mutant embryos (Fig. 4D, arrowheads), possibly because of the further development of physical impediments, such as intersomitic blood vessels.

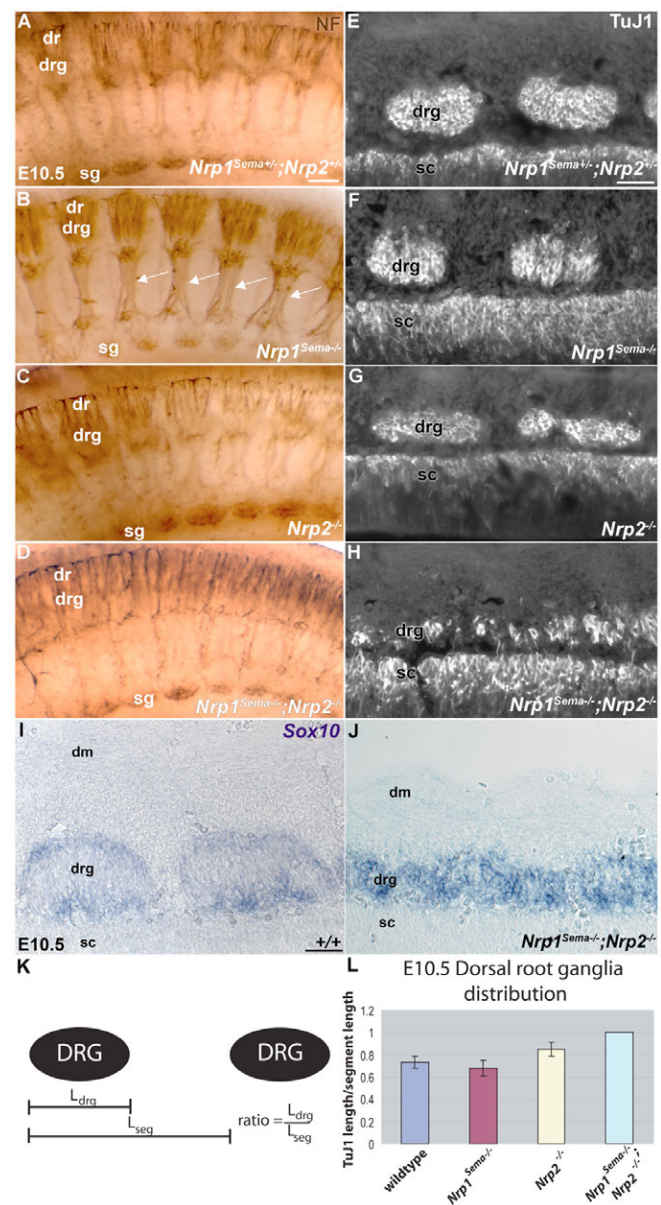
### Neuropilins pattern segmental motor axons and spinal nerves

In characterizing E10.5 neuropilin mutants, we noticed that *Nrp1<sup>Sema-/-</sup>* and *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* ventral roots were defasciculated (Fig. 3B,D, arrows) (Kitsukawa et al., 1997). Motor axons, like neural crest cells and DRGs, are segmentally arranged in the somites (Keynes and Stern, 1984). Because motor axon outgrowth through the somites takes place around E10, just prior to segmental DRG formation, we wondered whether neuropilin signaling was also required for the segmentation of non-neural crest-derived components of the PNS. *Nrp1* and *Nrp2* pattern and fasciculate distal spinal nerves into segmental intercostal bundles (Huber et al., 2005).

To this end, we examined neuropilin single and double mutant E10.5 motor axons and E11.5 mixed spinal nerves in TuJ1-immunostained sections. In wild-type embryos, motor axons emerged from the spinal cord in a segmental pattern just ventral to the DRGs, projecting through only the anterior sclerotome (Fig. 5A,  $n=9$ ; Fig. 6B). Within each segment, motor axons fasciculated into defined ventral roots that were visible in more ventral sections (Fig. 5A). These bundles then combined with sensory projections from the DRG to form the mixed spinal nerve. Spinal nerves in E11.5 wild-type embryos formed a single bundle that was also restricted to the anterior sclerotome (Fig. 5E,  $n=6$ ). The distribution of these projections and the number and arrangement of fascicles was quantified in Fig. 6.

*Nrp1<sup>Sema</sup>* mutant motor axonal projections emerged exclusively adjacent to the anterior sclerotome (Fig. 5C,  $n=3$ ) as they did in wild types (Fig. 5A), although these initial projections were more tightly packed than those of wild-type embryos (Fig. 6B;  $P=2.80 \times 10^{-4}$ ). As predicted from the whole-mount view (Fig. 3B), these projections failed to form a single ventral root fascicle, and instead clustered into multiple bundles, which were still restricted to the anterior sclerotome (Fig. 5C; Fig. 6C,D;  $P=2.79 \times 10^{-6}$ ). One day later, the mixed spinal nerves in *Nrp1<sup>Sema-/-</sup>* E11.5 embryos formed one elongated bundle or several bundles, which were also anteriorly restricted (Fig. 5G,  $n=3$ ). This was similar to the defasciculation observed at later stages in *Nrp1<sup>Sema-/-</sup>* intercostal nerves (Huber et al., 2005). Meanwhile, *Nrp2<sup>-/-</sup>* motor axons projected uniformly from the spinal cord and entered the sclerotome all along its length (Fig. 5B,  $n=12$ ; Fig. 6B;  $P=2.01 \times 10^{-4}$ ). The severity of this phenotype was variable, ranging from the mild effect shown in Fig. 5, to a more extreme loss of segmentation distal to the spinal cord (data not shown). Motor axons projected uniformly into the sclerotome from the time of their initial appearance at E10 (data not shown). Despite non-segmental outgrowth proximal to the spinal cord, *Nrp2<sup>-/-</sup>* motor axons still fasciculated into fairly cohesive ventral roots exclusively in the anterior sclerotome (Fig. 5B, lower panel; Fig. 6C,D,  $P=0.101$ ). *Nrp2<sup>-/-</sup>* mixed spinal nerves were also anteriorly restricted, although these bundles appeared less tightly packed than wild-type controls (Fig. 5F;  $n=4$ ).

Unlike DRG condensation, motor axon outgrowth was sensitive to neuropilin gene dosage. Motor axons initially projected along almost the entire length of the somite in *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>+/-</sup>* embryos, with less restriction than *Nrp1<sup>Sema-/-</sup>* motor axon projections, and still fasciculated into multiple clusters within the anterior sclerotome (compare Fig. 5C with Fig. S1E in the



**Fig. 3. *Nrp1* is essential for DRG formation.** (A–D) Lateral views of  $\alpha$ -neurofilament (NF)-stained E10.5 embryos. (E–H) Longitudinal sections of E10.5 embryos stained with neural-specific  $\beta$ -tubulin (TuJ1). Forming DRGs and DRs are segmentally arranged in control (*Nrp1<sup>Sema-/-</sup>;Nrp2<sup>+/-</sup>*; A,E) and *Nrp1<sup>Sema-/-</sup>* embryos (B,F). Ventral roots of *Nrp1<sup>Sema-/-</sup>* embryos are defasciculated (B, arrows). DRGs are segmental but elongated in *Nrp2<sup>-/-</sup>* embryos (C,G). Forming DRGs and DRs are continuous and non-segmental in double mutant *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos (D,H). (I,J) Sections of *Sox10* in situ hybridized embryos reveal segmental wild-type DRGs (I) and fused *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* DRGs (J). (K) Calculating the segment proportion occupied by DRGs. TuJ1 staining length ( $L_{drg}$ ) and segment length ( $L_{seg}$ ) were measured in ImageJ. Staining length was divided by segment length. The average was calculated for each genotype and statistical significance determined using Student's *t*-test. (L) E10.5 *Nrp2<sup>-/-</sup>* mutant DRGs are significantly closer together than DRGs in control embryos ( $P=4.65 \times 10^{-6}$ ), whereas double mutant DRGs are unsegmented ( $P=2.13 \times 10^{-14}$ ). *Nrp1<sup>Sema-/-</sup>* DRGs are significantly shorter than those in control embryos ( $P=0.0242$ ). Data are reported as mean  $\pm$  s.d. dm, dermomyotome; dr, dorsal root; drg, dorsal root ganglion; sc, spinal cord; sg, sympathetic ganglion. Anterior is to the left. Scale bars: in A, 100  $\mu$ m for A–D; in E, 50  $\mu$ m for E–J.

supplementary material,  $n=3$ ).  $Nrp1^{Sema+/-};Nrp2^{-/-}$  motor axon projections closely resembled those of  $Nrp2^{-/-}$  embryos (compare Fig. 5B with Fig. S1F in the supplementary material,  $n=5$ ).

In stark contrast to single mutants,  $Nrp1^{Sema+/-};Nrp2$  double mutant motor axons projected along the entire length of each segment (Fig. 5D, upper panel,  $n=3$ ; Fig. 6B,  $P=3.52 \times 10^{-12}$ ), and fasciculated into multiple bundles of 'ventral roots' throughout the anterior and posterior sclerotome (Fig. 5D, lower panel; Fig. 6C,D,  $P=1.16 \times 10^{-9}$ ). Mixed spinal nerves were also evenly distributed throughout the anterior and posterior sclerotome at E11.5 (Fig. 5H,  $n=5$ ). This phenotype resembled that of the intercostal nerves of E13.5  $Nrp1^{Sema+/-};Nrp2^{-/-}$  embryos (Huber et al., 2005), although the spinal nerves were largely continuous at E11.5 compared with those at later stages. Nerve bundles may avoid segment boundaries because of physical impediments, such as intersegmental blood vessels and, at later stages, ribs, which remain segmentally arranged

in neuropilin mutants. Together, these data show that neuropilins are essential for patterned motor axon outgrowth, with  $Nrp2$  directing the initial segmentation of motor axons and  $Nrp1$  guiding the fasciculation of ventral roots. Neuropilins also impose segmental character on the mixed spinal nerves.

## DISCUSSION

We have examined the role of  $Nrp2$ /Sema3F and  $Nrp1$ /Sema3A signaling in patterning PNS segmentation. We show that while  $Nrp2$ /Sema3F is required for segmental neural crest migration,  $Nrp1$ /Sema3A signaling is essential for segmental DRG formation, but only when neural crest cells migrate non-segmentally in an  $Nrp2$  mutant background. Additionally, we find that  $Nrp2$  guides motor axon outgrowth, whereas  $Nrp1$ /Sema3A signaling is essential for the fasciculation of these axons into ventral roots. Our work reveals a stepwise requirement for neuropilin/semaphorin signaling in the segmental patterning of two independently derived portions of the PNS (Fig. 7).

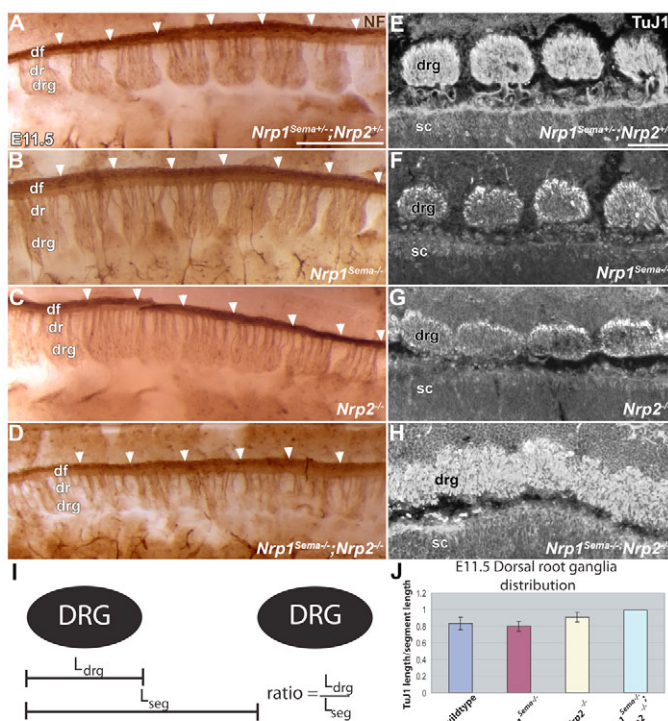
### Sensorimotor segmentation is a two-step process guided by neuropilins

It has classically been assumed that segmental neural crest migration generates the segmented, metamereric pattern of the peripheral ganglia (Bronner-Fraser, 1986; Kuan et al., 2004; Rickmann et al., 1985; Teillet et al., 1987). Our previous work showed that  $Nrp2$ /Sema3F guides segmental neural crest migration but not metamereric DRG formation (Gammill et al., 2006), indicating that additional, previously unappreciated steps were involved. We now identify  $Nrp1$ /Sema3A signaling as the crucial pathway downstream of  $Nrp2$  that segments DRG morphogenesis. Furthermore, we show that  $Nrp2$  and  $Nrp1$  are required to pattern both initial motor axon outgrowth and the fasciculation of these axons into ventral roots. Thus, both sensory and motor components of the PNS are segmentally arranged in separable, neuropilin-regulated steps.

What is the selective advantage of dividing sensory neuronal segmentation into two steps, especially given that the first phase is dispensable? Because different waves of neural crest migration create distinct subtypes of sensory neurons within the DRGs (Marmigere and Ernfrors, 2007), one possibility is that, although  $Nrp2^{-/-}$  DRGs are segmental, neurons are not properly patterned within them. Although  $Nrp2$  mutant mice can survive into adulthood, they do not breed well (data not shown) and could have neurological problems. Another possibility is that independently restricting both neural crest migration and gangliogenesis within the sclerotome provides back-up mechanisms to ensure that the PNS is properly segmented.

### Patterned motor axon outgrowth: a direct effect of neuropilin signaling

The role of neural crest cells in patterning motor axon outgrowth has been a topic of debate. Neural crest-derived boundary cap cells were originally thought to pattern motor exit points in the ventral spinal cord (Niederlander and Lumsden, 1996). Thus, non-segmental motor axon outgrowth in  $Nrp2$  mutants could reflect the abnormal, uniform distribution of boundary cap cells along the spinal cord. However, recent work has shown that boundary cap cells do not pattern motor axon outgrowth, but rather that they retain motor neuronal cell bodies in the spinal cord (Vermeren et al., 2003). In fact, boundary cap cells do not associate with the ventral spinal cord until after motor axons have emerged (Fraher et al., 2007). Together, these results indicate that motor axon projections are not patterned by boundary cap cells. Therefore, non-segmental motor axon



**Fig. 4. DRGs are fused when  $Nrp1$ /Sema3A and  $Nrp2$ /Sema3F signaling are lost.** (A–D) Lateral views of E11.5  $\alpha$ -neurofilament (NF)-stained embryos. Arrowheads indicate segment boundaries. (E–H) Longitudinal sections through E11.5 somites stained with neural-specific  $\beta$ -tubulin (TuJ1). DRGs and DRs are segmental in control ( $Nrp1^{Sema+/-};Nrp2^{+/+}$ ) embryos (A,E) and in  $Nrp1^{Sema+/-}$  embryos (B,F).  $Nrp2^{-/-}$  DRGs are segmental but more closely spaced than controls (C,G). DRGs and DRs are non-segmental and completely fused in double mutant  $Nrp1^{Sema+/-};Nrp2^{-/-}$  embryos (D,H). (I) Calculating the segment proportion occupied by DRGs. TuJ1 staining length ( $L_{drg}$ ) and segment length ( $L_{seg}$ ) were measured in ImageJ. Staining length was divided by segment length. The average was calculated for each genotype and statistical significance determined using Student's  $t$ -test. (J) E11.5 double mutant DRGs are unsegmented ( $P=1.78 \times 10^{-13}$ ), whereas  $Nrp2^{-/-}$  DRGs are significantly closer together than DRGs in control embryos ( $P=1.56 \times 10^{-5}$ ).  $Nrp1^{Sema+/-}$  DRGs are more widely spaced than those in control embryos ( $P=0.0498$ ). Data are reported as mean  $\pm$  s.d. df, dorsal funiculus; dr, dorsal root; drg, dorsal root ganglion; sc, spinal cord. Anterior is to the left. Scale bars: in A, 200  $\mu$ m for A–D; in E, 100  $\mu$ m for E–H.



outgrowth in *Nrp2* mutants cannot be a secondary consequence of non-segmental neural crest migration, and we conclude that neuropilin/semaphorin signaling directly guides motor axon outgrowth.

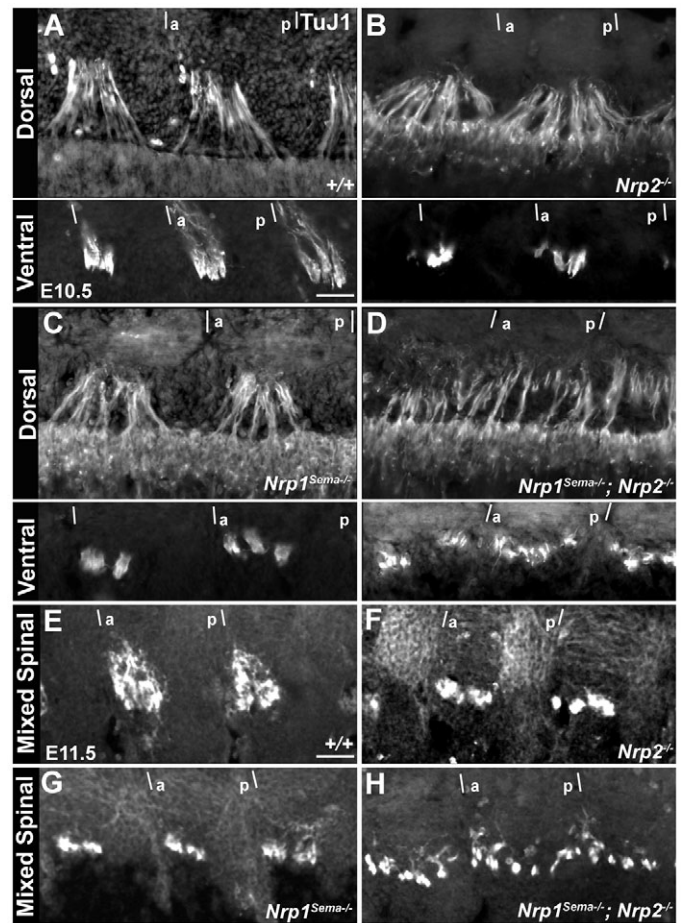
### Neuropilins in the PNS: redundant versus sequential signals?

Both *Nrp2*/Sema3F and *Nrp1*/Sema3A signaling are required for PNS segmentation. This dual requirement could reflect sequential use of *Nrp2* and *Nrp1* receptors in independent steps in this process, or it could indicate redundancy in these pathways. For a number of reasons, we favor the conclusion that sequential neuropilin signaling patterns DRG segmentation. First, although neural crest cells express both *Nrp1* and *Nrp2* receptors throughout neural crest migration and DRG morphogenesis (Fig. 1) (Chen et al., 2000; Gammill et al., 2006), within the sclerotome, *Sema3a* and *Sema3f* expression are temporally restricted. In particular, *Sema3f* is expressed in the posterior sclerotome during neural crest migration, but diminishes as DRGs condense (Fig. 1G,H; Fig. 7). Meanwhile, *Sema3a* is expressed in the posterior sclerotome only during DRG formation (Fig. 1D-F; Fig. 7). Second, DRGs do not exhibit intermediate segmentation phenotypes in single neuropilin mutants or in compound heterozygote/mutant combinations. This is not the case in the head, where the severity of neural crest guidance defects corresponds to the number of *Nrp1*<sup>Sema</sup> and *Nrp2* mutations: neural crest cells cross between cranial streams in *Nrp1*<sup>Sema</sup> or *Nrp2* single mutants, whereas the defects in compound heterozygote/mutant and double mutant animals are progressively more severe (Gammill et al., 2007; Schwarz et al., 2008). Third, E9.5 trunk *Nrp1*<sup>Sema</sup> and *Nrp2* mutant phenotypes are strictly additive, with double mutants exhibiting combined *Nrp1*<sup>Sema</sup> neural crest migration outside the somite (Fig. 2C) and non-segmental *Nrp2*<sup>-/-</sup> neural crest migration throughout the sclerotome (Fig. 2D). Together, these data suggest that *Nrp2*/Sema3F and *Nrp1*/Sema3A regulate separable, sequential steps during segmental trunk neural crest migration and DRG morphogenesis. We postulate that *Nrp1*<sup>Sema</sup> single mutants do not exhibit overt DRG segmentation defects because neural crest cells are already segmentally arranged by *Nrp2*/Sema3F signaling.

The sequential versus redundant use of neuropilin receptors during segmental motor axon outgrowth is less clear. Motor axons project through the sclerotome as the transition from *Sema3f* to *Sema3a* expression takes place (Fig. 1D-H). Furthermore, compound *Nrp2* heterozygote/*Nrp1*<sup>Sema</sup> mutant animals exhibit intermediate phenotypes (see Fig. S1 in the supplementary material), and there is no evidence for additive effects. However, the phenotype of *Nrp2* mutants is most apparent proximal to the spinal cord, whereas *Nrp1*<sup>Sema</sup> mutants are more strongly affected distally during the fasciculation of ventral roots. Thus, we favor a mechanism in which *Nrp2* guides initial motor axon outgrowth, whereas *Nrp1* is crucial for ventral root fasciculation, with some overlap in these requirements.

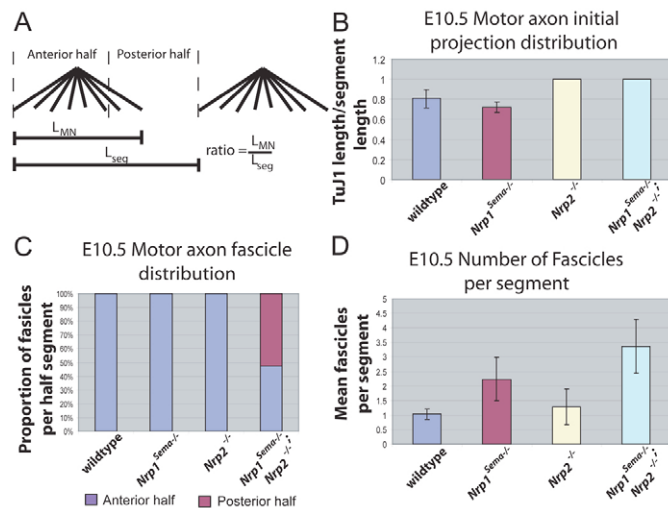
### Neuropilin signaling during DRG neurogenesis and morphogenesis

Neurogenesis is defective in *Nrp1*<sup>Sema</sup>;*Nrp2*<sup>-/-</sup> mutant DRGs. E10.5 neuropilin double mutant neural crest cells maintain multipotency, as indicated by *Sox10* expression (Kim et al., 2003), and TuJ1 expression is delayed compared with in wild types (Fig. 3H,I). By E11.5, the number of TuJ1-expressing cells has recovered (Fig. 4H). These results indicate that neurogenesis is impeded but not blocked in neuropilin double mutant embryos. This could be



**Fig. 5. *Nrp1* and *Nrp2* are required for segmentation of motor axons and spinal nerves.** (A-D) Longitudinal sections through E10.5 embryos stained with neural-specific  $\beta$ -tubulin (TuJ1). Top panels show initial motor axon projections from the spinal cord in dorsal sections, whereas the bottom panels show the fasciculation of the same projections in more ventral sections of the same embryos. (E-H) Ventral longitudinal sections through E11.5 embryos stained with TuJ1. Segment boundaries (short vertical lines) were determined using morphological markers, such as intersegmental blood vessels and epidermal bulges, lateral to the pictured area. (A) Wild-type motor axons project only into the anterior sclerotome, fasciculating into a single ventral root in each segment. The plane of section captured a length of the nerve. (B) *Nrp2*<sup>-/-</sup> mutant motor neuron projections initiate along the entire length of the somite, but fasciculate into a single ventral root. (C) *Nrp1*<sup>Sema</sup> motor axons project adjacent to the anterior somite, but ventral roots are defasciculated within the anterior sclerotome. (D) *Nrp1*<sup>Sema</sup>;*Nrp2*<sup>-/-</sup> motor neurons initiate projections all along the spinal cord and these projections are defasciculated throughout the somite. (E) Spinal nerves are organized in a single bundle in the anterior sclerotome of wild-type embryos. The plane of section captured a length of the nerve. (F) *Nrp2* mutant embryos exhibit a single bundle of spinal nerves in the anterior somite. (G) *Nrp1*<sup>Sema</sup> spinal nerves fasciculate into multiple bundles within the anterior sclerotome. (H) Double mutant embryos contain multiple spinal nerve bundles distributed throughout the length of each segment. a, anterior; p, posterior. Anterior is to the left. Scale bars: in A, 50  $\mu$ m in A-D; in E, 100  $\mu$ m in E-H.

because *Nrp1*/Sema3A signaling regulates DRG compaction (Kawasaki et al., 2002), and so these cells fail to adhere or to experience outside-inside distinctions properly. Cell sorting or

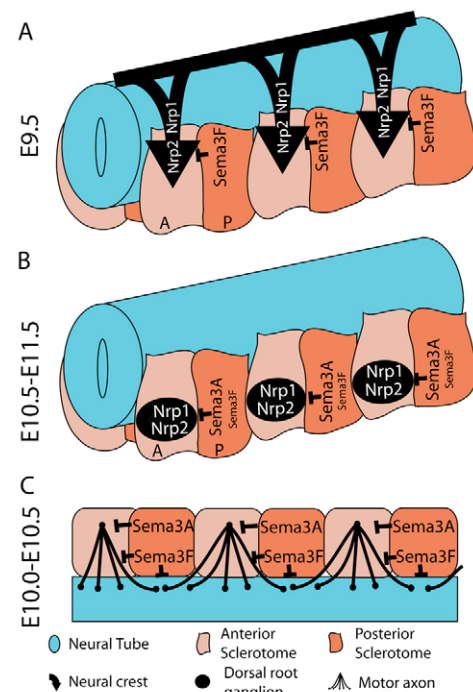


**Fig. 6. Quantitation of motor axon defects.** (A) Calculating the segment proportion invaded by motor axons. TuJ1 staining length ( $L_{MN}$ ) and segment length ( $L_{seg}$ ) were measured in ImageJ. Staining length was divided by segment length. The average was calculated for each genotype and statistical significance determined using Student's *t*-test. Motor axon fascicle distribution was determined by dividing sclerotomes into equal anterior and posterior halves and counting the number of bundles in each half. Additionally, the number of bundles was determined in each segment. (B) Wild-type motor axons project into anterior sclerotome only, whereas  $Nrp2^{-/-}$  and  $Nrp1^{Sema3A-/-};Nrp2^{-/-}$  projections are distributed throughout the length of the sclerotome ( $Nrp2^{-/-}$ ,  $P=2.01 \times 10^{-4}$ ;  $Nrp1^{Sema3A-/-};Nrp2^{-/-}$ ,  $P=3.52 \times 10^{-12}$ ).  $Nrp1^{Sema3A-/-}$  motor axons are restricted to a smaller space within the anterior sclerotome ( $P=2.80 \times 10^{-4}$ ). (C) Control,  $Nrp1^{Sema3A-/-}$  and  $Nrp2$  mutant motor axons are contained in the anterior half, whereas neuropilin double mutant motor axons project throughout each segment. (D) Control and  $Nrp2$  mutant motor axons fasciculate into a single bundle per segment, whereas  $Nrp1^{Sema3A-/-}$  and  $Nrp1^{Sema3A-/-};Nrp2^{-/-}$  motor axons form more than one bundle per segment ( $Nrp1^{Sema3A-/-}$ ,  $P=2.79 \times 10^{-6}$ ;  $Nrp1^{Sema3A-/-};Nrp2^{-/-}$ ,  $P=1.16 \times 10^{-9}$ ). Data are reported as mean  $\pm$  s.d. in B and D.

interactions within the DRGs could also be impaired or delayed (Marmigere and Ernfors, 2007). Alternatively, neuropilins could be directly required for differentiation.

Strikingly, unsegmented double mutant DRGs are not uniform along the length of the ganglia but instead have periodic bulges (Fig. 4H) that resemble fused DRGs in chick embryos implanted with cranial half-somites (Kalcheim and Teillet, 1989). This non-uniform shape may be imposed by the somites, as previous reports indicate that anterior sclerotome is less dense than posterior sclerotome (Christ et al., 2004) and is mitogenic to DRGs (Goldstein et al., 1990). Thus, anterior sclerotome is a more hospitable environment and might lead to thicker regions within the unsegmented ganglion. Alternatively, posterior sclerotome could be less favorable to DRG development because neuropilin-semaphorin signaling can stimulate apoptosis (Gagliardini and Fankhauser, 1999; Guttman-Raviv et al., 2007).

Since semaphorins regulate apoptosis, it is possible that neuropilin double mutant DRGs fuse because of an excess of sensory neuronal precursors. Previous work indicates that *Sema3A* induces apoptosis in sensory neurons (Gagliardini and Fankhauser, 1999), whereas both *Sema3A* and *Sema3F* promote neuropilin receptor-dependent apoptosis in endothelial cells (Guttman-Raviv et al., 2007). Similarly, *Sema3A* promotes cell death in cultured



**Fig. 7. Sequential neuropilin signaling patterns trunk neural crest migration and PNS segmentation.** A model for PNS patterning.

(A) *Sema3F* in the posterior sclerotome repels *Nrp2*-expressing migratory neural crest cells, restricting their migration to the anterior sclerotome at E9.5. Neural crest cells express *Nrp1* at this stage, but *Sema3A* is not yet produced by the sclerotome. (B) *Sema3A* in the posterior sclerotome positions anterior DRG formation through *Nrp1* on neural crest cells at E10.5 and E11.5. Although neural crest cells continue to express *Nrp2*, *Sema3F* is downregulated in the posterior sclerotome. (C) At E10.0, *Sema3F* in the posterior sclerotome restricts anterior-only initial motor axon projections. More laterally at E10.5, *Sema3A* in the posterior sclerotome bundles these axons into segmental ventral roots.

DRG neurons, although this effect requires the *Sema3A* receptor plexin A3 (Ben-Zvi et al., 2008). Curiously, metameric DRGs form in plexin A3 mutant embryos (Waimsey et al., 2008). Thus, segmental patterning and regulation of cell death might be separable semaphorin-dependent functions during PNS development, a possibility that warrants further exploration.

### Neuropilin-semaphorin signaling does not pattern SG formation

Like DRGs, SGs are segmented in a two-step process. First, neural crest cells migrate segmentally through the anterior sclerotome and emerge ventrally at the level of the dorsal aorta, where they undergo extensive mixing as far as two segments rostrally and two segments caudally (Kasemeier-Kulesa et al., 2005; Yip, 1986). Subsequently, sympathetic precursors re-segment into metameric SGs as a result of Eph-ephrin repulsion and N-cadherin-mediated adhesion (Kasemeier-Kulesa et al., 2006). *Nrp1* is also required for neural crest cells to stop at the dorsal aorta, and for their compaction into ganglia (Kawasaki et al., 2002).

Although DRGs and SGs are both neural crest-derived, segmentally arranged components of the PNS, neuropilin-semaphorin signaling is not required for SG segmentation. In



contrast to completely unsegmented DRGs, initially unsegmented DRs (Figs 3, 4), and uniformly projecting motor axons (Fig. 5), SGs were patterned normally in *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* mice (Fig. 3D). Although sympathetic neurons have been shown to differentiate in ectopic locations and extend processes across the midline in *Nrp1* mutant mice (Kawasaki et al., 2002), the position of SGs within each segment was not affected in either *Nrp1<sup>Sema-/-</sup>* or *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos. Thus, neither Nrp2/Sema3F nor Nrp1/Sema3A signaling segments SGs. As VEGF is also a ligand for Nrp1, Nrp1/VEGF signaling is likely to account for the SG defects observed in *Nrp1* mutant mice.

Our data also show that segmental neural crest migration is dispensable for SG segmentation. Non-segmentally migrating neural crest cells in *Nrp2<sup>-/-</sup>* or *Nrp1<sup>Sema-/-</sup>;Nrp2<sup>-/-</sup>* embryos form metameric SGs. Although the autonomy of SG segmentation from the pattern of neural crest migration has been assumed by the extensive intersegmental mixing of sympathetic precursors in wild-type embryos (Kasemeier-Kulesa et al., 2005), this is the first time it has been demonstrated directly.

### The role of other PNS guidance molecules

The molecular basis for segmental neural crest migration and motor axon outgrowth has been a key question since these phenomena were first observed (Bronner-Fraser, 1986; Keynes and Stern, 1984; LeDouarin and Kalcheim, 1999; Rickmann et al., 1985; Teillet et al., 1987). Since that time, a variety of signaling pathways have been proposed to dictate segmentation, including Eph/ephrins, Nrp1/Sema3A and F-spondin (reviewed by Kuan et al., 2004). However, mouse mutants have forced a re-evaluation of results from other vertebrates. Other than *Nrp2* mutants, in which segmental neural crest migration is completely abolished (Gammill et al., 2006), only ephrin B2 mutants exhibit transient trunk neural crest migration defects accompanied by loss of somite polarity, so the autonomy of the phenotype cannot be discerned (Davy and Soriano, 2007). Segmental trunk neural crest migration defects are not apparent in any other available mouse mutants (Adams et al., 2001; Davy et al., 2004; Hrabe de Angelis et al., 1997; Kawasaki et al., 2002; Orioli et al., 1996; Wang and Anderson, 1997; Wang et al., 1998), although it is technically impossible to generate mice mutant for all Eph/ephrin receptors. As all alternative signaling pathways are intact in *Nrp2* mutant mice, none is sufficient to restrict migratory neural crest cells to the anterior somite (Gammill et al., 2006). However, neural crest cells respond to these cues in vitro, and function blocking antibodies or interfering constructs for Nrp1, F-spondin and ephrin B1 do lead to neural crest migration defects (Debby-Brafman et al., 1999; Eickholt et al., 1999; Krull et al., 1997; Wang and Anderson, 1997). Rather than patterning segmental neural crest migration, it is more consistent with existing data across species if these signaling pathways regulate other aspects of PNS development. In other words, the relevant receptor might be expressed by neural crest cells but used for a purpose other than segmental guidance. For example, we find that Nrp1/Sema3A is required both to restrict migratory neural crest cells to the sclerotome, and for DRG segmentation. Eph/ephrins initially repel neural crest cells from the dorsolateral pathway and later stimulate dorsolateral migration of neural crest cells destined to become melanoblasts (Santiago and Erickson, 2002). Eph/ephrin signaling might also indirectly impact neural crest migration because it regulates anteroposterior patterning of the somite (Davy and Soriano, 2007). As an extracellular matrix molecule, F-spondin is likely to determine the substrate preference of migratory neural crest cells, as it affects neural crest cell morphology (Debby-Brafman et

al., 1999). Thus, whereas segmental neural crest migration is affected by interfering with these pathways, and neural crest cells avoid these cues in vitro because the relevant receptors are expressed, they are not essential for segmental neural crest guidance. Although it is possible that different vertebrates employ unique guidance cues in the neural crest, given the evolutionary conservation and importance of PNS segmentation, this possibility is unlikely in our opinion.

Reports of guidance cues that segment initial motor axon outgrowth are less conflicting. Nrp1 restricts the exit points and branching of single segmental motor axons in zebrafish (Feldner et al., 2005), and both Nrp1 and Nrp2 are required distally for segmentation of the intercostal nerves (Huber et al., 2005). Although not required for segmental motor axon outgrowth (Koblar et al., 2000), ephrin Bs do account for growth cone collapsing activity in the posterior somite (Vermeren et al., 2000). Although Sema3A depletion does not diminish this activity, this might reflect the requirement for Nrp1/Sema3A more distal to the spinal cord (Fig. 5C).

In summary, we have ascribed precise roles to Nrp2/Sema3F and Nrp1/Sema3A during PNS segmentation. We show that Nrp2/Sema3F is required for segmental neural crest migration and initial segmental motor axon outgrowth, while Nrp1/Sema3A is essential to restrict neural crest cells to the sclerotome, for metameric DRG formation in the absence of segmental neural crest migration, and for the fasciculation of ventral roots. Surprisingly, neither neuropilin is required for metameric SG formation. These results shed new light on the interplay of neuropilin signaling during development, and provide a stepwise mechanism for the establishment of the metameric vertebrate PNS.

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### Supplementary material

Supplementary material available online at  
<http://dev.biologists.org/cgi/content/full/136/11/1879/DC1>

### References

- Adams, R. H., Betz, H. and Puschel, A. W. (1996). A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. *Mech. Dev.* **57**, 33-45.
- Adams, R. H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U. and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69.
- Ben-Zvi, A., Manor, O., Schachner, M., Yaron, A., Tessier-Lavigne, M. and Behar, O. (2008). The Semaphorin receptor PlexinA3 mediates neuronal apoptosis during dorsal root ganglia development. *J. Neurosci.* **28**, 12427-12432.
- Bronner-Fraser, M. (1986). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115**, 44-55.
- Bronner-Fraser, M. and Stern, C. (1991). Effects of mesodermal tissues on avian neural crest cell migration. *Dev. Biol.* **143**, 213-217.
- Chen, H., Bagri, A., Zupicich, J. A., Zou, Y., Stoeckli, E., Pleasure, S. J., Lowenstein, D. H., Skarnes, W. C., Chedotal, A. and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron* **25**, 43-56.
- Christ, B., Huang, R. and Scaal, M. (2004). Formation and differentiation of the avian sclerotome. *Anat. Embryol. (Berl.)* **208**, 333-350.
- Davy, A. and Soriano, P. (2007). Ephrin-B2 forward signaling regulates somite patterning and neural crest cell development. *Dev. Biol.* **304**, 182-193.
- Davy, A., Aubin, J. and Soriano, P. (2004). Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev.* **18**, 572-583.
- De Bellard, M. E., Ching, W., Gossler, A. and Bronner-Fraser, M. (2002). Disruption of segmental neural crest migration and ephrin expression in delta-1 null mice. *Dev. Biol.* **249**, 121-130.

- Debby-Brafman, A., Burstyn-Cohen, T., Klar, A. and Kalcheim, C. (1999). F-Spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. *Neuron* **22**, 475-488.
- Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S. and Doherty, P. (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-2189.
- Feldner, J., Becker, T., Goishi, K., Schweitzer, J., Lee, P., Schachner, M., Klagsbrun, M. and Becker, C. G. (2005). Neuropilin-1a is involved in trunk motor axon outgrowth in embryonic zebrafish. *Dev. Dyn.* **234**, 535-549.
- Fraher, J. P., Dockery, P., O'Donoghue, O., Riedewald, B. and O'Leary, D. (2007). Initial motor axon outgrowth from the developing central nervous system. *J. Anat.* **211**, 600-611.
- Gagliardini, V. and Fankhauser, C. (1999). Semaphorin III can induce death in sensory neurons. *Mol. Cell. Neurosci.* **14**, 301-316.
- Gammill, L. S., Gonzalez, C., Gu, C. and Bronner-Fraser, M. (2006). Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. *Development* **133**, 99-106.
- Gammill, L. S., Gonzalez, C. and Bronner-Fraser, M. (2007). Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. *Dev. Neurobiol.* **67**, 47-56.
- Giger, R. J., Wolfer, D. P., De Wit, G. M. and Verhaagen, J. (1996). Anatomy of rat semaphorin III/collapsin-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. *J. Comp. Neurol.* **375**, 378-392.
- Giger, R. J., Cloutier, J. F., Sahay, A., Prinija, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S. et al. (2000). Neuropilin-2 is required *in vivo* for selective axon guidance responses to secreted semaphorins. *Neuron* **25**, 29-41.
- Goldstein, R. S., Teillet, M. A. and Kalcheim, C. (1990). The microenvironment created by grafting rostral half-somites is mitogenic for neural crest cells. *Proc. Natl. Acad. Sci. USA* **87**, 4476-4480.
- Gu, C., Rodriguez, E. R., Reimert, D. V., Shu, T., Fritzsche, B., Richards, L. J., Kolodkin, A. L. and Ginty, D. D. (2003). Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev. Cell* **5**, 45-57.
- Guttmann-Raviv, N., Shraga-Heled, N., Varshavsky, A., Guimaraes-Sternberg, C., Kessler, O. and Neufeld, G. (2007). Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J. Biol. Chem.* **282**, 26294-26305.
- Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* **386**, 717-721.
- Huber, A. B., Kania, A., Tran, T. S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T. M., Ginty, D. D. and Kolodkin, A. L. (2005). Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron* **48**, 949-964.
- Kalcheim, C. and Teillet, M. A. (1989). Consequences of somite manipulation on the pattern of dorsal root ganglion development. *Development* **106**, 85-93.
- Kasemeier-Kulesa, J. C., Kulesa, P. M. and Lefcort, F. (2005). Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. *Development* **132**, 235-245.
- Kasemeier-Kulesa, J. C., Bradley, R., Pasquale, E. B., Lefcort, F. and Kulesa, P. M. (2006). Eph/ephrins and N-cadherin coordinate to control the pattern of sympathetic ganglia. *Development* **133**, 4839-4847.
- Kawasaki, T., Bekku, Y., Suto, F., Kitsukawa, T., Taniguchi, M., Nagatsu, I., Nagatsu, T., Itoh, K., Yagi, T. and Fujisawa, H. (2002). Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. *Development* **129**, 671-680.
- Keynes, R. J. and Stern, C. D. (1984). Segmentation in the vertebrate nervous system. *Nature* **310**, 786-789.
- Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron* **38**, 17-31.
- Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T. and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19**, 995-1005.
- Koblar, S. A., Krull, C. E., Pasquale, E. B., McLennan, R., Peale, F. D., Cerretti, D. P. and Bothwell, M. (2000). Spinal motor axons and neural crest cells use different molecular guides for segmental migration through the rostral half-somite. *J. Neurobiol.* **42**, 437-447.
- Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E. and Bronner-Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7**, 571-580.
- Kuan, C. Y., Tannahill, D., Cook, G. M. and Keynes, R. J. (2004). Somite polarity and segmental patterning of the peripheral nervous system. *Mech. Dev.* **121**, 1055-1068.
- Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-250.
- LeDouarin, N. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge: Cambridge University Press.
- Leitges, M., Neidhardt, L., Haenig, B., Herrmann, B. G. and Kispert, A. (2000). The paired homeobox gene *Uncx4.1* specifies pedicles, transverse processes and proximal ribs of the vertebral column. *Development* **127**, 2259-2267.
- Mansouri, A., Voss, A. K., Thomas, T., Yokota, Y. and Gruss, P. (2000). *Uncx4.1* is required for the formation of the pedicles and proximal ribs and acts upstream of *Pax9*. *Development* **127**, 2251-2258.
- Marmigere, F. and Ernfor, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. *Nat. Rev. Neurosci.* **8**, 114-127.
- Nelson, B. R., Matsushashi, S. and Lefcort, F. (2002). Restricted neural epidermal growth factor-like like 2 (NELL2) expression during muscle and neuronal differentiation. *Mech. Dev.* **119 Suppl.** **1**, S11-S19.
- Niederlander, C. and Lumsden, A. (1996). Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves. *Development* **122**, 2367-2374.
- Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). *Sek4* and *Nuk* receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J.* **15**, 6035-6049.
- Puschel, A. W., Adams, R. H. and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron* **14**, 941-948.
- Rao, M. S. and Anderson, D. J. (1997). Immortalization and controlled *in vitro* differentiation of murine multipotent neural crest stem cells. *J. Neurobiol.* **32**, 722-746.
- Rickmann, M., Fawcett, J. W. and Keynes, R. J. (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Embryol. Exp. Morphol.* **90**, 437-455.
- Santiago, A. and Erickson, C. A. (2002). Ephrin-B ligands play a dual role in the control of neural crest cell migration. *Development* **129**, 3621-3632.
- Schwarz, Q., Vieira, J. M., Howard, B., Eickholt, B. J. and Ruhrberg, C. (2008). Neuropilin 1 and 2 control cranial gangliogenesis and axon guidance through neural crest cells. *Development* **135**, 1605-1613.
- Serbedzija, G. N., Fraser, S. E. and Bronner-Fraser, M. (1990). Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. *Development* **108**, 605-612.
- Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouché, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, M. et al. (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J. Clin. Invest.* **109**, 327-336.
- Stern, C. D. and Keynes, R. J. (1987). Interactions between somite cells: the formation and maintenance of segment boundaries in the chick embryo. *Development* **99**, 261-272.
- Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki, J. I., Hirota, S., Kitamura, Y. et al. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. USA* **99**, 3657-3662.
- Teillet, M. A., Kalcheim, C. and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. *Dev. Biol.* **120**, 329-347.
- Tosney, K. W. (1988). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: partial and complete deletion of the somite. *Dev. Biol.* **127**, 266-286.
- Vermeren, M. M., Cook, G. M., Johnson, A. R., Keynes, R. J. and Tannahill, D. (2000). Spinal nerve segmentation in the chick embryo: analysis of distinct axon-repulsive systems. *Dev. Biol.* **225**, 241-252.
- Vermeren, M., Maro, G. S., Bron, R., McGonnell, I. M., Charnay, P., Topilko, P. and Cohen, J. (2003). Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. *Neuron* **37**, 403-415.
- Waimey, K. E., Huang, P. H., Chen, M. and Cheng, H. J. (2008). Plexin-A3 and plexin-A4 restrict the migration of sympathetic neurons but not their neural crest precursors. *Dev. Biol.* **315**, 448-458.
- Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.
- Wang, H. U. and Anderson, D. J. (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-396.
- Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.
- Weskamp, G. and Reichardt, L. F. (1991). Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron* **6**, 649-663.
- Wright, D. E., White, F. A., Gerfen, R. W., Silos-Santiago, I. and Snider, W. D. (1995). The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. *J. Comp. Neurol.* **361**, 321-333.
- Yip, J. W. (1986). Migratory patterns of sympathetic ganglioblasts and other neural crest derivatives in chick embryos. *J. Neurosci.* **6**, 3465-3473.