



COLEC12 and TRAIL Signaling Confine Cranial Neural Crest Cell Trajectories and Promote Collective Cell Migration

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**Colec12 and Trail Signaling Confine Cranial Neural Crest Cell Trajectories
and Promote Collective Cell Migration**

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Keywords: Colec12, Trail, agent-based modelling, chick, neural crest, inhibition, collective cell migration, developmental biology

Running title: Colec12 and Trail promote collective cell migration

ABSTRACT

Background: Collective and discrete neural crest cell (NCC) migratory streams are crucial to vertebrate head patterning. However, the factors that confine NCC trajectories and promote collective cell migration remain unclear.

Results: Computational simulations predicted that confinement is required only along the initial one-third of the cranial NCC migratory pathway. This guided our study of Colec12 (Collectin-12, a transmembrane scavenger receptor C-type lectin) and Trail (tumor necrosis factor-related apoptosis-inducing ligand, CD253) which we show expressed in chick cranial NCC-free zones. NCC trajectories are confined by Colec12 or Trail protein stripes *in vitro* and show significant and distinct changes in cell morphology and dynamic migratory characteristics when co-cultured with either protein. Gain- or loss-of-function of either factor or in combination enhanced NCC confinement or diverted cell trajectories as observed *in vivo* with 3D confocal microscopy, respectively, resulting in disrupted collective migration.

Conclusions: These data provide evidence for Colec12 and Trail as novel NCC microenvironmental factors playing a role to confine cranial NCC trajectories and promote collective cell migration.

INTRODUCTION

Vertebrate development critically relies on the long distance migration of loosely connected neural crest cells (NCCs) that are sculpted into discrete streams. After reaching peripheral targets throughout the face and neck, cranial NCCs give rise to multiple cell types including bone, cartilage, and neurons (LeDouarin and Kalcheim, 1999). Failure to maintain discrete NCC migratory streams may lead to improper anterior-to-posterior craniofacial patterning, resulting in birth defects termed neurocristopathies (Vega-Lopez et al., 2018; Siismets and Hatch, 2020). Repair of neurocristopathies may result in significant costs associated with successive corrective surgeries (Cerrizuela et al., 2020; Ibarra and Atit, 2020). Thus, a better understanding of the mechanisms that underlie the confinement of cranial NCC trajectories and promotion of collective cell migration would provide insights into the root causes of neurocristopathies and inform emerging stem cell-based tissue repair strategies (Liu and Cheung, 2016).

Despite the discovery of chemical signals that attract NCCs towards peripheral targets (McLennan et al., 2010; Kasemeier-Kulesa et al., 2010; Olesnick-Killian et al., 2009; Theveneau et al., 2013), several unanswered questions remain. In this paper, we address the following questions: (i) what are the signals that confine cranial NCC trajectories to stereotypical migratory pathways; and (ii) how do these signals promote collective NCC migration? In the head, cranial NCCs emerge all along the vertebrate axis of the hindbrain and are sculpted to exit into the paraxial mesoderm adjacent to even-numbered rhombomeres (denoted by r). The tissue origins and molecular components of the signals within the hindbrain that regulate cranial NCC behaviors immediately after dorsal neural tube exit have been well-studied (reviewed in Trainor et al., 2000; Kulesa et al., 2004). For example, repulsive signals present in the dorsal neural tube microenvironment during r3 neuroepithelium and r3 surface ectoderm interactions prevent cranial NCCs from turning anterior or posterior shortly after exiting from the midline of either r2 or r4, through Erbb4 receptor and Neuregulin ligand interactions (Golding et al., 2000). After NCCs exit into the paraxial mesoderm, disruption of Semaphorin-Neuropilin signaling either by overexpression of soluble Neuropilin-1/2 Fc in chick (Osborne et al., 2005) or in mice carrying null mutations for either Neuropilin-2 or its ligand Sema3F (Gammill et al., 2006) results in diversion of r4 NCCs away from the stereotypical migratory pathway. This loss of NCC confinement immediately adjacent to the neural tube (within 0-200 um from the dorsal midline) results in the formation of narrow, single cell-wide cellular bridges between the r2 and r4 NCC migratory streams. Together, these signals prevent uncontrolled NCC invasion shortly after

neural tube exit and suggest the presence of other inhibitory signals further away from the influence of the dorsal hindbrain that confine NCC trajectories.

Signals that confine cranial NCC trajectories near the target branchial arches 1-4 (ba1-4) are well-studied. Complementary expression of EphA4/EphB1 receptors and Ephrin-B2 ligand on migrating *Xenopus* cranial NCCs restricts intermingling of closely juxtaposed r4 and r6 streams to ensure proper invasion into ba2 and ba3, respectively (Smith et al., 1997). More recently, Versican has been reported to confine *Xenopus* cranial NCC migration. However, its knockdown by morpholino reveals that migrating NCCs do not undergo widespread intermingling between neighboring streams as expected, complicating its role as a permissive or inhibitory factor (Szabo et al., 2016). Moreover, *Versican* expression is present in *Xenopus* placodal tissues near the branchial arches, suggesting a later role to prevent NCC stream mixing prior to branchial arch entry (Szabo et al., 2016). In the chick, examination of Eph/ephrin expression in cranial NCC streams that are more widely separated by NCC-free zones revealed EphA3, EphA7, and EphB3 expression in migrating r4 cranial NCCs (Mellott and Burke, 2008). Although EphB2 and Ephrin-B1 confine migrating chick cranial NCCs in protein stripe assays, their *in vivo* expression in the mesoderm lateral to the otic vesicle (EphB2) and within the target ba2 (Ephrin-B1) make these factors unlikely candidates to confine *in vivo* cranial NCC trajectories between the dorsal neural tube and branchial arch entrances. Together, these static studies implicate microenvironmental signals within the head mesoderm in confining NCC trajectories near the dorsal neural tube exit or branchial arch entrances. However, the identification of microenvironmental factors along the cranial NCC migratory pathways and their function in regulating dynamic *in vivo* cell behaviors to promote collective migration remain unclear.

Computational models are well-equipped to investigate the role of confining signals in collective cell migration. Szabo and colleagues (2016) used agent-based modeling in the context of tightly coupled *Xenopus* NCC clusters to predict that the optimal two-dimensional confinement width (coinciding with the antero-posterior length along the axis from which cells emigrate) is proportional to the number of migrating cells. From this work, a more detailed mechanochemical model of *Xenopus* NCC cluster migration integrating cell polarity signaling through Rac1 and RhoA (Merchant et al., 2018; 2020), confirmed these predictions. Szabo and colleagues (2019) then integrated their data on *Versican* expression to simulate the interaction between migrating

NCC clusters with distally located placodal cells. They interpreted their experimental results and model simulations to speculate that confinement of NCC trajectories promoted collective cell migration by keeping cells close to each other, allowing for proper functioning of contact inhibition of locomotion (CIL)/Co-attraction (Co-A) (Woods et al., 2014). However, the width of this corridor must be optimized since the CIL/Co-A mechanism breaks down when cells are confined to a narrow corridor (Merchant et al., 2020).

In contrast to modeling the migration of tightly clustered NCCs, agent-based models of loosely connected NCC streams have elucidated the leader-follower mechanism of collective cell migration; leader cells readout guidance signals and communicate them to follower cells (McLennan et al., 2012; 2015). The discovery of the BMP-antagonist Dan (Differential Screening-Selected Gene Aberrant in Neuroblastoma) present in the chick paraxial mesoderm and *in vitro* confinement of cranial NCCs in stripe assays posed the question of its *in vivo* function (McLennan et al., 2017). By using this modeling framework that relaxed the previous two-dimensional reflecting boundary conditions present in both leader-follower (McLennan et al., 2012, 2015) and CIL/Co-A (Woods et al., 2014) models and replaced them with a Dan-region, simulations showed that slowing leader cell migration through the Dan-region resulted in robust collective cell migration (Schumacher et al., 2016). Thus, computational modeling frameworks offer a powerful and rapid approach to determine the mechanisms and parameters of cell confinement and collective cell migration and are, therefore, well-placed to guide experimental investigation.

In this study, we integrated computational modeling and experiments to study the requirements of spatial confinement of NCC trajectories and how this confinement promotes collective cell migration. Model simulations focused on determining the minimum requirements of a “spatial confinement boundary” along the anterior and posterior borders of a two-dimensional migratory domain extending in the distal direction away from the neural tube and to maintain a discrete NCC stream over long distances. This helped to direct experimental analyses of previously undescribed factors in the embryonic NCC microenvironment as Colec12 (Collectin-12 (or CL-12), a transmembrane scavenger receptor C-type lectin; Ma et al., 2015) and Trail (tumor necrosis factor-related apoptosis-inducing ligand, CD253; Fleten et al., 2016), part of a subset of genes we identified by profiling chick cranial mesoderm (McLennan et al., 2017). By using our integrated RNAscope fluorescence *in-situ* hybridization (FISH), immuno-histochemistry, and

tissue clearing method (Morrison *et al.*, 2017a), we analyzed the three-dimensional expression patterns of Colec12 and Trail with respect to migrating cranial NCCs in the intact chick embryo. Protein stripe assays and co-culture in the presence of either Colec12 or Trail protein allowed us to evaluate the confinement of cranial NCC trajectories and dynamic changes in cell morphology and migratory characteristics. After *in vivo* gain- or loss-of-function of single or multiple combinations of these factors, we measured the extent of NCC diversion away from stereotypical migratory streams and into typical NCC-free zones, using confocal time-lapse imaging to visualize changes in cell behaviors. Together, our results predict the spatial requirements to confine cranial NCC trajectories to discrete streams, characterize Colec12 and Trail expression as consistent in space and time with computer model predictions, and demonstrate their functional roles to confine NCC trajectories and promote collective cell migration.

RESULTS

In our previous work, we focused our computational modeling of cranial NCC migration solely on the corridor connecting r4 to ba2 with boundary conditions adjacent to (rostral and caudal borders) along the entire length (proximal-to-distal) of the migration corridor to ensure that cells stay confined to the corridor (Fig. 1). Here, we relax this assumption and, instead consider the larger domain that also includes the nearest neighboring corridors emanating adjacent to r3 and r5. In this case, the corridor of interest (r4 to ba2) is now a subset of this larger domain (Fig. 1A). Within this larger domain, we will consider internal boundary conditions that confine cells to the corridor of interest by imposing zero flux boundary conditions along part of the r4-ba2 corridor (Fig. 1A). We refer to such cases as an internal confinement boundary.

Model simulations predict the full length of an internal confinement boundary is not required to maintain discrete streams over long distances

We use our computational model to explore the effects of spatial confinement on the cranial NCC migratory pattern. The model is of hybrid off-lattice (agent-based) type, in which cells are agents whose motility is coupled to a continuum, reaction-diffusion model of the dynamics of the known cranial NCC chemoattractant vascular endothelial growth factor VEGF (McLennan *et al.*, 2010) on a growing rectangular two-dimensional domain (described in Experimental Procedures). In the model, cells adopt one of two possible phenotypes, leader or follower, and

here we specify a fixed number of leader cells. The cells may change their phenotypes based on their position within the migratory stream (see Experimental Procedures). Leader cells sample the microenvironment through the extension of three filopodia and move in the direction of the highest concentration of chemoattractant sensed, provided it is higher by a threshold value than that at the position of the center of the cell (the cell moves randomly if there is no measured difference). If follower cells are in a stream, defined as a group of cells that are close to each other with at least one of the cells a leader, they move in the same direction as the leader cell in that stream. If follower cells are in a tunnel, they move along the tunnel defined as a directed path created by a leader cell. If follower cells are not in a stream or tunnel, the cells move randomly.

In simulations of the full length of an internal confinement boundary with follower cells guided by stream and/or tunnels, we find cells reach the target without a breakdown of collective cell migration (that is, the majority of follower cells are in streams and/or tunnels) (Fig. 1A). In contrast, without an internal confinement boundary, cells may migrate away from the stereotypical pathway, resulting in a considerable breakdown of collective migration (Fig. 1B). To compare the migratory patterns of cells in the model for increasing the extent of an internal confinement boundary (Fig. 1C), we quantified two different stream characteristics. We first consider the likelihood of a breakdown of collective migration, defined as the fraction of cells not in streams or tunnels at the end of a simulation (Fig. 1D,E). We determined that breakdown of collective migration is most likely when the internal confinement is short (0 – 200 μ m) in both model scenarios: 1. When follower cells may only form streams (Fig. 1C,D); 2. When cells can form streams and move through leader-created tunnels (Fig. 1C,E). This result is to be expected because when the cells are free to travel in an unrestricted wide region, it is more difficult for them to find streams or tunnels, resulting in breakdown of collective migration. The likelihood of breakdown of collective migration decreases as the extent of an internal confinement boundary is increased, again, as expected (Fig. 1C-E). Unexpectedly, the model predicts that even with a relatively short spatial confinement length of 0-600 μ m (Fig. 1D; streams) or 0-500 μ m (Fig. 1E; stream and/or tunnels) the likelihood of breakdown in collective migration is low (that is, efficient collective cell migration), implying the critical importance of an internal confinement boundary as cells exit the neural tube and start the first phase of migration (Fig. 1D-F). The average likelihood of breakdown in collective migration is almost zero for an internal confinement boundary of length greater than or equal to 600 μ m (Fig. 1D,E). We conclude that the cell confinement along the entire length of the migratory domain is not necessary to avoid

breakdown in collective migration. To this end, biological inhibitory signals are only required in the approximately first one-third of the migratory domain.

We also investigated to what extent the cells spread out in the y-direction when different lengths of an internal confinement boundary are considered (Fig. 1G-I). To this end, we defined three subregions, recorded the y-coordinate of each cell at the end of a simulation and calculated the fractions of cells in different subregions. Subregion 1 corresponds to the observed NCC migratory domain (width - 120um, Fig. 1G; R1). Subregion 2 corresponds to a weak deviation from the biologically realistic migratory domain (width - 240, Fig. 1G; R2); subregion 3 corresponds to a large deviation from the biologically realistic NCC migratory domain (width - 360um, Fig. 1G; R3). We find that there is a monotonic decrease in the fractions of cells in subregions 2 and 3 when the length of the internal confinement is increased in both model scenarios (Fig. 1H,I). Taken together, simulations of our model, which considers chemotactic cell movement and various cell-cell interactions with varying levels of cell confinement, demonstrate that unknown inhibitory signals within the neural crest microenvironment play a crucial role in the collective migration of NCCs from neural tube exit through approximately the first one-third of the stereotypical r4 migratory pathway.

Colec12 and Trail are present in the chick cranial mesoderm and show restricted expression within cranial NCC-free zones

COLEC12 has been characterized in vascular endothelial cells and its knockdown in zebrafish causes severe defects in vasculogenesis and development (Fukuda et al., 2011). TRAIL and its receptors, TRAIL-R1 and -R2, selectively trigger apoptotic cell death in tumor cells and has been extensively studied as a target of cancer treatment (Micheau et al., 2013; Snajdauf et al., 2021). However, the expression of both factors has not been examined in the embryonic NCC microenvironment and there is no known functional role for either factor in collective cell migration.

To first determine the mRNA expression patterns of Colec12 and Trail with respect to migrating cranial NCC positions, we performed a detailed analysis across chick developmental stages corresponding to NCC exit from the neural tube throughout migration to the branchial arches

(stages HH11, HH13, and HH15; using Hamburger and Hamilton (HH), 1951). Using multiplexed fluorescence *in situ* hybridization on whole (HH11) or half mount embryo heads (HH13,15), we determined that both *Colec12* and *Trail* expression is enhanced within the presumptive NCC-free zone adjacent to r3 (Fig. 2A,B). Specifically, we find that the rostral and caudal borders of *Colec12* expression at HH11-13 within the tissue adjacent to r3 are juxtaposed to the r1-r2 and r4 NCC migratory streams marked by *Sox10* expression (Fig. 2A). *Trail* expression in the subregion adjacent to r3 is slightly diffuse in comparison to *Colec12* (Fig. 2B) with *Sox10* expression marking the migrating NCCs (Fig. 2B, open circle). Transverse sections through the hindbrain clearly show that *Colec12* mRNA is present in the mesoderm adjacent to the neural tube and extending to approximately 400um away from the neural tube (Fig. 2C).

Cranial neural crest cells avoid *Colec12* and *Trail* protein *in vitro* stripe assays

To test whether NCCs avoid *Colec12* and/or *Trail* protein in culture, we explanted cranial neural tubes onto stripe assays and performed both static and time-lapse analyses (Fig. 2D-F). Static analysis of NCC positions after 12 hours of incubation on the stripe assays showed a dramatic difference in the number of cells on either *Colec12*-, or *Trail*-containing stripes (Fig. 2D,E). Migrating NCCs mostly avoided *Colec12*- or *Trail*-containing stripes; only around 25% of migrating NCCs were found on these protein stripes, in comparison to the negative control that showed a nearly equal distribution of migrating NCCs on and off stripes (Fig. 2D,E). Time-lapse imaging of dynamic NCC behaviors revealed distinct responses depending on the presence of either *Colec12* or *Trail* protein (Fig. 2E). