Published in final edited form as:

Neuroscience. 2009 September 15; 162(4): 1106–1119. doi:10.1016/j.neuroscience.2009.05.035.

The retinoic acid inducible Cas-family signaling protein Nedd9 regulates neural crest cell migration by modulating adhesion and actin dynamics

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Summary

Cell migration is essential for the development of numerous structures derived from embryonic neural crest cells (NCCs), however the underlying molecular mechanisms are incompletely understood. NCCs migrate long distances in the embryo and contribute to many different cell types, including peripheral neurons, glia and pigment cells. In the present work we report expression of Nedd9, a scaffolding protein within the integrin signaling pathway, in non-lineage restricted neural crest progenitor cells. In particular, Nedd9 was found to be expressed in the dorsal neural tube at the time of neural crest delamination and in early migrating NCCs. To analyze the role of Nedd9 in neural crest development we performed loss- and gain-of-function experiments and examined the subsequent effects on delamination and migration in vitro and in vivo. Our results demonstrate that loss of Nedd9 activity in chick NCCs perturbs cell spreading and the density of focal complexes and actin filaments, properties known to depend on integrins. Moreover, a siRNA dose-dependent decrease in Nedd9 activity results in a graded reduction of NCCs migratory distance while forced overexpression increases it. Retinoic acid (RA) was found to regulate Nedd9 expression in NCCs. Our results demonstrate in vivo that Nedd9 promotes the migration of NCCs in a graded manner and suggest a role for RA in the control of Nedd9 expression levels.

Keywords

HEF1; Cas-L; cytoskeleton; focal adhesion; multipotency; RA

The emergence of vertebrates and their success can, in part, be attributed to the appearance of the neural crest which provides the embryological basis for the formation of vertebrate-specific features such as cranial skeleton, myelinated nerves, peripheral neurons and skin pigmentation (Baker and Bronner-Fraser, 1997; Glenn, 2005; Northcutt and Gans, 1983). The most prominent feature of neural crest cells (NCCs) is their high motility which makes them able to migrate extensively and populate tissues distant to their site of origin (Bronner-Fraser, 1995). While detaching from the dorsal neural tube – a process called delamination – NCCs adopt a

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Section Editor: Weimin Zhong, for 'Developmental Neuroscience'

mesenchymal morphology which allows them to invade the underlying mesoderm. Conversion of NCCs from a non-motile into a motile state requires interactions with various extracellular matrix (ECM) components and the acquisition of specific competences to properly sense these cues. While a number of factors regulating migratory properties of NCCs have been identified, the molecular framework that governs the movement of NCCs in vivo is just beginning to emerge.

Cell migration involves a concerted action of different mechanical forces within the cell. Cell adhesion to a substrate is necessary for cell spreading and motility and it requires the assembly of small focal contacts which link the actin cytoskeleton to the ECM (Huttenlocher et al., 1996; Small and Kaverina, 2003). Such focal adhesions and focal complexes are believed to provide the adhesive force necessary for traction at the leading edge of migrating cells. Integrin receptors are found clustered at focal adhesions and recruit a large complex of signaling molecules responsible for regulating actin dynamics and cell motility (Brunton et al., 2004). Numerous studies have demonstrated that the ECM functions as a scaffold onto which the NCCs migrate and that integrins play a prominent role in this process (Perris and Perissinotto, 2000). In vitro integrin signaling has been found to play a significant role in NCC spreading and migration onto a variety of ECM components (Desban and Duband, 1997; Erickson and Perris, 1993). Other studies have demonstrated that interfering with the function of integrins or their ligands, including laminin, is sufficient to perturb migration of NCCs (Bronner-Fraser, 1985; Lallier and Bronner-Fraser, 1993; Kil et al., 1996; Strachan and Condic, 2004; Coles et al., 2006; Strachan and Condic, 2008). Nedd9/CasL/HEF1 (hereafter referred to as Neural precursor cell expressed, developmentally down-regulated 9, Nedd9) is a scaffolding protein of the Cas family (O'Neill et al., 2000). Nedd9 is a member of the $\beta 1$ -integrin signaling pathway found to be necessary for glioblastoma and Jurkat T cell motility (Natarajan et al., 2006; O'Neill et al., 2000; van Seventer et al., 2001). Investigations on other Cas family members suggest that this class of adaptor proteins might serve as key bridges for the assembly of actin-cytoskeleton signaling complexes (O'Neill et al., 2000).

In the present work we investigated the mechanisms through which NCC migration is regulated. Nedd9 was identified by sequencing of mRNAs expressed in the early dorsal root ganglia (DRG) and its expression in migratory NCCs prompted further studies. Our results suggest that the motile capacity of multipotent NCCs is governed by a RA-controlled dynamic regulation of Nedd9 levels which influence cell adhesion and cytoskeleton properties.

Experimental procedures

All experimental procedures were approved by the Swedish National Board for Laboratory Animals (N17/2003, N404/2003 and N293/05).

In-situ hybridization

Embryos were fixed in 4% paraformaldehyde/PBS and sectioned at 14- μ m thickness. The mouse probe (498bp) was amplified from mouse cDNA using the following primers Forward 5'- CCCAACAGCATCATGAACTCA-3'; Reverse 5'- CTGAGCTGACGCAGCTGAA-3', corresponding to nucleotide 1705 to 2203 in the mouse Nedd9 CDS. The chick probe was amplified from chick cDNA using the following primers, Forward 5'- ATGAAGTACAAGAATCTTATGGCAA-3'; Reverse 5'- GTCTTTTGTCTGTGTAGTTGA-3', corresponding to nucleotide 1 to 1191 in the chick Nedd9 CDS. Plasmids containing specific probes for mouse or chicken Nedd9 were used to synthesize digoxigenin-labeled antisense riboprobes according to the supplier's instructions (Roche). *In-situ* hybridization (ISH) was performed as previously described (Hjerling-Leffler et al. 2005).

