Development 135, 4113-4122 (2008) doi:10.1242/dev.023119

Directing pathfinding along the dorsolateral path – the role of EDNRB2 and EphB2 in overcoming inhibition

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Neural crest cells that become pigment cells migrate along a dorsolateral route between the ectoderm and the somite, whereas most other neural crest cells are inhibited from entering this space. This pathway choice has been attributed to unique, cell-autonomous migratory properties acquired by neural crest cells when they become specified as melanoblasts. By shRNA knockdown and overexpression experiments, we investigated the roles of three transmembrane receptors in regulating dorsolateral pathfinding in the chick trunk. We show that Endothelin receptor B2 (EDNRB2) and EphB2 are both determinants in this process, and that, unlike in other species, c-KIT is not. We demonstrate that the overexpression of EDNRB2 can maintain normal dorsolateral migration of melanoblasts in the absence of EphB2, and vice versa, suggesting that changes in receptor expression levels regulate the invasion of this pathway. Furthermore, by heterotopic grafting, we show that neural crest cell populations that do not rely on the activation of these receptors can migrate dorsolaterally only if this path is free of inhibitory molecules. We conclude that the requirement for EDNRB2 and EphB2 expression by melanoblasts is to support their migration by helping them to overcome repulsive or non-permissive cues in the dorsolateral environment.

KEY WORDS: Melanoblast, Pigment cell, EDNRB2, ET3, c-KIT, EphB2, Ephrins, Pathfinding, Migration, Chick, Neural crest

INTRODUCTION

One way that signaling molecules influence embryonic patterning is by controlling cellular pathfinding. Neural crest cells (NCCs) are a good model system for studying pathfinding because they migrate to many different positions within the embryo along stereotypical pathways. In the chick trunk, NCCs of the peripheral nervous system emerge from the neuroepithelium first and migrate ventrally between the neural tube and somite. A day later, pigment cell precursors, or melanoblasts, emigrate and invade the dorsolateral (DL) space between the dermamyotome and the ectoderm. Melanoblasts are the only cells that occupy the DL pathway, and they will invade this space precociously if transplanted into embryos undergoing ventral migration (Erickson and Goins, 1995; Reedy et al., 1998a). This implies that melanoblasts are specified prior to invasion of the DL path and that this specification gives them the unique ability to access this space. The switch from ventral to DL migration might be regulated through the upregulation of subpopulation-specific receptors that respond to differentially localized guidance cues present in these pathways (Wehrle-Haller and Weston, 1997).

In support of this idea, EphB receptors mediate a repulsive response to ephrin ligands in neuronal NCCs and an attractive response in melanoblasts (Santiago and Erickson, 2002). It is unknown how NCCs modulate EphB signaling to produce these two outcomes, but one possibility is that each NC subpopulation expresses a particular EphB receptor that dictates their migratory ability. EphB3 is expressed by neuronal NCCs and participates in the segmental patterning of the peripheral nervous system (Krull et al., 1997). Thus, EphB3 is a good candidate for maintaining the ventral migration of neuronal precursors; however, an EphB receptor specific to melanoblasts has yet to be indentified.

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Accepted 15 October 2008

Melanoblasts require a number of receptors during their dissemination, as revealed by mouse and zebrafish mutant analysis. These include the type III receptor tyrosine kinase encoded by the *Kit* gene, also known as c-KIT, W or dominant white (Qiu et al., 1988; Bernex et al., 1996; Kunisada et al., 1998; Parichy et al., 1999; Jordan and Jackson, 2000), and a G-protein-coupled receptor encoded by the *Endothelin receptor B* gene (EDNRB) (Shin et al., 1999; Lee et al., 2003; Pla et al., 2005). In the mouse, *Kit* is necessary to maintain the survival of melanoblasts, and for their consequent dispersal onto the DL pathway (Steel et al., 1992; Bernex et al., 1996; Wehrle-Haller et al., 2001). The KIT receptor is activated by cell-surface and soluble forms of Steel factor (SLF; Kitl – Mouse Genome Informatics), which is produced by the dermatome flanking the DL space (Wehrle-Haller and Weston, 1995; Wehrle-Haller et al., 2001). EDNRB, by contrast, is required later, when melanoblasts are traversing the length of the dermamyotome, and during their differentiation (Shin et al., 1999; Lee et al., 2003; Hou et al., 2004). Despite the almost complete absence of pigmentation in *Ednrb-s*^{l} mouse mutants, melanoblasts lacking this receptor do not undergo overt apoptosis, suggesting that EDNRB maintains the melanogenic fate (Lee et al., 2003). Thus, in mouse, melanoblasts require KIT during their entry into the DL path and depend on EDNRB to maintain their melanogenic properties. However, the molecular mechanism by which KIT drives DL pathfinding only in the melanogenic neural crest population has not been elucidated by studying mouse mutants. Melanoblasts respond chemokinetically to SLF (Jordan and Jackson, 2000), which suggests that although KIT may increase the rate at which melanoblasts encounter the DL entryway, other determinants are required for directional guidance.

In birds, there are deviations in the expression patterns of these receptors and their ligands in comparison to mouse. First, avian melanoblasts do not express c-KIT until they are well into the DL pathway, at a time coincident with the upregulation of SLF by the epidermal ectoderm (Lecoin et al., 1995; Reedy et al., 2003). Second, aves have acquired a melanoblast-specific endothelin receptor subtype, EDNRB2 (Lecoin et al., 1998). *EDNRB2* is

upregulated in melanoblasts prior to entering the DL space, and *endothelin3* (*ET3*), its ligand, is expressed by cells of the ectoderm and dermamyotome at a similar time (Lecoin et al., 1998; Nataf et al., 1998; Nagy and Goldstein, 2006). Mouse embryonic stem cells producing EDNRB2 have an increased ability to migrate dorsolaterally after being grafted into the migration staging area (MSA) of a chick embryo, whereas those expressing c-KIT do not (Beauvais-Jouneau et al., 1999; Pla et al., 2005). This suggests that EDNRB2 is a more likely candidate than c-KIT to mediate DL pathfinding in the chick.

Repulsive cues present in the DL path that restrict the migration of neuronal precursors include the ephrins, slits, spondins, chondroitin sulfate proteoglycans and PNA-binding molecules (Oakley and Tosney, 1991; Oakley et al., 1994; Debby-Brafman et al., 1999; Santiago and Erickson, 2002; Jia et al., 2005). However, some of these inhibitory molecules remain in the DL path as melanoblasts invade this space. How melanoblasts achieve this robust, stereotypical migration in the presence of such inhibitory cues is not adequately understood. The identification of a receptor involved in the chemotactic guidance of melanoblasts could explain the unique ability of a melanoblast to access this pathway. In order to identify specific determinants in DL pathfinding in the chick, we investigated the role of c-KIT, EDNRB2 and Eph receptors. We determined that EDNRB2 and EphB2, but not c-KIT, drive melanoblast invasion of the DL path. EphB2 and EDNRB2 both regulate a chemotactic response and, moreover, signaling from these receptors is additive because the overexpression of one receptor can rescue the loss of the other. Additionally, we demonstrate that EDNRB2 and EphB2 signaling is essential for selection of the DL path, but only when inhibitory molecules are present at high levels within this space. Our findings provide new insights into when and why instructive interactions provided by positive guidance cues are essential for the DL pathfinding of specific NC subpopulations.

MATERIALS AND METHODS

Experimental animals

Fertilized White Leghorn chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) eggs were obtained from the Avian Science Research Facility (ASRF, UC Davis). Eggs were incubated at 38.5°C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992).

White Rooster Serum (WRS) was obtained from a de-pigmented Jungle Fowl rooster located at the ASRF. Three mls of blood were drawn, clotted overnight and spun at 2000 ***g*** for 15 minutes. The supernatant was used for immunolabeling.

Quail trunk NC cultures

Quail neural tubes were dissected and cultured as described previously (Erickson and Goins, 1995). For melanoblasts/cytes, neural tubes were cultured for 12-24 hours and then replated. The NCCs that migrate from these replated tubes will differentiate primarily into pigment cells (Reedy et al., 1998a). For immunocytochemistry, melanoblasts were fixed 48 hours post-replating and melanocytes (indicated by melanin production) at 5 days.

Quantitative RT-PCR

Quail neural tubes were cultured for 8 hours to produce early outgrowths. These neural tubes were replated, and after 24 hours clusters of melanoblasts had formed. These clusters were picked and allowed to disperse on a fresh dish. Seventy-two hours after initial replating the mRNA from early outgrowths and melanoblast cell cultures was purified (Micro-FastTrack, Invitrogen). Fifty nanograms of mRNA were reverse transcribed using the TaqMan Gold RT-PCR kit (Applied Biosystems).

Quantitative PCR was performed using Applied Biosystems SYBR Green Master Mix at an annealing temperature of 62°C for 40 cycles and a primer concentration of 200 nM. The primer sequences were as follows: GAPDH, 5′-AAAGTCCAAGTGGTGGCCATC-3′, 5′-TTTCCCGTTCT-CAGCCTTGAC-3′; EphB2, 5′-CCGCAACATCCTGGTCAAC-3′, 5′-TGCGCTGGTGTAAGTGGGA-3′. Samples were performed in triplicate and normalized to the GAPDH results.

Western blotting

Chick embryo fibroblasts (CEFs) were harvested from a day-10 chick embryo. The torso was shredded with forceps and digested in TrypLE Express (Gibco). The trypsinized tissue was diluted in DMEM supplemented with 10% bovine growth serum and 1% penicillin/streptomycin, and filtered through lens paper using a Swinny adaptor. CEFs were spun down at 200 ***g***, resuspended in DMEM and grown in a 75-ml flask. CEFs were grown to 80-90% confluence and transiently transfected by applying an 8:2 ratio of Fugene (Roche):DNA (1 μg each c-KIT-HA and c-KIT-pSilencer or scramble-pSilencer) to the cells for 30 hours. The c-KIT expression construct (c-KIT-HA) contains a truncated fragment of chick c-*KIT* cDNA coupled to three HA epitope tags in pMES (M. Reedy, Creighton University).

Twenty micrograms of cell lysate from day-9 chick, stage-25 quail embryos or CEFs were mixed with sample buffer (2× Laemmli, 5% β-mercaptoethanol) and boiled for 5 minutes. Proteins were separated on a 4%/12% stacked SDS-PAGE polyacrylamide gel, transferred to an Immobilon-P PDVF membrane (Millipore), and blotted with WRS, Smyth line serum (a gift from Ray Boissy, University of Cincinnati) or an HA-probe antibody (Santa Cruz Biotechnology).

Immunolabeling, in situ hybridization and TUNEL

Embryos were fixed in 4% paraformaldehyde for 3 hours at room temperature (RT), cryoprotected in 15% sucrose, and frozen in Histoprep (Fisher). Embryos were cut into 12-μm sections and immunohistochemistry was performed as described (Hall and Erickson, 2003). Cells used for immunocytochemistry were fixed in 4% paraformaldehyde for 10 minutes at RT and permeabilized with 0.1% Triton X-100 for 20 minutes. Antibodies were applied as described above.

We used the following primary antibodies: WRS (1:400 dilution); HNK1 and QCPN (1:1, supernatants from hybridomas, Developmental Studies Hybridoma Bank); anti-laminin (1:400, Sigma); EphB2 (1:20, gift from E. Pasquale, Burnham Institute for Medical Research). To identify the location of peanut agglutinin (PNA)-binding molecules, sections were incubated for one hour at RT in blocking solution containing PNA-lectin conjugated to FITC (1:200, Vector Laboratories).

Transgene and endogenous mRNA expression was analyzed by whole-mount in situ hybridization, as described previously (Kos et al., 2001). DIG-labeled riboprobes were produced by in vitro transcription of EDNRB2 and EDNRB vectors: the EDNRB2 vector contains a partial sequence amplified from E9 chick cDNA (5′-CCATTGTGCTTGCAGTCCCTGA-3′; 5′-TCC-TGCCCATTGGCTTTCCACT-3′); the EDNRB vector was a gift from Herve Kempf (Harvard Medical School).

Whole embryos treated for TUNEL staining were processed as if for in situ hybridization up to the hybridization step. After 30 minutes in equilibration buffer (Promega), embryos were treated with rTdT solution [90% equilibration buffer, 5 μM DIG-11-dUTP, 10 μM dATP, 1 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 30U rTdT] for 3 hours at 37°C. Embryos were washed overnight at RT in 2×SSC. DIG label was detected using anti-DIG-AP antibodies (0.375U/ml, Roche) and visualized with NBT/BCIP.

In ovo electroporation

Trunk neuroepithelium was transiently transfected with experimental or control vectors (Table 2) by electroporation (Kos et al., 2003) and incubated for 18-72 hours. Expression vectors producing c-KIT, EDNRB2, EphB2 and control short hairpin RNA (shRNA, Table 1) were constructed using the pSilencer 1.0-U6 siRNA Expression Vector (Ambion) or the siSTRIKE-hMGFP U6 Hairpin Cloning System (Promega). A circularized non-hairpin siSTRIKE construct (siSTRIKE-empty) was used as a negative control and as a GFP reporter construct. pSilencer contains no internal fluorescent marker and was co-electroporated with siSTRIKE-empty. The EDNRB2 expression vector contains a 4.2 kb fragment of quail *ENDRB2* cDNA in pCAGGS-Hyg (L. Larue, Institut Curie). The EphB2 expression vector contains the full-length EphB2 seqeunce in pMES (K. Cramer, UC Irvine).

Table 1. Oligonucleotides for shRNA expression constructs

Gene	Insert sequence	shRNA vector
c-KIT	5′-GAATCCTCGCTGGTTGTGATTCAAGAGATCACAACCAGCGAGGATTCTTTTTT-3′ 5′-AATTAAAAAAGAATCCTCGCTGGTTGTGATCTCTTGAATCACAACCAGCGAGGATTCGGCC-3′	pSilencer
pSilencer scramble	5′-GTCAGTCTGGTCGAGTTCATTCAAGAGATGAACTCGACCAGACTGACTTTTTT-3′ 5′-AATTAAAAAAGTCAGTCTGGTCGAGTTCATCTCTTGAATGAACTCGACCAGACTGACGGCC-3′	pSilencer
EDNRB2	5′-ACCGGAAAGAAACTGATCTCAATTCAAGAGATTGAGATCAGTTTCTTTCCTTTTTC-3′ 5′-TGCAGAAAAAGGAAAGAAACTGATCTCAATCTCTTGAATTGAGATCAGTTTCTTTC-3′	siSTRIKE
EphB2	5′-ACCGGCGGGTGATGAAGATTAATTCAAGAGATTAATCTTCATCACCCGCCTTTTTC-3′ 5′-TGCAGAAAAAGGCGGGTGATGAAGATTAATCTCTTGAATTAATCTTCATCACCCGC-3′	siSTRIKE
siSTRIKE-empty	5′-ACCGTGAGACGCTACGC-3′ 5′-TGCAGCGTAGCGTCTCA-3′	siSTRIKE

Cryosections were cut along an 870-μm length of the trunk and immunolabeled as described above. WRS^+ (or $MITF^+$)/GFP^+ cells were counted in every other section and categorized based on their postion: DL, the region between the ectoderm and dermamyotome; MSA, the entire space dorsal to the neural tube between the left and right dermamyotome lips (in contrast to the traditional definition of MSA, we included in our counts any cells dorsal to the neural tube); or V (ventral), the region between the neural tube and the somite. The DL path was also divided into smaller sections: medial DL, lateral DL and lateral mesenchyme (see Fig. S3 in the supplementary material). All results are presented as actual counts and significance was determined by using a Student's *t*-test after a $\log_{10}$ transformation of the data.

Transfection efficiency was determined in embryos electroporated with EDNRB2-siSTRIKE or siSTRIKE-empty at stage 16 and assessed at stage 21. At this stage, no experimental effects were anticipated, as endogenous EDNRB2 has just begun to be upregulated. Counts (WRS^+:GFP^+ cells/total WRS^+ cells) demonstrated that a similar percentage of melanoblasts were transfected in control (46.8±6.5%) and experimental (41.8±10.3%) animals. This indicates that comparable transfection rates were achieved at least for these vectors.

Migration assay

Transfilter migration chambers were comprised of 8-μm-pore PET inserts (BD Falcon), coated on the top and bottom with 10 μg/ml fibronectin in PBS (1 hour at 37°C), placed in a 12-well tissue culture plate (Corning). ET3 (1-10 nM) or PBS was diluted in enriched F12 media (the same as was used for NC cultures) and placed into the bottom well. Melanoblasts from quail neural tube explants were harvested in 1 mM EDTA, resuspended in enriched F12 medium (with or without ET3), and added to the upper wells at a concentration of ~5×10^4 cells/well. Cells were allowed to migrate for 6 hours at 37°C in a 5% CO_2 incubator before being fixed in a solution of 20% methanol and 0.5% crystal violet. The inserts were rinsed in tap water and the upper wells cleaned with a cotton swab. The bottom-side of the filter was imaged across its diameter and the number of attached melanoblasts estimated by determining the percentage area covered using ImageJ software (rsbweb.nih.gov/ij).

Quail-chick chimeras

Eggs were windowed on their side and the embryo visualized using a subectodermal injection of India ink. The vitelline layer was removed and the neural tube excised using tungsten needles. Quail donor neural tubes (isolation technique described above) were positioned dorsal-side up at the site of the excised neural tube in host embryos. The egg was sealed with tape and incubated to the appropriate stage at 37°C.

RESULTS

Melanoblasts upregulate EphB2 and EDNRB2

Melanoblasts reside transiently in the MSA prior to invading the DL path (Weston, 1991; Erickson et al., 1992). Thus, determinants of melanoblast pathway choice must be upregulated during this time and preferentially expressed by this NC subpopulation. Previously we demonstrated that both neuronal and melanogenic NCCs express *EphB2* and *EphB3* (Santiago and Erickson, 2002); however, by quantitative RT-PCR of NCCs cultured from quail neural tubes, we demonstrate that melanoblasts express *EphB2* at significantly higher levels than do neuronal cells (Fig. 1A). This supports the idea that the switch from ventral to DL is mediated by subpopulation-specific Eph receptors.

Based on the expression of *EDNRB2* in sections (Lecoin et al., 1998), we hypothesized that this receptor also plays a role in melanoblast pathfinding. In whole embryos, $EDNRB2^+$ cells were observed in the MSA of the forelimb at stage 19 (Fig. 1C-C″). At stage 20, $EDNRB2^+$ cells were distributed along the length of the trunk and had begun to invade the DL pathway (Fig. 1D-D″). By stage 22, $EDNRB2^+$ cells were present along the length of the embryo and spanned the width of the dermamyotome (Fig. 1E). In transverse sections of the stage-22 embryo, $EDNRB2^+$ staining colocalized with the NC marker HNK1 in cells present along the DL pathway (Fig. 1E′). As *EDNRB2* is upregulated first in the NCCs that are found in the MSA, yet rarely in cells dorsal to the neural tube (Fig. 1C-C″), we speculated that EDNRB2 initiates DL migration.

EphB2 and EDNRB2 are both required for DL migration

To elucidate the role of EphB2 and EDNRB2 in melanoblast pathfinding we used an shRNA-induced knockdown technique. Knockdown was achieved by electroporating *EphB2*-specific

Table 2. Vector combinations for shRNA and overexpression experiments

Experiment	Experimental vectors (1 μg/μl each)	Control vectors (1 μg/μl each)
c-KIT knockdown	c-KIT-pSilencer/siSTRIKE-empty	scramble-pSilencer/siSTRIKE-empty
EphB2 overexpression	EphB2-pMES/siSTRIKE-empty	pBluescript/siSTRIKE-empty
EDNRB2 overexpression	pCAGG-$E2^+$/siSTRIKE-empty	pBluescript/siSTRIKE-empty
EDNRB2 knockdown	EDNRB2-siSTRIKE	siSTRIKE-empty
EphB2 knockdown	EphB2-siSTRIKE	siSTRIKE-empty
EDNRB2 overexpression/EphB2 knockdown	pCAGG-$E2^+$/EphB2-siSTRIKE	siSTRIKE-empty
EphB2 overexpression/EDNRB2 knockdown	EphB2-pMES/EDNRB2-siSTRIKE	siSTRIKE-empty

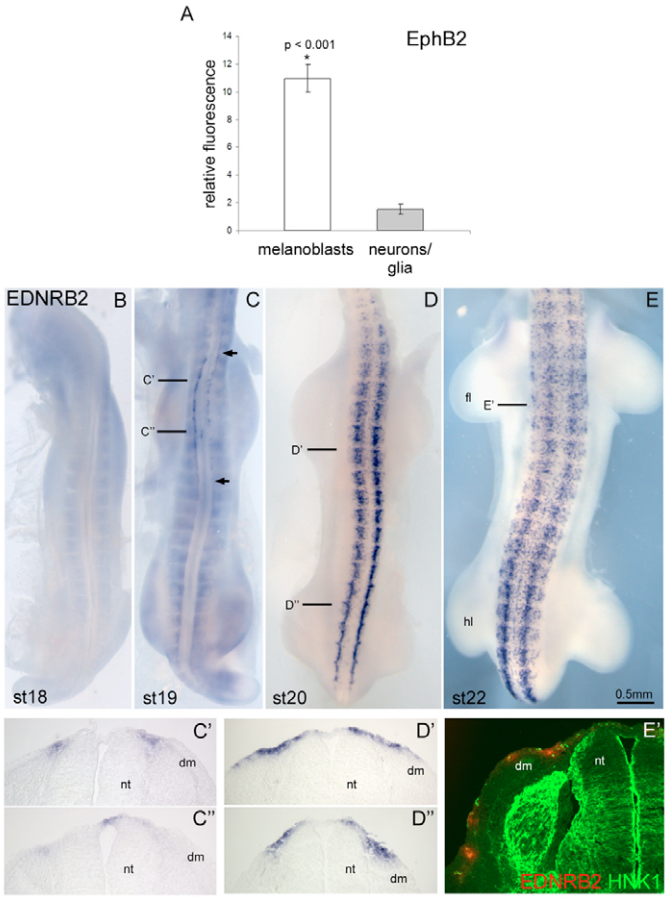

Fig. 1. EphB2 and EDNRB2 expression. (**A**) Quantitative RT-PCR of quail NCCs reveals an increase in *EphB2* expression in melanoblasts. (**B-E′**) Dorsal views of chick embryos processed for in situ hybridization of *EDNRB2*. Horizontal lines in C-E correspond to transverse sections C′-E′. *EDNRB2* is not present at stage 18 (B). At stage 19, *EDNRB2* extends the length of the forelimb (C, between black arrows), is localized to the MSA (C′), and is only rarely observed dorsal to the neural tube (C″). At stage 20, *EDNRB2* expression extends into the tailbud (D) and the DL path (D′,D″). By stage 22, *EDNRB2* is detected along the entire trunk (E), and over the dermamyotome (E′). *EDNRB2* (red, pseudo-colored) colocalizes with the NC marker HNK-1 (E′, green). fl, forelimb; hl, hindlimb; nt, neural tube; dm, dermamyotome.

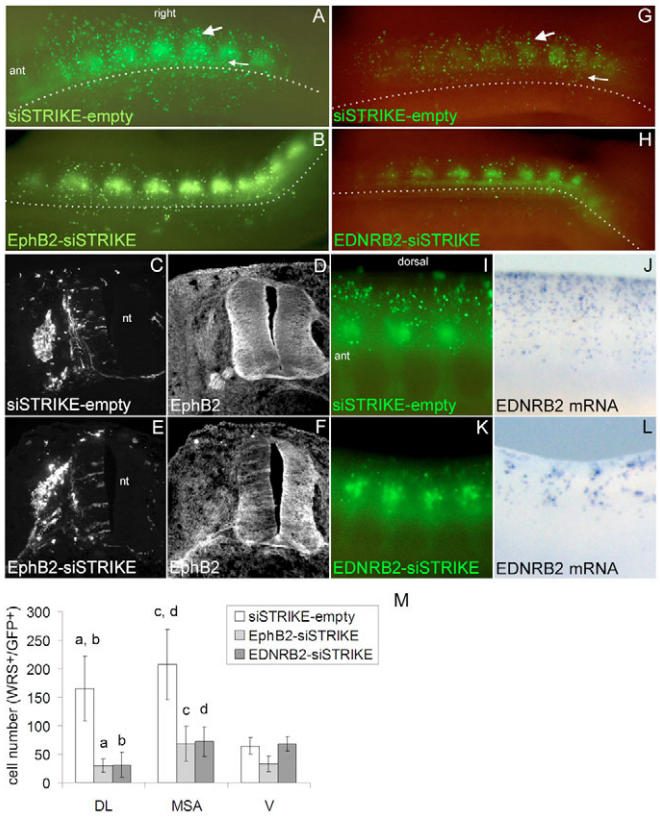

Fig. 2. Loss of EDNRB2 or EphB2 reduces DL migration. (**A**,**B**,**G**,**H**) Dorsal views of chick embryos electroporated with siSTRIKE-empty (A,G), EphB2-siSTRIKE (B) or EDNRB2-siSTRIKE (H) at stage 16 and assayed for melanoblast migration at stage 24. Dotted line marks the region of the dorsal neural tube. In siSTRIKE-empty embryos (A,G), GFP fluorescence is observed ventrally (diffuse, typical of neuronal cells) and laterally (punctuate, typical of melanoblasts) within the MSA (small arrow) and the DL path (large arrow). By contrast, embryos electroporated with EphB2-siSTRIKE (B) or EDNRB2-siSTRIKE (H) lack most dorsolaterally migrating GFP$^+$ cells. (**C-F**) By immunohistochemistry, the EphB2 antibody detects cells of the neuroepithelium, ventral root and DRG, and is unaffected by the expression of siSTRIKE-empty (C,D). EphB2-siSTRIKE effectively knocks down EphB2 protein production (F) on the electroporated side of the neural tube (E). (**I-L**) In lateral views of the trunk, EDNRB2-siSTRIKE embryos (K,L) have a severe reduction in *EDNRB2* expression (by in situ hybridization) in comparison to siSTRIKE-empty embryos (I,J). (**M**) Quantification of WRS$^+$/GFP$^+$ cells counted in transverse sections of siSTRIKE-empty, EphB2-siSTRIKE and EDNRB2-siSTRIKE embryos at stage 24. Data are presented as mean±s.d., with significance set at $P \leq 0.05$. Identical letters above the bars indicate those categories that, when compared, were significantly different (a, $P=0.006$; b, $P=0.047$; c, $P=0.023$; d, $P=0.021$). ant, anterior.

(EphB2-siSTRIKE), *EDNRB2*-specific (EDNRB2-siSTRIKE) or control (siSTRIKE-empty) short hairpin RNA (shRNA) constructs into the trunk neuroepithelium just prior to melanoblast emigration (stage 16). The position of melanoblasts in the DL pathway, ventral pathway or MSA was assayed at stage 24. To detect melanoblasts, we developed a robust marker for these cells derived from the serum of a Jungle Fowl rooster undergoing amelanosis (White Rooster Serum or WRS; see Fig. S1A in the supplementary material). WRS contains antibodies that recognize melanoblasts in quail neural crest culture and in chick tissue sections (Fig. S1B-G). Immunoblot analysis showed that WRS and another melanoblast marker, the Smyth line serum, identify proteins of similar molecular weight (see Fig. S1H in the supplementary material), suggesting that both recognize antigens of tyrosinase-related protein 1 (Austin and Boissy, 1995). WRS specifically labels melanoblasts, melanocytes and the RPE, and was used as the primary melanoblast marker throughout this study.

By stage 24 in embryos transfected with siSTRIKE-empty, GFP$^+$ cells populated the region between the MSA and the lateral-most edge of the dermamyotome. By contrast, in EphB2-siSTRIKE embryos, GFP$^+$ cells were reduced in number and localized primarily to the MSA and the dorsomedial portion of the DL path (Fig. 2A,B). By immunohistochemistry, our EphB2 antibody detects protein only in the neural tube, ventral roots and dorsal root ganglia (Fig. 2C,D). Loss of EphB2 on the electroporated side of the neural tube was evident in sections of embryos treated with EphB2-siSTRIKE, confirming the efficacy of the siRNA effect (Fig. 2E,F).

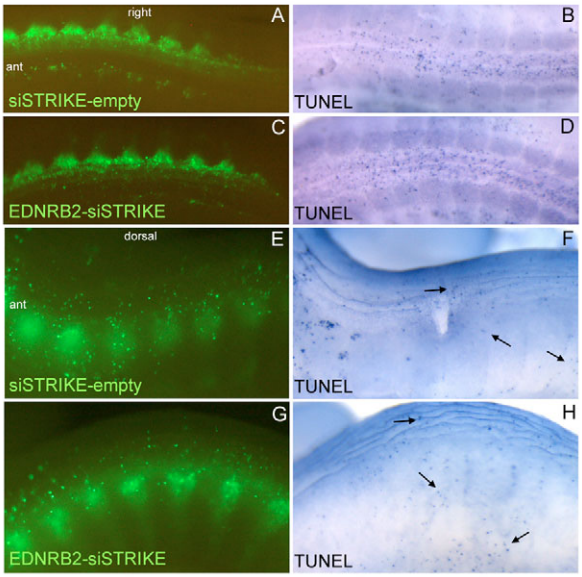

Fig. 3 Loss of EDNRB2 does not lead to increased apoptosis. (**A-H**) Embryos electroporated with siSTRIKE-empty (A,B,E,F) or EDNRB2-siSTRIKE (C,D,G,H) exhibit qualitatively similar numbers of TUNEL-positive cells (indicated by arrows) along their trunks at stage 21 (A-D; dorsal views) and stage 24 (E-H, lateral views). ant, anterior.

EDNRB2-siSTRIKE-treated embryos produced a similar phenotype to those transfected with EphB2-siSTRIKE (Fig. 2H). As mRNA interference is thought to produce gene knockdown by inciting degradation of the targeted mRNA (McManus and Sharp, 2002), we assessed the presence of *EDNRB2* mRNA by in situ hybridization, and demonstrate that EDNRB2-siSTRIKE-treated embryos exhibit a loss of *EDNRB2*$^+$ cells in comparison to control-treated embryos (Fig. 2I-L). Counts of WRS$^+$/GFP$^+$ cells from transverse sections revealed that the number of WRS$^+$/GFP$^+$ cells in the DL path and MSA was significantly higher in control embryos than in EphB2- or EDNRB2-siSTRIKE-treated embryos (Fig. 2M).

In embryos transfected with EphB2- and EDNRB2-siSTRIKE, the reduction of WRS$^+$/GFP$^+$ cells in the DL path did not result in a concomitant increase in the number of transfected melanoblasts in the MSA or ventral pathway (Fig. 2M). Potentially, these receptors are required for melanoblast survival. In particular, ET3, the ligand for EDNRB2, is known to promote the survival of melanoblasts in culture, and thus may play a similar role in vivo (Lahav et al., 1998). TUNEL staining, however, revealed that the numbers and general location of apoptotic cells were similar in control and EDNRB2-siSTRIKE embryos both at the onset of DL migration (Fig. 3A-D) and after melanoblasts had invaded the DL path (Fig. 3E-H).

c-KIT is not required for early melanoblast migration or survival

Significant evidence favors the hypothesis that c-KIT is not involved in the early migration of melanoblasts in aves. However, one observation suggests that c-KIT is worth additional investigation: c-KIT$^+$ melanoblasts are detected migrating from quail neural tube explants at a time that correlates with DL migration in ovo (Luo et al., 2003). We assessed the involvement of c-KIT by co-electroporating c-KIT-pSilencer or scramble-pSilencer and siSTRIKE-empty (as a GFP reporter) into the trunk neuroepithelium at stage 16. In the absence of c-KIT, melanoblasts migrated to a similar extent to those in embryos transfected with scramble-pSilencer (see Fig. S2A,B in the supplementary material). Quantification of WRS$^+$/GFP$^+$ cells revealed no significant difference in the number of shRNA-expressing melanoblasts in the DL path in these embryos (Fig. S2C in the supplementary material). By western blot, we confirmed that c-KIT pSilencer produces shRNA that effectively knocks down ectopic c-KIT-HA expression in CEFs (see Fig. S2D in the supplementary material).

EphB2 and EDNRB2 are both sufficient to induce DL migration of non-melanogenic cells

Transcripts for the ligands of EphB2 and EDNRB2, ephrin Bs and *ET3*, respectively, are detected in tissues flanking the DL pathway preceding melanoblast invasion (Santiago and Erickson, 2002; Nagy and Goldstein, 2006). If EphB2 and EDNRB2 mediate DL entry by responding chemotactically to these cues, then misexpressing these receptors in neuronal precursors should result in their ectopic DL migration. We co-electroporated chick *EphB2* (EphB2$^+$-pMES) or quail *EDNRB2* (pCAGG-E2$^+$) with siSTRIKE-empty (as a GFP reporter) into the trunk neuroepithelium of stage-12 chick embryos and evaluated NC migration at stage 16. In control embryos, neuronal precursors populate the dorsal root ganglia and migrate in distinct segmented streams (Fig. 4A). In embryos that ectopically express *EphB2* or *EDNRB2*, GFP$^+$ cells are instead observed in subectodermal positions (Fig. 4B,C). The response is particularly robust in embryos that misexpress *EDNRB2*; GFP$^+$ cells form a uniform, non-segmented wave (Fig. 4C), typical of melanoblasts (Erickson et al., 1992). In transverse sections, we confirmed that these ectopic GFP$^+$ cells are within the DL pathway and are not melanoblasts because they do not co-label with the melanoblast

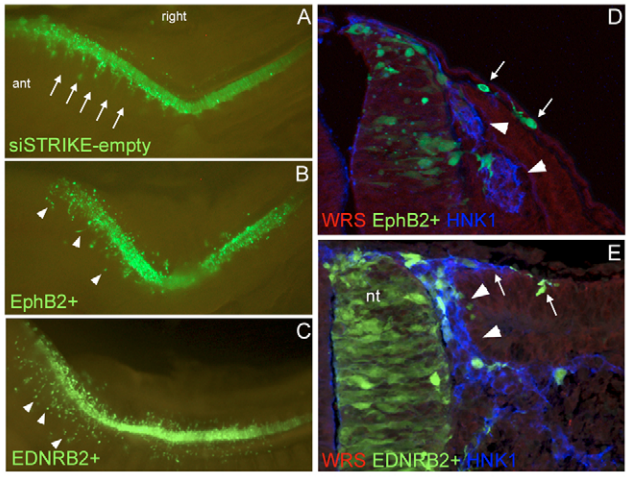

Fig. 4. Overexpression of EphB2 or EDNRB2 in the neurogenic crest leads to ectopic DL migration. (**A-C**) Dorsal views of stage-16 chick embryos co-electroporated at stage 12 with pBluescript/siSTRIKE-empty (A), EphB2-pMES/siSTRIKE-empty (B) or pCAGG-E2$^+$/siSTRIKE-empty (C). In comparison to pBluescript/siSTRIKE-empty control embryos (A), in which cells are organized in segmental streams typical of ventral migration (arrows), GFP$^+$ cells in EphB2-pMES and pCAGG-E2$^+$/GFP reporter embryos (B,C) appear in a subectodermal, non-segmented wave typical of DL migration (arrowheads). (**D**,**E**) Transverse sections of EphB2-pMES/siSTRIKE-empty (D) and pCAGG-E2$^+$/siSTRIKE-empty (E) embryos confirm neural crest cell migration (HNK1$^+$, blue) in both the ventral (arrowheads) and DL pathways (arrows). Cells in the DL path are not recognized by the melanoblast marker WRS (red). ant, anterior; nt, neural tube.

marker WRS (Fig. 4D,E). Although it is established in the chick that only melanogenic NC precursors acquire the appropriate cell autonomous properties to access the DL path (Erickson and Goins, 1995), the misexpression of *EphB2* or *EDNRB2* is sufficient to drive non-melanogenic cells dorsolaterally.

Upregulation of EDNRB2 by melanoblasts determines the timing of DL invasion through a chemotactic response to ET3

At the forelimb, melanoblasts are in the MSA as early as stage 18, but do not invade the DL pathway until stage 20 (Erickson et al., 1992; Reedy et al., 1998a). The upregulation of EDNRB2 just prior to invasion suggests that this receptor is responsible for defining when melanoblasts access the DL path. To test this, pCAGG-E2$^+$ and siSTRIKE-empty (as a GFP reporter) were co-electroporated into stage-16 chick embryos just prior to melanoblast emigration from the neural tube. At stage 19, in embryos transfected with pCAGG-E2$^+$, melanoblasts entered the DL path precociously (Fig. 5B-D). Melanoblasts that do not express EDNRB2 at this stage remained clustered in the MSA (arrowhead, Fig. 5C). When pCAGG-E2$^+$-treated embryos were evaluated at later stages for their position along the DL path (medial DL, lateral DL and lateral mesenchyme; see Fig. S3A in the supplementary material), we found that melanoblasts had reached positions that were more lateral than those in control embryos and that they had increased in number (see Fig. S3B-G in the supplementary material). This latter observation suggests not only that EDNRB2 participates in pathfinding but also that it might act as a mitogen, which is consistent with the effect ET3 exerts on melanoblasts in cell culture (Lahav et al., 1996).

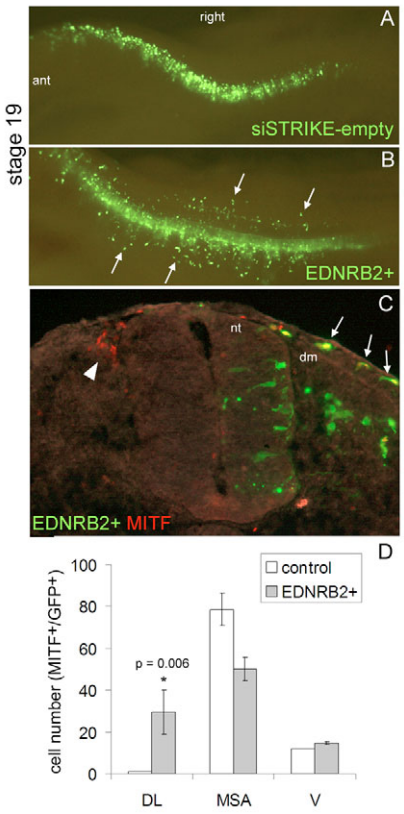

Fig. 5. Overexpression of EDNRB2 in melanoblasts leads to precocious DL migration. (**A**,**B**) Dorsal views of stage-19 chick embryos co-electroporated at stage 15 with pBluescript/siSTRIKE-empty (A) or pCAGG-E2$^+$/siSTRIKE-empty (B). At stage 19, GFP$^+$ cells are seen just dorsal to the neural tube in pBluescript/siSTRIKE-empty control embryos (A). In pCAGG-E2$^+$/GFP reporter embryos, GFP$^+$ cells precociously invade the DL path (B, arrows). (**C**) Transverse section of a stage-19 pCAGG-E2$^+$/GFP reporter embryo in which MITF$^+$ (red) melanoblasts expressing pCAGG-E2$^+$ (green) are observed along the DL path (arrows), whereas those that do not express pCAGG-E2$^+$ remain clustered in the MSA (arrowhead). (**D**) Quantification of MITF$^+$/GFP$^+$ cells in transverse sections confirms the precocious DL migration of melanoblasts in pCAGG-E2$^+$-treated embryos. Data are presented as mean±s.d., with significance (*) set at $P \leq 0.05$. ant, anterior; dm, dermamyotome; nt, neural tube.

Previous work has shown that melanoblasts respond chemotactically to ephrin B1 ligands (Santiago and Erickson, 2002); however, it is not known how EDNRB2/ET3 mediates melanoblast migration. We therefore assessed the role of ET3 as a chemoattractant. Melanoblasts were placed in the upper well of a trans-well filter and challenged to migrate in response to ET3. The presence of 1 nM and 5 nM ET3 significantly increased the ability of melanoblasts to migrate toward the bottom chamber; however, 10 nM ET3 diminished this response (Fig. 6A). When ET3 was placed in the top chamber, melanoblast migration was not significantly different from in medium alone, demonstrating that ET3 does not increase migration chemokinetically (Fig. 6B).

We demonstrated above that the loss of EphB2 or EDNRB2 disrupts proper DL invasion. If these receptors both respond chemotactically to cues present in the DL path it remains to be explained why the presence of the remaining receptor is insufficient to guide melanoblasts. One possibility is that these receptors act additively to overcome DL inhibitory cues. To test this hypothesis, we investigated whether overexpression of one receptor can compensate for the loss of the other. When we knocked down EphB2 and simultaneously overexpressed EDNRB2, the EphB2-siSTRIKE phenotype was ameliorated (Fig. 6C,D). In whole-mount, GFP$^+$ cells were observed at a similar density and position in these embryos to in control embryos (compare to Fig. 2A). Overexpression of EDNRB2 also restored counts of GFP$^+$ melanoblasts back to control levels, rather than to those seen in EphB2 knockdown embryos (compare with Fig. 2M; average cell count±s.d.: DL, 215±62.39; MSA, 129±22.5; V, 32±3.6). Immunolabeled sections of these embryos demonstrated that ectopic EDNRB2 does not rescue the EphB2 migratory defect by directly affecting EphB2 protein levels (Fig. 6E,F). The converse experiment – knockdown of EDNRB2 while misexpressing EphB2 – produced a modest rescue phenotype in two out of six embryos. In these two embryos, GFP$^+$ cells were not as numerous as in EDNRB2$^+$/EphB2-siSTRIKE embryos, and they appeared somewhat rounded (Fig. 6G).

EDNRB2 and EphB2 signaling is specific only for the DL migration of melanoblasts

At the vagal axial level (somite 1-7), there are two waves of NCCs that migrate dorsolaterally (Reedy et al., 1998b). The first occurs at stage 10 and involves the cells that will populate the pharyngeal arches and heart; the second occurs at stage 18 and involves the melanoblasts (Kuratani and Kirby, 1991; Reedy et al., 1998b). Because both subpopulations use the same migratory pathway, we predict that they also share signaling programs. However, by in situ hybridization analysis, *EDNRB2* was not detected in cells that migrate during the first DL wave at the vagal level (Fig. 7A). These cells instead expressed *EDNRB* (Fig. 7B,B′). *EDNRB2* was upregulated later, in the melanoblasts that migrate during the second DL wave (data not shown). This demonstrates that EDNRB2 is not

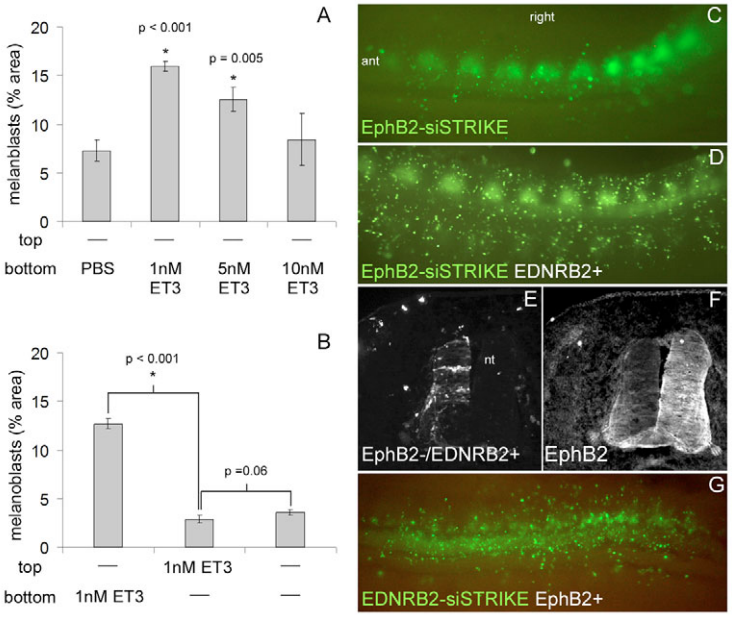

Fig. 6. EDNRB2 acts as a chemoattractant and can rescue loss of EphB2. (**A**,**B**) Melanoblasts placed in the top well of a trans-filter migration chamber migrate to the bottom side of the filter in response to 1 and 5 nM ET3; however, this effect is diminished at 10 nM (A). Melanoblasts only migrate to the bottom side of the filter when in the presence of a chemotactic gradient of ET3. When ET3 is in the top well, migration into the bottom well does not vary from that of control wells, showing that ET3 does not produce a chemokinetic effect (B). (**C-F**) Dorsal views of chick embryos electroporated along the trunk with EphB2-siSTRIKE or EphB2-siSTRIKE/pCAGG-E2$^+$ at stage 16 and assayed at stage 24. Embryos electroporated with EphB2-siSTRIKE (C) have reduced numbers of GFP$^+$ cells that migrate into the DL path; however, this loss can be rescued by the overexpression of pCAGG-E2$^+$ (D, compare with Fig. 2A). Overexpression of EDNRB2 in combination with EphB2 knockdown does not rescue the EphB2 phenotype by affecting EphB2 protein levels (E,F). (**G**) Dorsal view of a chick embryo electroporated along the trunk with EDNRB2-siSTRIKE/EphB2-pMES at stage 16 and assayed at stage 24. EphB2 overexpression mitigated EDNRB2 knockdown in two out of the six embryos analyzed. In these embryos, GFP$^+$ cells appear more rounded than in embryos electroporated with EphB2-siSTRIKE/pCAGG-E2$^+$. ant, anterior; nt, neural tube.

specific for DL migration, and is required only by melanoblasts. We also found that, at the vagal level, little to no *ephrin B1* (Baker and Antin, 2003) or PNA-binding (Fig. 7C,D) was detected along the DL path during the first wave of migration. We hypothesize that, although vagal NCCs do not benefit from the positive influence of EDNRB2 or EphB2 activation (lack of ephrin B1 as a chemoattractant), they invade the DL path because the repulsive factor ephrin B1 and the non-adhesive PNA-binding molecules are not present in their local environment.

To investigate this, we challenged the first wave of vagal NCCs to migrate in the presence of DL inhibitory cues at the trunk axial level. We removed the neural tube from a stage-13/14 chick host from the region of the last three somites and replaced it with a stage-10 quail neural tube from the axial level of somites 1-3 (Fig. 8A). In these chimeras, quail NCCs (QCPN$^+$/HNK1$^+$) migrate to the dorsal root and sympathetic ganglia, but none are found in the DL path (Fig. 8B,B′). Next we assessed the ability of neuronal precursors, which normally populate ventral structures and do not express EphB2 or EDNRB2 at high levels, to migrate dorsolaterally at the vagal axial level. We removed the neural tube from a stage-10 chick host from the region of somites 1-3 and replaced it with a stage-13/14 quail neural tube from the axial level of the trunk (Fig. 8C). In these chimeras, quail NCCs were observed on the DL path (Fig. 8D,D′).

DISCUSSION

Migration barriers in the DL pathway are believed to be the primary reason that neuronal and glial NCCs are forced to migrate along the ventral pathway. If the dermamyotome is ablated, NCCs enter the DL path precociously (Erickson et al., 1992; Oakley et al., 1994). This suggests that the tissues that comprise the DL path possess inhibitory properties. However, because melanoblasts eventually access this space, the DL path must be inhibitory only transiently. Two mechanisms by which this inhibition might be alleviated are the loss of repulsive molecules in the environment and/or cell-autonomous changes in the repertoire of receptors expressed by melanoblasts that stimulate their migration. In support of the former, repellants such as chondroitin-6-sulfate, PNA-binding molecules and F-spondin are all present in the DL space during ventral NC migration, but recede at the onset of melanoblast migration (Oakley et al., 1994; Debby-Brafman et al., 1999). In support of the latter, Slit1 and Slit2, secreted chemorepellants, remain indefinitely in the DL space, and melanoblast migration is coincident with the downregulation of the Robo receptors that mediate repulsion (Jia et al., 2005). However, permissiveness of the DL path owing to the loss of and inability of melanoblasts to respond to DL inhibitory molecules does not adequately explain why these cells

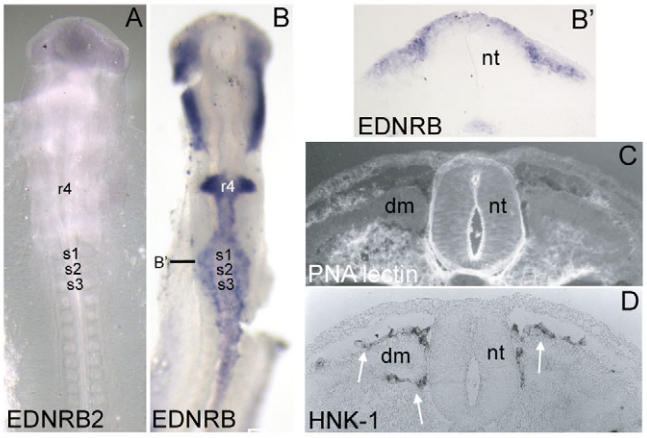

Fig. 7. Absence of *EDNRB2* and PNA-binding molecules at the vagal axial level. (**A**,**B**) In situ hybridization reveals a complete lack of *EDNRB2* at the vagal axial level of a stage-11 chick embryo (A). Conversely, *EDNRB* is observed in the head mesenchyme, in rhombomere 4, over the dorsal neural tube and extending laterally from the first three somites (B). (**B′**) In a transverse section, *EDNRB* is localized to the region dorsal to the neural tube and the DL path. (**C**,**D**) A transverse section of a stage-13 embryo exhibits high levels of PNA-binding in the sclerotome and low levels in the lateral DL pathway, and is absent from the dorsal portion of the DL pathway (C). HNK1$^+$ NCCs (arrows) migrate into regions of the DL and ventral pathways that are not occupied by PNA glycoconjugates (D). r, rhombencephalon; s, somite; nt, neural tube; dm, dermamyotome.

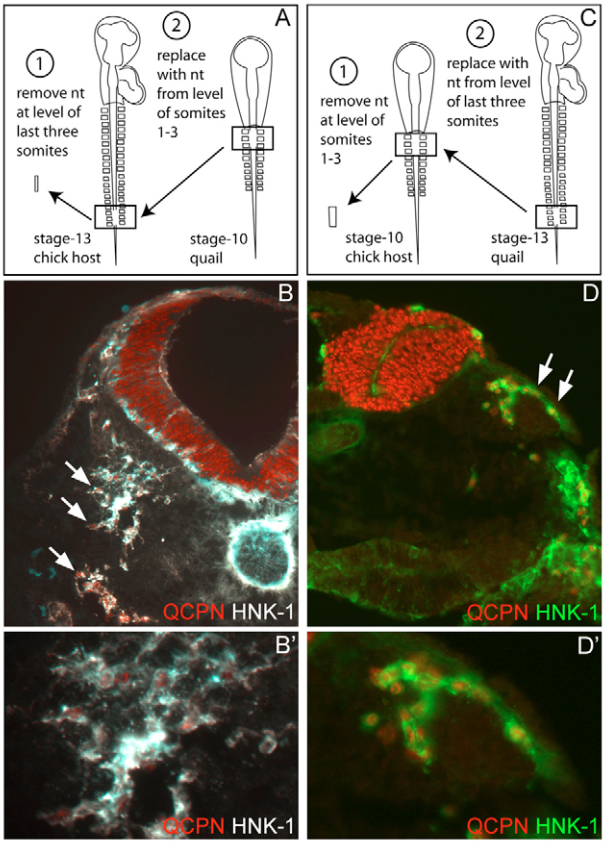

Fig. 8. Vagal neural crest cells do not migrate dorsolaterally at the trunk axial level; neuronal cells migrate dorsolaterally at the vagal axial level. (**A**,**C**) Schematics depicting the heterotopic/chronic transplantation of quail neural tube (donor) into a chick host. (**B**,**B′**) Transverse section of a quail/chick chimera in which vagal NCCs are transplanted into a trunk environment. QCPN$^+$ (red nuclei) NCCs (HNK1$^+$, white) have migrated into the ventral pathway (arrows), but none are observed along the DL pathway. High magnification of the ventral path is shown in B′. (**D**,**D′**) Transverse section of a quail/chick chimera in which neuronal cells are transplanted into a vagal environment. QCPN$^+$ (red nuclei) NCCs (HNK1$^+$, green) are observed on the DL pathway (arrows). High magnification of the dorsolateral path is shown in D′.

stereotypically prefer the DL path over the ventral one. Nor does it explain how melanoblasts grafted into an early embryo will take the DL pathway precociously before these inhibitory molecules recede (Erickson and Goins, 1995).

EphB2 in DL pathfinding

We speculate that the pre-specification of melanoblasts results in the upregulation of receptors that determine their pathfinding. This notion is supported by our previous discovery that melanoblasts respond chemotactically to soluble ephrin B1 ligands in trans-well migration assays and that substratum-adsorbed ephrin B1 increases their attachment to fibronectin (Santiago and Erickson, 2002). However, until now, the specific EphB receptor that mediates this attraction was unknown. By RT-PCR, we show that EphB2 is expressed at higher levels in melanoblasts than in neurogenic precursors. We also demonstrate that EphB2 is necessary and sufficient for the dispersal of NCCs on the DL path. These data, in combination with in situ studies showing that EphB3 is detected in ventrally migrating NC (Krull et al., 1997), suggest that ephrin B ligands initiate the collapse response in the neurogenic crest through EphB3 and the stimulatory response in melanoblasts through EphB2. Thus, pathway choice could simply be controlled by the modulation of a cell's response to ephrins through differential Eph receptor expression.

Despite the attractive simplicity of this model there is evidence that other signaling systems contribute to the transition between migratory pathways. Tosney (Tosney, 2004) has shown that emerging dermis provides a long-distance, chemoattractive cue, and that this dermis, if placed near the MSA in young embryos, results in precocious melanoblast migration. One explanation for this phenomenon is that the dermis produces matrix metalloproteases that cell-bound ephrin B ligands require to create a soluble gradient to which melanoblasts respond. However, we have shown that neuronal cells misexpressing EphB2 will migrate ectopically into the DL space independently of the emergence of the dermis. This suggests that the DL path generates a different, as yet unidentified, attractive substance.

EDNRB2 in DL pathfinding

Pigmentary defects in *Kit* and *Ednrb* mutants of mouse and zebrafish demonstrate the importance of these receptors in melanoblast morphogenesis. Thus, their ligands are good candidates for dorsolaterally derived guidance cues. However, the extent to which these receptor/ligand pairs are involved in melanoblast pathfinding across species is variable. In the chick, it is not until melanoblasts enter the DL space that they upregulate c-KIT and, as predicted, we have shown that embryos treated with *c-KIT* shRNA do not exhibit defects in early melanoblast migration.

ET3, however, has proven more promising. *ET3* is expressed by the ectoderm and dermamyotome during NC cell migration (Nataf et al., 1998; Nagy and Goldstein, 2006), and functions as a chemotactic factor (shown here). Furthermore, avian melanoblasts uniquely express the endothelin receptor EDNRB2. EDNRB2 is required for normal pigment patterning in adult birds (Miwa et al., 2007), and we have shown that it contributes to the specific migratory behavior of melanoblasts. In aves, melanoblasts migrate along the DL pathway 24 hours after neurogenic NCCs commence their migration ventrally (Loring and Erickson, 1987; Teillet et al., 1987; Serbedzija et al., 1989). This delay has been attributed to the late emigration of melanoblasts from the neural tube (Reedy et al., 1998a) and their transient accumulation in the MSA prior to invading the DL path (Weston, 1991; Le Douarin and Kalcheim, 1999). From our observations that EDNRB2 is upregulated in melanoblasts in the MSA and can induce the ectopic or premature DL migration of neurogenic NCCs and melanoblasts, respectively, we further propose that EDNRB2 acts as the gatekeeper to trigger the advance of melanoblasts from the MSA to the DL path.

Integrating the roles of EphB2 and EDNRB2

Our finding that both EphB2 and EDNRB2 are necessary for the recruitment of melanoblasts into the DL path raises the question of how these signaling pathways are integrated. We demonstrate by shRNA-mediated downregulation of EDNRB2 that endogenous levels of EphB2 are not sufficient to maintain DL melanoblast migration. Similarly, EDNRB2 cannot compensate for the loss of dorsolaterally migrating melanoblasts after EphB2-siSTRIKE knockdown, except when overexpressed. This suggests that the expression levels of receptors that positively influence migration must exceed a certain threshold in order to induce DL invasion. This model is reinforced by two observations: (1) overexpression of

EDNRB2 in non-melanogenic NC results in a switch in their migration from ventral to DL; and (2) although both melanoblasts and neurogenic crest express EphB2, the latter expresses EphB2 at a level that is not sufficient to drive this subpopulation dorsolaterally. Pathway selection must therefore result from the combination of receptors produced by NCCs and their expression levels.

Still, it remains to be understood why overexpression of EphB2 only occasionally compensates for the loss of EDNRB2. Previous reports show that endothelin signaling participates in melanoblast proliferation (Lahav et al., 1996), in defining the number of melanoblast precursors (Van Raamsdonk et al., 2004), and in melanoblast fate maintenance (Dupin et al., 2000; Dupin et al., 2003). Therefore, without EDNRB2 the effects of EphB2 rescue on pathfinding may be obscured by the overall loss of melanoblasts by any of these means.

A model for the DL migration of melanoblasts

We have developed a model for DL pathfinding that states that in order for NCCs to invade the DL path their response to attractive cues must exceed their response to inhibitory cues. In order to substantiate our model, we studied NC migration at the vagal level, where NCCs invade the DL space in the absence of EDNRB2 and EphB2 activation. Here, there is an absence of the non-permissive/repulsive cues that are found in the DL space at other axial levels. When first-wave, vagal NCCs are challenged to migrate at the trunk axial level they populate only ventral structures. As NCCs at the vagal axial level begin expressing EphB3 as early as stage 8 (Baker et al., 2001), we predict that their inability to migrate dorsolaterally in the trunk is due to their response to the repulsive ephrin B ligands and PNA-binding molecules that are present there. Conversely, we demonstrate that in an environment that is free of inhibitory molecules, like at the vagal axial level, neuronal NCCs access this space freely.

Conclusion

We have explored the role of c-KIT, EphB2 and EDNRB2 in DL pathfinding, and have found that only the last two are required for the invasion of the DL pathway by melanoblasts. We show that melanoblasts respond chemotactically to ET3, as they do to ephrin B1, and that these ligands together, by the activation of their receptors, define the unique spatiotemporal migratory pathway taken by melanoblasts. Furthermore, we demonstrate that these receptors appear to be required by NCCs to initiate DL migration only when this space contains inhibitory molecules.

In the future, we will want to investigate the specific threshold of EphB2 and EDNRB2 expression levels (and/or loss of inhibitory cues) that is sufficient to switch NC cell migration from the ventral to the DL pathway. It will also be interesting to examine whether this model applies to other systems, such as the mouse, where subpopulations of NCCs migrate onto their respective pathways simultaneously.

We thank Lionel Larue, Herve Kempf, Ray Boissy, Jim Weston, Mark Reedy, Elena Pasquale and Karina Cramer for providing us with the EDNRB2 expression construct, *EDNRB* cDNA probe, Smyth line serum, 7B3 antibody, c-KIT expression construct, EphB2 antibody and EphB2 expression construct, respectively. We are also grateful to Jackie Pisenti for bringing the White Rooster to our attention, and for providing blood samples and photo opportunities. This work is supported by grants from the NIH and the American Heart Association to C.A.E.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4113/DC1

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