Immunohistochemistry and immunocytochemistry

Cryoprotected tissue was sectioned at 14- μ m thickness and mounted on slides. Sections were processed as previously described (Aquino et al. 2006). Primary antibodies specific to β -III-tubulin (Tuj1, mouse, IgG, 1 μ g/ml, Promega), Brn3a (mouse, IgG, 1:50, Chemicon), HEF1 (Nedd9, 2G9, mouse, IgG, 1 μ g/ml for mouse tissue and 2 μ g/ml for chicken, Abcam), Islet1 (39.4D5, mouse, IgG, 11 μ g/ml, Developmental Studies Hybridoma Bank), Sox2 (rabbit, IgG, 1:3000, from Dr. Thomas Edlund), Sox10 (guinea pig, IgG, 1:1000, from Dr. Michael Wegner) and Paxillin (mouse, IgG, 5.6 μ g/ml, Upstate and mouse, IgG, 2.5 μ g/ml, BD Transduction Laboratories, clone 349) were used. The secondary antibodies were Cy3, Cy2 and Cy5-conjugated (Jackson ImmunoResearch). Immunocytochemistry was performed following previously published protocols (Aquino et al., 2006). For phalloidin staining: cells were permeabilized with 0.1% Triton-X 100 (Merck, 10 minutes), washed and incubated with Alexa Fluor 546 or Alexa Fluor 647-phalloidin (Molecular Probes, 1:50 in PBS, 2 hours, room temperature). Pictures were taken using a Zeiss LSM 510 confocal microscope. Measurements were obtained from pictures, taken with same settings from sections that were simultaneously processed for immunohistochemistry (IHC). For quantification purposes, 4.7 μ m optical slice pictures (using an air 20x objective) were used. Only cells sectioned across their nuclei were included in the analyses. Optic density measurements were obtained using the ImageJ software (NIH) as follows: individual cells were carefully scanned, at least twice, by manually moving the cursor over the whole cell surface and maximum score obtained was registered for quantification. For in vivo cell counting, sections were first stained for Sox10 and Is11 and number of EGFP⁺ cells was determined by counting number of nuclei which were either Sox10⁺ or Is11⁺. For this purpose, at least one slide with several serial sections (every 5th section taken) per embryo was used and only sections through the core of migrating cells/DRG were considered for quantification. Criteria of inclusion of border DRG cells were as followed: Sox10⁺ cells located within a two cells-thick layer at the periphery of the DRG.

In ovo electroporation

For Nedd9 overexpression experiments, cDNAs encoding mouse Nedd9 (Law et al., 1998) and EGFP were separately sub-cloned into an expression vector driven by the chick actin promoter (pCA). Constructs were injected into the neural tube of either HHst10–11 or HHst13–14 chick embryos following published procedures (Marmigere et al., 2006).

For loss-of-function experiments, naked siRNA constructs (1.5 μ g/ μ l) were electroporated in combination with the EGFP expression vector (0.5 μ g/ μ l). The siRNA sequences were as follows: Nedd9 siRNA 1, 5'-UCAACCAACAGAAGAUCUAUCAAGUGC-3' (MWG Biotech); and Nedd9 siRNA 2, 5'-AUUUAAGGGACUAAGCAUACCCUCC-3' (Invitrogen); corresponding to Nedd9 coding sequence (from NCBI database, accession # AF104246.1) starting from nucleotides 261 and 332, respectively. As control experiments, either EGFP alone (0.5 μ g/ μ l) or in combination with a control (scramble) siRNA (1.5 μ g/ μ l; sequence: 5'-UCAACCAACAGAAGAUCUAUCAAGUGC-3'; Invitrogen) were used. E3.5: $n=5$ Nedd9 siRNA 1.5 μ g/ μ l and control; $n=3$ Nedd9 siRNA 0.5 μ g/ μ l. E4: $n=3$. E5: $n=4$. In Nedd9 siRNA plus mouse Nedd9 overexpression experiment, both the construct and siRNA (1.5 μ g/ μ l each) together with the EGFP construct (0.5 μ g/ μ l) were co-electroporated ($n=4$); as control condition pCA empty vector (1.5 μ g/ μ l) was used instead of mouse Nedd9 full-length ($n=4$). For quantifications, only sections showing similar pattern and intensity of EGFP expression in the neural tube including the roof plate, were considered. Around 20 sections were analyzed for each experimental condition. Electroporation efficiency was 15% (measured in EGFP-electroporated E4 chicken as proportion of targeted cells within the DRG).

Chick neural tube explants

Chick E3 (24 hours after electroporation) neural tubes between, but not including, forelimbs and hindlimbs were dissected, cut into 2–3 somite long pieces and cleaned of somites. Neural tube explants were individually plated on 24-well plates coated with PDL and fibronectin (20 µg/ml, Sigma) in N2 medium supplemented with B27, BDNF (50 ng/ml), bFGF (40 ng/ml) and chicken embryo extract (0.5%, a generous gift from Dr. Hannu Sariola).

After 16–24 hours, as indicated, cultures were either fixed or processed for NCC dissociation. After dissociation, NCCs were re-plated at 3000 cells/well, in 24-well plates on cover-slips coated with either PDL and laminin (10 µg/ml, $n=3$ wells), PDL alone or heat-denatured laminin (10 µg/ml, 5' at 99°C, $n=3$ wells/condition) in a medium containing N2, B27 and all-trans RA (5 ng/ml). After 4 hours, cultures were fixed and phalloidin or paxillin stained. A cell was considered to have spread when a flattened morphology and at least one process larger than its soma diameter was noted. The spreading area (or whole cell area) of EGFP co-transfected individual cells was measured using the EGFP signal: for this purpose the threshold signal was increased to cover the whole cell surface (ImageJ software). For value calculations, the average area score obtained from non-spread cells plated in PDL and denatured laminin was subtracted from individual cell area values.

Density measurements of phalloidin and paxillin stainings were calculated by obtaining from confocal images (using 60 x objectives; phalloidin: stack projections from five 1 µm optic sections; paxillin: single 1.25 µm thick optic sections) the immunostained area value of individual cells and dividing it by total cell area (assessed using EGFP staining, Image J software).

Cell spreading area: $n=3$ wells per condition. Density of paxillin staining: $n=6$ Nedd9 siRNA; $n=17$ control siRNA. Density of phalloidin staining: $n=7$ Nedd9 siRNA; $n=6$ control siRNA. Migratory index = [1/distance between each individual EGFP⁺ cell leading edge and the front of NCC migration as indicated by paxillin staining, in arbitrary units; $n=4$ explants in Nedd9 siRNA; $n=5$ explants in control].

Boundary cap neural crest stem cell-neurosphere migration assay

Boundary cap neural crest stem cell (bNCSC) neurospheres were derived as previously described (Aquino et al. 2006). Neurospheres were incubated with cDNAs for Nedd9 or empty cassette in combination with EGFP (1.5 µg each), cloned in pcDNA3 expression vector, overnight with Lipofectamine 2000 in OPTIMEM medium including factors contained in the neurosphere propagation medium (Aquino et al., 2006), according to manufacturer's recommendations (Invitrogen Life Technologies). Spheres were then washed and placed in 24 well-plates pre-coated with PDL (50 µg/ml) and laminin (20 µg/ml) and incubated overnight in N2, B27, GDNF (20 ng/ml), NGF (50 ng/ml) and β 1-herregulin (50 ng/ml). Cells that migrated out from spheres were quantified ($n=9$ spheres in EGFP; $n=7$ spheres in Nedd9).

Boundary cap neural crest stem cell-derived Schwann cell migration assay

Schwann cells were derived from bNCSCs according to Aquino et al. (2006). cDNAs for Nedd9 and EGFP or EGFP and empty cassette (1.5 µg each), cloned in pcDNA3 expression vector, were electroporated into Schwann cells according to Marmigere et al. (2006). Cells were plated at low density (5000 cells/well) in one lateral compartment of Campenot chambers already placed into 12-well plates, pre-coated with PDL (50 µg/ml) and laminin (20 µg/ml). Cells were cultured overnight using previously published conditions (Aquino et al., 2006). Inner chamber limits were marked, Campenot systems removed and plates returned to incubator for 24 hours. EGFP⁺ cells that passed the inner limit of the compartment were counted. For statistical analyses, values obtained were adjusted according to percentage of transfection efficiency/

proliferation rate whenever differences were found between conditions. Migrating Schwann cells: $n=3$ wells Nedd9 + EGFP; $n=4$ wells pcDNA3 empty vector + EGFP.

Mouse neural tube explants and RA injection in pregnant females

Mouse E9.5 neural tubes, between forelimbs and hindlimbs, were dissected out, cut into 2–3 somite long pieces and cleaned of somites. Neural tube explants were individually plated on 24-well plates coated with PDL and fibronectin (20 μ g/ml, Sigma) in N2 medium supplemented with B27, BDNF (50 ng/ml) and bFGF (40 ng/ml).

After 10 hours, culture medium was replaced by a defined medium consisting of Ham's F14 supplemented with glutamine, bovine serum albumin, progesterone, putrescine, L-tyroxine, sodium selenite, triiodo-thyronine plus any of the following factors: all-trans RA (5 ng/ml or 500 ng/ml, in DMSO, Sigma), citral (0.1M, Sigma), BMP4 (50 ng/ml, R&D), noggin (100 ng/ml, R&D), FGF8 (25 ng/ml, R&D), Wnt3a (100 ng/ml, R&D), Wnt5a (400 ng/ml, R&D), Shh (400 ng/ml, R&D), Wnt3a plus BMP4 or none (control). $n=4$ explants per condition. Neural tube segments from same embryo were never treated with same factor. After 6 hours, cultures were fixed and stained with Nedd9 antibody. Pictures were taken using a Zeiss Axioplan 2 microscope (Germany), a ProgRes C14 CCD camera (Jenoptik/Jena, Germany) and Openlab software (Improvision Ltd., England). Up to 20 values of optic density (OD) were obtained per explant from every 10th Nedd9⁺-cell, using the ImageJ software (NIH). Counting followed the same criteria in all cases. OD values were obtained as previously described (see Immunohistochemistry and immunocytochemistry section) from regions wherein single layer of cells could be observed, avoiding areas in which cells were superposed (i.e. in close proximity to neural tube).

RA (60 mg/kg animal, in olive oil) was injected i.p. into pregnant females at 9.25 days of gestation. In the control condition a similar volume of oil was injected.. Embryos ($n=5$ control; $n=6$ RA) were obtained and fixed 6 hours after single injections. Tissue was sectioned and immunostained for Nedd9 as described above.

Statistical analyses were carried out by applying *t* student or Mann-Whitney tests.

Results

A rapid and dynamic regulation of Nedd9 expression in migrating multipotent Sox10⁺ trunk NCCs

In order to identify molecules involved in NC migration, we sequenced mRNA transcripts from the early DRG. Nedd9 was identified and confirmed to be expressed in the trunk neural tube of the mouse embryo at E9, at which point the epithelium has adopted a pseudo-stratified aspect (data not shown). Thereafter, expression of Nedd9 within the neural tube was confined to the ventricular zone (Aquino et al., 2008). Between E9 and E9.5, both Nedd9 mRNA and protein were transiently expressed in the roof plate of the neural tube while NCCs were delaminating from this area (Fig. 1A,J and not shown). Shortly after NCC delamination, Nedd9 was down-regulated from the roof plate (Fig. 1B,K). At E9.5–E10.5 Nedd9 mRNA and protein were still highly expressed in delaminated NCCs, with a peak at trunk levels where somites dissociate into the dermamyotome and sclerotome, the developmental stage at which NCCs start migrating down through the rostral half of the somite (Fig. 1C,D,K and S1). All migrating Sox10⁺ NCCs analyzed at the dorsal neural tube in the trunk expressed Nedd9 at all time points analyzed. NCCs at a more ventral location of the prospective DRG showed decreased Nedd9 expression with levels below detection limit at the level of the ventral spinal cord (Fig. 1C,E,K and S1C–F).

In migrating NCCs of the dorsal paraxial mesoderm and in the early condensed DRG, Nedd9 mRNA (Fig. S1C–F) and immunoreactivity (Fig. 1) were almost exclusively present in Sox10⁺ NCCs (percent Sox10⁺ cells expressing Nedd9; paraxial mesoderm: 97.35±1.57%, *n*=4; early condensed DRG: 93.56±1.86%, *n*=8). Sox10 marks multipotent NCCs (Kim et al., 2003). Nedd9⁺ cells in the condensed ganglion were primarily “border cells” which are located at the periphery of the nascent ganglion and known to retain multipotency (Montelius et al., 2007) (81.44±2.69% of Nedd9⁺ cells were located within a 2 cell layer of the DRG perimeter, *n*=7; Fig. 1G–I,L). Isl1⁺/Brn3a⁺/Foxs1⁺/Sox10[−] post-migratory and neuronally committed progenitor cells of the DRG were negative for Nedd9 expression (percent Isl1⁺ cells lacking expression of Nedd9; 98.75±1.25%, *n*=8; Fig. 1G,I and not shown). At E12.5, when the NCCs are committed to either glial or neuronal fates in the ventral migratory pathway, Nedd9 was no longer expressed in the neural crest/neural crest-derived cells (Aquino et al., 2008). A similar developmental sequence of Nedd9 expression in NCCs was observed in the chick (Fig. S1G,H). Based on these results, we conclude that Nedd9 is expressed in delaminating and emigrating NCCs of the ventral migratory pathway and in post-migratory multipotent Sox10⁺ NCCs of the DRG.

Attenuation of Nedd9 expression results in reduced trunk NCC number and migration deficits

To examine the involvement of Nedd9 on NCC migration in vivo, we electroporated chick embryos with Nedd9 siRNA in ovo and analyzed them at E3.5 (1.5 days after electroporation). The efficiency of the siRNA construct in knocking-down Nedd9 was assessed in vivo by immunohistochemistry (Fig. 2). Similar results were observed by analyzing Nedd9 mRNA levels with in situ hybridization (data not shown). Further confirmations of siRNA specificity were determined by applying a second independent Nedd9 siRNA which resulted in similar phenotype and by gain of function rescue through co-electroporation with a mouse Nedd9 overexpression construct.

Chick embryos were first electroporated into the trunk region at HHst13–14, before NCCs emigration. Loss of Nedd9 function caused a marked reduction in the number of delaminated EGFP⁺ NCCs (31% of control; Fig. 3A–C). However, this phenotype was only found at hindlimb levels of the trunk. We therefore performed the same analysis on chicken embryos electroporated at an earlier stage (HHst10–11) confirming a defect in EGFP⁺ delaminated NCCs also at more rostral levels of the trunk (data not shown), indicating a direct correlation between the rostro-caudal developmental timing and a time-dependent effect of Nedd9 depletion on NCCs delamination. In addition, Nedd9 depletion did not affect apoptosis in transfected NCCs, as assessed by the lack of activated caspase-3 immunostaining in EGFP⁺ cells at NC level in Nedd9 siRNA condition at HHst16–18 (15–25 hours after electroporation at HHst10–11) (data not shown). Furthermore, in embryos transfected with Nedd9 siRNA, a striking accumulation of EGFP⁺ cells at the dorsal neural tube was observed at E3.5, with some of them expressing Sox10, a phenotype never observed in the control condition (Fig. 4). Collectively, our data suggest that down-regulation of Nedd9 in the NC leads to a decrease in the ability of NCCs to delaminate and further emigrate from the dorsal neural tube.

The role of Nedd9 in neural crest migratory ability was analyzed at the hindlimb level of the trunk from E3.5 chicken embryos. The number of transfected EGFP⁺ NCCs per section that were able to reach at least the dorsal edge of the prospective DRG were measured and normalized to the total number of delaminated EGFP⁺ NCCs in the same section (Fig. 3D). Nedd9 siRNA caused a marked reduction in the number of EGFP⁺ migrating NCCs to nearly 1/3rd of control conditions. A second independent Nedd9 siRNA construct confirmed the specificity of these effects (data not shown).

We next explored the functional consequences of different levels of Nedd9 expression during development on neural crest development. Nedd9 levels were reduced by electroporating

Nedd9 siRNA at different concentrations (0.5 $\mu\text{g}/\mu\text{l}$ and 1.5 $\mu\text{g}/\mu\text{l}$) and embryos were analyzed at the hindlimb region of the trunk. Nedd9 siRNA dose-dependently reduced the number of EGFP⁺ delaminated NCCs at E3.5 (Fig. 3H). Consistently, Nedd9 siRNA also induced a dose-dependent reduction in NCC migration as noted by the lower percentage of EGFP⁺ cells that were able to reach at least the dorsal DRG (Fig. 3I).

In order to address whether Nedd9 expression would be required for early migration of NCCs we exploited the fact that at the time of transfections, HHst13–14, NCC delamination is not affected at trunk levels rostral to the hindlimbs. NCC migration was studied *in vitro* by explanting E3 neural tubes (transfected at HHst13–14) taken from trunk regions between fore- and hindlimbs. The NCCs were cultured in a medium favoring maintenance of undifferentiated NCCs; nevertheless, inclusion in the analysis of some cells undergoing early stages of differentiation can not be excluded. Nedd9 siRNA targeted NCCs migrated significantly less than control cells in all explants analyzed at 24 hours *in vitro* (Fig. 3E–G). Neural tube explants kept for 3 days in culture were consistent with the deficiency in migration of Nedd9 siRNA targeted NCCs (average distance of migration; control siRNA: $216.7 \pm 17.14 \mu\text{m}$ vs. Nedd9 siRNA: $148.0 \pm 6.92 \mu\text{m}$; $n=7$, $P=0.0013$, *t*-test). The number of EGFP⁺ cells that migrated out from neural tube explants was similar between conditions (control: 177.0 ± 18.03 , siRNA: 148.8 ± 19.45 ; $n=7$, $P=0.4036$, *t*-test).

A reduction in Nedd9 activity affects NCC spreading, adhesion and cytoskeleton properties

Cas family proteins have been proposed to act as scaffolding proteins participating in the assembly of focal adhesions and cytoskeleton dynamics (O'Neill et al., 2000). To examine the impact of Nedd9 on NCC morphology, adhesion and cytoskeleton properties, Nedd9 siRNA was introduced in the chick by *in ovo* electroporation and transfected cells were identified by EGFP expression. NCCs migrating from either Nedd9 or control siRNA electroporated chick neural tube explants were dissociated and re-plated at low density on laminin (a ligand for integrins), poly-D-lysine (PDL) or denatured laminin (which is unable to act as a ligand for integrins) and kept in culture for 4 hours. To exclude effects of cell-cell interactions, cells found within one-cell distance were excluded in the analysis. In both experimental and control siRNA conditions, about 70% of cells were found to spread when plated on laminin whereas this proportion was significantly reduced to 10–30% when plated on PDL or denatured laminin (Fig. 5A,B). There was no significant statistical difference between the percentage of spread cells of Nedd9 siRNA and control siRNA conditions (laminin: $P=0.59$; PDL: $P=0.25$; denatured laminin $p=0.12$, *t*-test). However, the area of spreading was significantly reduced in the Nedd9 siRNA condition (Fig. 5A,C), suggesting that Nedd9 is required for cells to efficiently spread in an integrin-dependent context.

To further explore the role of Nedd9 in NCC morphology and adhesion properties, we examined for the dependence of actin and paxillin expression profile on Nedd9. The density of both phalloidin binding to actin and paxillin was decreased in Nedd9 siRNA targeted NCCs compared to the control condition (Fig. 6A,B). In addition, Nedd9 siRNA targeted NCCs were often lacking thick phalloidin⁺ stress fibers which are normally seen as straight bundles of microfilaments crossing the cell body (Fig. 6F–H) (Pellegrin and Mellor, 2007), and were observed in a significant proportion of control NCCs ((Wright J et al., 1988) (Fig. 6C–E). These are believed to be essential for changes in cell morphology during migration (Hotulainen and Lappalainen, 2006). It seems likely that the absence or decrease in the number of stress fibers in Nedd9 knock-down condition is the main cause of the observed decrease in phalloidin density. Unlike Nedd9 siRNA targeted cells, control siRNA cells often extended elaborated cellular protrusions (Fig. 6C–L). We have then analyzed further differences in the migratory phenotype of NCCs between the control (control siRNA, $n=31$) and the Nedd9 loss-of-function (Nedd9 siRNA, $n=25$) conditions, by applying a previously described method based on NCC

morphological criteria after phalloidin staining (migratory cells i.e. polarized cells with a leading lamella and rearward positioned nucleus: 61.29% vs 28%; ambiguous cells i.e. cells that were not symmetrically spread but with no clear axis of migration: 35.48% vs. 20%; stationary cells, i.e. symmetrically spread cells with a central nucleus, circumferential lamella or ring of filopodia: 3.23% vs. 52%, respectively, control vs. Nedd9 siRNAs) (Strachan and Condic, 2003). Combined, these results demonstrated that Nedd9 is necessary for efficient NCC spreading in an integrin-dependent context and suggests that Nedd9 participates in the dynamics of focal complexes and actin filaments required for cytoskeleton remodeling and cell motility (DeMali and Burrridge, 2003; Ridley et al., 2003). These cytoskeletal and morphological changes may contribute to the failure of NC delamination and early migration when Nedd9 expression is down-regulated.

Elevated expression of Nedd9 causes increased migration of trunk NCCs

To confirm a dose-dependent effect of Nedd9 activity in NCCs, we next examined whether Nedd9 overexpression influences migration. We have previously shown that co-electroporation of EGFP and RFP vectors results in 95% of cells co-expressing both proteins (Marmigere et al., 2006). Chicks were electroporated with an expression construct for the mouse full-length Nedd9 together with EGFP (Nedd9) or with EGFP alone (EGFP) as control, and migrating NCCs were analyzed 48 hours later (E4). As mentioned above, NCCs contributing to sympathetic neurons and Schwann cells of the skeletal nerves, migrate earlier and a longer distance than those of the sensory lineage (Fig. 7). The total number of migrated EGFP⁺ cells of the ventral migratory pathways (DRG + nerve trunks + sympathetic ganglia) did not significantly differ between the control and the experimental conditions (Nedd9: 61.66 ± 17.95; control: 68.81 ± 21.99; $n=3$, $P=0.6852$, t -test). Significantly more EGFP⁺ prospective Schwann and sympathetic cells and less EGFP⁺ DRG cells were found after Nedd9 overexpression (Fig. 7B,D,F,G) when compared to the control condition (Fig. 7A,C,E,G). These results suggest that increased or maintained Nedd9 expression leads to enhanced migration in cells that would otherwise have contributed to the DRG.

It is worth noting that the EGFP⁺ neuronal-to-non neuronal ratio was unaffected by Nedd9 overexpression in the DRG (Fig. 7H), a phenotype also obtained in the Nedd9 siRNA condition when compared to the control condition ($p>0.05$), suggesting that Nedd9 does not seem to be involved in NCC fate determination.

We next addressed whether Nedd9 overexpression could rescue the migration defects observed following endogenous Nedd9 depletion. Embryos were electroporated with Nedd9 siRNA and a mouse Nedd9 expression construct or with Nedd9 siRNA and empty vector, as control. At 48 hours after electroporation, overexpression of Nedd9 rescued the migratory deficit in the Nedd9 siRNA condition (percentage of cells which migrated farther than DRG levels: control, 16.1 ± 5; Nedd9, 40.2 ± 5.9; Nedd9+Nedd9 siRNA, 25 ± 5.82; empty vector+Nedd9 siRNA, 10.12 ± 3.64. Nedd9 siRNA+empty vector vs. Nedd9 siRNA+Nedd9, $P=0.007$; control vs. Nedd9 siRNA+empty vector, $P=0.03$). In contrast, the decreased number of delaminated NCCs observed in the Nedd9 siRNA condition could not be rescued by mouse Nedd9 exogenous expression. Our Nedd9 expression pattern analysis indicates that Nedd9 is first present in cells of the NC lineage coincident with NC delamination. siRNA treatment may therefore eliminate the onset of Nedd9 expression while the maximal levels following overexpression occur approximately 24 hours after delivery. Hence, the rescue by overexpression of Nedd9 of later developmental roles, but not of delamination, may reflect temporal aspects related to the experimental procedure.

The effect of forced expression of Nedd9 on migration was also examined in vitro both in mouse bNCSC neurosphere and bNCSC-derived Schwann cell cultures (Aquino et al., 2006). In the gain-of-function condition, comparing EGFP⁺ to control cells, significantly more neural

crest stem cells and Schwann cells migrated from neurospheres and from a Campenot chamber respectively (Fig. 8). Thus, we can conclude from these results that forced expression of full-length Nedd9 in NCCs and neural crest-derived Schwann cells increases their migration also in the mouse.

Nedd9 expression in NCCs is regulated by RA

In order to identify the signals inducing Nedd9 expression, NCCs migrating out from mouse neural tube explants were exposed for 6 hours to a number of candidate factors known to be produced at the time and location of migrating NCCs (RA, FGF8, Wnt3a, Shh, BMP4, Wnt5a, Wnt3a+BMP4 and Noggin; Fig. 9). Of all factors tested, RA produced the most robust and dose-dependent up-regulation of Nedd9 expression (Fig 9A,B,D). Consistently, inhibition of RA biosynthesis by incubation with citral (Kikonyogo et al., 1999; Song et al., 2004) led to markedly decreased levels of Nedd9 (Fig. 9C,D). In order to confirm that Nedd9 expression was regulated by RA in vivo, RA was injected into pregnant mouse females at 9.25 days of gestation and embryos were analysed 6 hours later. A statistically significant increase in Nedd9 immunoreactivity was observed in NCCs upon RA treatment when compared to the control condition (Fig. 9E–G).

Discussion

In the neural crest, as well as in the developing brain, the onset of cell migration is timed with extracellular cues determining the path of migration (Lopez-Bendito et al., 2006; Shah et al., 1996). Significant advances have been made in recent years in understanding how extracellular cues with attractive/repulsive properties determine cell migration. In the present work, we propose that the ability of NCCs to migrate is regulated by changes in their responsiveness to ECM. NCCs are known to remain clustered at the level of the dorsal neural tube for several hours before they start migrating down through the anterior half of the sclerotome (Erickson and Perris, 1993). Ca^{2+} dependent cell-cell adhesions through cadherins were found to be involved in keeping pre-migratory NCCs clustered and to prevent their migration (Newgreen and Gooday, 1985). Monier-Gavelle and Duband showed that a raise in intracellular Ca^{2+} levels, likely due to integrin activation, is able to trigger intracellular signaling events through serine-threonine kinases which in turn mediate both prevention of intercellular contacts through cadherins and release from the extracellular matrix, thus promoting NCC migration (Monier-Gavelle and Duband, 1997). This signaling was found to be modulated by tyrosine kinases and phosphatases (Brennan *et al.*, 1999; Monier-Gavelle and Duband, 1995). Nevertheless, key signaling molecules involved in these pathways have been unexplored. In the present study, we describe the expression of Nedd9, a member of the Cas family proteins which can be phosphorylated by serine-threonine kinases after cell adhesion (O'Neill et al., 2000; Zheng and McKeown-Longo, 2006) and modulated by tyrosine kinases and phosphatases (Cote *et al.*, 1998; Manie *et al.*, 1997), in premigratory and migratory NCCs. In addition, by means of in vitro and in vivo experiments we present evidence that Nedd9 expression levels, controlled by RA, participate in determining the migratory properties of NCCs. Thus, Nedd9 appears likely to be an important intracellular scaffolding molecule determining responsiveness to extracellular cues which in turn determine NCC migration. Our data indicate that Nedd9 is present in limiting quantities since overexpression results in enhanced migration. Therefore, dynamic changes in Nedd9 expression levels might result in functional consequences on NCC migration in vivo.

A role for Nedd9 in delamination and early migration processes of neural crest cells

Delamination of NCCs and their emigration from the dorsal neural tube represents critical processes in which premigratory NCCs undergo changes in cell shape and cell-cell interactions

and acquire the ability to properly interpret extracellular cues before initiating their dispersion through the embryo.

The results shown here suggest that Nedd9 activity is involved in the delamination and early migratory events of NCCs. Nedd9 was found to be expressed in the neural dorsal roof at the time of NCC delamination. Nedd9 expression is gradually induced in delaminating NCCs. Interestingly, Nedd9 expression in NCCs remains highly expressed at the axial level at which the somites differentiate into the sclerotome and dermamyotome, and at the dorsal aspects of the dermamyotome, suggesting that Nedd9 might be implicated in facilitating NCC migration down through the sclerotome. At the stages analyzed, including early stages of NCC delamination and migration (E9–E10), we were unable to find any section showing significant numbers of Sox10⁺/Nedd9⁺ cells.

Upon NCC migration through the sclerotome, Nedd9 expression was found to decrease and eventually disappear in NCCs as well as in neuronal and glial progenitors. Because virtually all migratory NCCs were found to express Nedd9 at the dorsal neural tube, this result implies that sympathetic progenitors and ventrally migrating Schwann cell precursors down-regulate Nedd9 once migrating ventrally beyond the neural tube. Nedd9 was found to be expressed dorsally to the coalescing DRG. Thus, Nedd9 down-regulation in neuronally committed progenitors might be linked to their limited migratory capacity and likely contributes to DRG condensation (Ma *et al.*, 1999; Montelius *et al.*, 2007). Altogether, the temporal and spatial expression patterns of Nedd9 suggest that this signaling protein might play a critical function during neural crest delamination and early migration through the dorsal part of the embryo. The lack of Nedd9 expression in NCCs ventral to the DRG imply the involvement of other Nedd9-independent mechanisms for migration beyond the neural tube of NCC progenitors of sympathetic and Schwann cell lineages.

To directly assess the role of Nedd9 in neural crest delamination and migration, we used a combination of loss- and gain-of-function approaches. The dose-dependent reduction in Nedd9 expression in NCCs *in vivo* resulted in a graded deficiency of NCC delamination and migration. The rescue by forced expression of a mouse Nedd9 cDNA provides evidence for the specificity of the knock-down strategy. These *in vivo* findings were corroborated by analyzing NCCs migration using an *in vitro* neural tube explant assay, in which depletion of Nedd9 expression in NCCs was shown to induce a significant loss of NCC migratory properties.

Nedd9 is a scaffolding protein which localizes to focal adhesions upon integrin activation (Fashena *et al.*, 2002; O'Neill *et al.*, 2000). The correct balance of cell adhesiveness and detachment by actin cytoskeleton and focal adhesion dynamics is required for cell migration (Giannone *et al.*, 2007). We found that the reduction in Nedd9 levels in NCCs reduced F-actin and paxillin densities of staining. This is likely to contribute to the significant reduction in cell spreading we demonstrate in Nedd9 deficient cells. Nedd9 might therefore participate in the formation of focal adhesions and the assembly of actin fibers in NCCs, which are known to be required for NCC migration.

Consistent with the knock-down phenotype, overexpression of Nedd9 *in vivo* resulted in alterations in the NCC migration. Indeed, forced expression of Nedd9 in delaminating and migrating NCCs increased the proportion of cells contributing to the sympathetic ganglion and nerves at the expense of DRG cell numbers. These results suggest that Nedd9 overexpression in NCCs increases migration resulting in a shift towards more distant homing sites (e.g. sympathetic ganglion and nerves). This conclusion was supported by *in vitro* experiments. The fact that Nedd9 was also shown to increase migration capacity in cells which have already down-regulated endogenous protein levels, i.e. Schwann cells, support a role for Nedd9 in

rendering cells sensitive to extracellular cues stimulating cell migration. Taken together, our results suggest that a dynamic regulation of Nedd9 expression is important for NCC migration.

A rapid and dynamic regulation of Nedd9 expression by RA

Nedd9 was found to be induced in NCCs in close proximity to the dorsal lip of the dermamyotome and to be rapidly down-regulated as migrating cells reach levels of the ventral neural tube. This observation suggested that signals from the dermamyotome might be involved in the induction/regulation of Nedd9 expression. A number of signaling factors have been described in the trunk, including RA (Haselbeck et al., 1999), fibroblast growth factors (Karabagli et al., 2002), BMP, Wnt, Noggin (Marcelle et al., 1997) and Shh (Echelard et al., 1993). In our studies, the ability of these factors to induce and/or modulate Nedd9 expression was assessed using mouse neural tube explants. Among the different signals inducing Nedd9 (RA, FGF, Wnt and Shh), RA appeared to be the best inducer. The down-regulation on Nedd9 expression by inhibition of RA signaling further supports a specific role for RA as an inducible/regulatory factor of Nedd9, which is consistent with previous studies (Merrill et al., 2004).

Retinaldehyde dehydrogenase type 2 (RALDH-2), a major RA generating enzyme, is expressed in the somites at the time of NCCs migration (Blentic et al., 2003; Haselbeck et al., 1999). Furthermore, Raldh2 mRNA expression peaks at the axial level in which somites dissociate into dermamyotome and sclerotome (Haselbeck et al., 1999). This coincides with the NCCs onset of migration through the rostral half of the somite (Erickson and Perris, 1993); Shoval *et al.*, 2007; our results), strengthening the view that Nedd9 expression in NCCs is under the control of RA. In agreement with this, the Nedd9 promoter contains a RA response element (RARE) that interacts with the RA receptor (Merrill et al., 2004).

Combined, the data presented here supports a model whereby dynamic regulation of Nedd9, along the dorso-ventral axis by RA produced in the dermamyotome, orchestrates NCC migration. In light of our data, it is conceivable that some of the phenotypes described for NCC-derived structures in conditions of vitamin A or retinoid insufficiency (Dickman et al., 1997; Lohnes et al., 1995) might be explained by deficiencies in Nedd9 expression levels required for NCC migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

List of abbreviations

NCC	neural crest cell
RA	retinoic acid
ECM	extracellular matrix
DRG	dorsal root ganglion
HHst	Hamburger/Hamilton stage
bNCSC	boundary cap neural crest stem cells

Acknowledgments

We thank Ernest Arenas for valuable experimental suggestions and Ruani Fernando for critical reading of the manuscript. We also thank Thomas Edlund and Michael Wegner for Sox2 and Sox10 antibodies, Hannu Sariola for chicken embryo extract and Anna Stenqvist for experimental help. We acknowledge Claudia Tello and Johnny Söderlund for technical assistance. This work was supported by the Swedish Medical Research Council, the Swedish

Foundation for Strategic Research (CEDB and DBRM grants), ERC advanced grant 232675 and NIH RO1 CA63366. J.B.A. was supported by European Union (Marie Curie RTN-CT-2003-504636). F.L. was partially supported by the Swedish Medical Research Council (K2007-77PK-20285-01-6) and the European Union (Marie Curie MEIF-CT-2006-039237).

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Fig. 1. Expression pattern of Nedd9 in the roof plate and in migrating NCCs

(A–C) Sections taken from E9.5 mouse embryos from more caudal (A) to more rostral levels of the trunk (C) immunostained for Nedd9 (red) and Sox10 (green). Asterisks: the dorsal roof of the neural tube express Nedd9 in (A) but not in (B). Arrowhead in A: delaminating NCCs, emerging from the neural tube. Arrows in A,B: early migratory NCCs at the epithelial somite level of the trunk. Arrows in C: NCCs, at the somite dissociation axial level, migrating down through the sclerotome. Curved arrows in C: migrating NCCs at the ventral neural tube level. (D) Quantitative data showing changes in Nedd9 expression between different trunk levels (epithelial somite or dermamyotome; $n=44$ and 17 , respectively; intensity values correspond to a scale from 1 to 250; all Sox10⁺ cells present in sections were included in the analysis; data are represented as mean $\pm$ S.E.M.; *** $P<0.0001$, Mann-Whitney test). (E) Nedd9 (red), Sox2 (green) and Sox10 (blue) immunostained caudal trunk E10.5 mouse embryo section showing that Nedd9 protein is expressed in migrating NCCs (arrowheads) but is down-regulated when cells reach levels of the ventral neural tube (curved arrows). (F–H) Images of Nedd9 (red) and

Sox10 (blue) immunostained sections taken from (F) lumbar (showing migrating NCCs) or (G,H) thoracic axial levels. (I) Adjacent section to G immunostained for Islet1 (red), Sox10 (blue) and FoxS1 (green). Curved arrows in (F,G) indicate Sox10⁺ that show reduced Nedd9 expression levels. Arrowheads in I: cells with decreased Sox10 expression contain low Islet1 levels. (J–L) Schematic representation of dynamics of Nedd9 expression in NCCs. DRG: dorsal root ganglion; NT: neural tube; RP: roof plate. Scale bars: A,B,F 10µm; C,E,G–I 20 µm.

Fig. 2. Nedd9 siRNA down-regulates Nedd9 protein expression *in vivo*

HH13–14 chick were electroporated *in ovo* with either EGFP+control siRNA (control; A) or EGFP+Nedd9 siRNA (siRNA; B) and embryos were collected 36 hours later (E3.5). Nedd9 down-regulation was confirmed in siRNA transfected NCCs from regions just rostral to the hindlimb axial levels (arrows), when compared to control transfected NCCs in dorsal areas of condensing DRG (arrows). Arrowhead in B: one siRNA-EGFP co-transfected NCC shows normal levels of Nedd9 expression. Scale bars: 10 μ m. (C) Quantification of Nedd9 immunoreactivity (IR) in EGFP⁺ migrating NCCs in control and siRNA Nedd9 conditions; *** $P < 0.001$, *t*-test.

Fig. 3. Nedd9 knock-down results in reduction of NCC number and decreased migration

(A–B) Representative images taken from E3.5 transversal trunk sections at the hindlimb level of chick embryos electroporated with Nedd9 siRNA+EGFP (B) or control siRNA+EGFP (A), stained for Sox10 (blue) and Islet1 (red). Arrows indicate similar dorso-ventral levels in migrating NCCs for both figures. (C,D) Quantitative data showing total number of EGFP⁺ NCCs found per section in control or Nedd9 siRNA conditions (C), and percentage of EGFP⁺ NCC which have reached the prospective DRG level, normalized to the total number of EGFP⁺ NCCs in each condition (“migrating NCCs”; D). (E–F) Nedd9 siRNA and control electroporated chicken neural tube explants containing NCCs were fixed after 24 hours in vitro and stained for paxillin (red). Distance migrated by Nedd9 siRNA targeted EGFP⁺ NCCs

(including some transfected NT cells) was significantly reduced when compared to control (nt: neural tube). (G) Quantitative data obtained from neural tube explant experiments showing changes in migratory index of the transfected NT/NC cells (see Experimental procedures) between control and Nedd9 siRNA conditions. (H,I) Quantitative data showing the effect of different doses of Nedd9 siRNA (0.5 $\mu\text{g}/\mu\text{l}$ or 1.5 $\mu\text{g}/\mu\text{l}$) in NCC behavior in vivo at E3.5 after in ovo electroporation. (H) The total number of EGFP⁺ NCCs per section per condition is shown as percentage of control; (I) percentage of “migrating EGFP⁺ NCCs”, which were able to reach the dorsal DRG, shown as percent of control. Note that NCC number (H) and migration (I) deficiencies are strongest at the highest levels of Nedd9 siRNA used. All in vivo quantifications were done from sections at the hindlimb level of the trunk. * vs. Control; § vs. siRNA 0.5 $\mu\text{g}/\mu\text{l}$. *, § $P < 0.05$; **, §§ $P < 0.01$; *** $P < 0.001$. ** in (G,I) *t*-test; Mann Whitney test in all other comparisons. Data are represented as mean $\pm$ S.E.M. Scale bars: A,B 20 μm ; E,F 50 μm .

Fig. 4. Alterations in Nedd9 siRNA transfected dorsal neural tube tissue suggest NCC delamination defects

(A,B) Representative EGFP-DIC images from control (A) and Nedd9 loss-of-function (B) chick embryos (at E3.5) showing appearance of cell clusters extruding dorsally from Nedd9 siRNA targeted neural tubes (dashed lines in B). (C) A confocal microscope projection image in the X,Y and Z axes showing Sox10⁺ (red) NCCs present in cell clusters in the dorsal neural tube. Scale bars: A,B 20 μm; C 10 μm.

Fig. 5. Integrin ligands and Nedd9 are both required for an efficient NCC spreading
 (A) Photomicrographs of NCCs receiving control or Nedd9 siRNA plated onto laminin (“lam”) (an integrin ligand) or PDL or denatured laminin (“denat lam”) (non-ligands for integrins). Confocal images were acquired at 0.8μm optical slices through the entire depth of cells and reconstructed on the Z-plane (small images on top of each X–Y plane image). (B) Quantification of the number of cells that spread. (C) Quantification of the cell spread area in arbitrary units (au). The spreading area of Nedd9 siRNA targeted cells was significantly reduced when compared to control siRNA transfected cells. (B) ** $P < 0.01$, t -test, vs. laminin. (C) ** $P < 0.01$, t -test, vs. control siRNA. Data are represented as mean $\pm$ S.E.M. Scale bars: 10 μm.

Fig. 6. A reduction of Nedd9 levels results in deficits of focal complexes and actin cytoskeleton
 Chick NCCs electroporated with control or Nedd9 siRNA were plated onto the integrin ligand laminin and fixed 4 hours after plating for cell adhesive property studies. (A,B) Quantitative data showing differences in density of phalloidin reactivity (A) or paxillin staining (B) between control and Nedd9 siRNA conditions. Density of staining has been normalized to the total cell area and is expressed as percentage of control siRNA. * $P < 0.05$, t -test. Data are represented as mean $\pm$ S.E.M. (C–H) Representative pictures showing phalloidin staining (red) in control (C–E) or Nedd9 (F–H) siRNA transfected NCCs. Note that some control siRNA transfected cells present stress fibers crossing through the center of the cell while none of the Nedd9

depleted cells show this feature. (I–L) Representative photomicrographs showing paxillin staining (red) in control (I,J) or Nedd9 (K,L) siRNA transfected NCCs. Scale bars: 5 μ m.

Fig. 7. *In vivo* Nedd9 overexpression increases migration of trunk NCCs
 (A–F) Chicks were in ovo electroporated with EGFP (control; A,C,E) or Nedd9+EGFP (B,D,F) and fixed 48 hours later (E4). Trunk sections were stained for Sox10 (red) and Islet1 (blue). In Nedd9 gain-of-function, more EGFP⁺ cells were found in both the spinal nerves (arrows in B; arrowheads in C,D) and sympathetic ganglia (E,F; arrowheads) when compared to control. (G) Quantitative data showing the percent of transfected EGFP⁺ cells present in DRG, along the nerve (prospective Schwann cells = SC) and in sympathetic chain (S), for both Nedd9 overexpressing (Nedd9) and control (EGFP) conditions. ** $P < 0.01$, t -test. (H) Quantitative data showing percentage of transfected cells in DRG that were Islet1⁺ (neurogenetic progenitors)

or Sox10⁺ (glial + NCC multipotent progenitors) in experimental and control conditions, *t*-test. Data are represented as mean $\pm$ S.E.M. Scale bars: A,B 50 μ m; C–F 20 μ m.

Fig. 8. *In vitro* overexpression of Nedd9 increases migration of bNCSCs stem cell-derived Schwann cells

(A) Boundary cap neural crest stem cell (bNCSC)-neurospheres were plated onto laminin after overnight transfection with EGFP+Nedd9 expression vector (Nedd9) or with EGFP+empty expression cassette (EGFP). No significant differences in transfection rate were found between conditions ($P=0.1480$; t -test). Significantly more EGFP⁺ bNCSCs migrated out from the neurospheres in Nedd9 overexpressing condition when compared to control. (B) bNCSC-derived Schwann cells were dissociated from differentiation cultures and electroporated with EGFP and empty expression cassette (EGFP) or EGFP and Nedd9 expression vector (Nedd9). Schwann cells were plated into one chamber of Campenot systems placed in 12 well plates, after cell sedimentation inner chamber limits were marked, the system removed and cells kept for 20 hours in culture. Significantly more EGFP⁺ Schwann cells crossed the chamber limits in Nedd9 overexpressing condition when compared to control. * $P < 0.05$; t -test. Data are represented as mean $\pm$ S.E.M.

(A–D) Mouse neural tube (NT) explants containing NCCs were kept overnight in proliferation media (see Experimental procedures) and thereafter exposed for 6 hours to different signals known to be present in the trunk during NCC migration. (A–C) Representative pictures taken from Nedd9 immunostained (red) explants pulsed with all-trans RA, citral or without addition of any of them (control). (D) Quantitative data showing levels of Nedd9 expression in NCCs (without excluding some NT cells) after 6 hours of incubation with different indicated factors in vitro. (E,F) Nedd9 expression (red) in sections of E9.5 mouse embryos exposed in vivo to exogenous RA for 6 hours (F) or vehicle (control, E). (G) Quantitative data of Nedd9 levels after in vivo exposure of embryos to RA. Values in D,G correspond to Nedd9 immunostaining

intensity (scale: 1–250). * vs. control; § vs. FGF; ^τ vs. Wnt3a; ^f vs. RA low and Shh. *, ^f $P < 0.05$; **, ^{ττ} $P < 0.01$; ***, ^{§§§} $P < 0.001$; *t*-test. Data are represented as mean ± S.E.M. Scale bars: A–C 25 μm; D,E 20 μm.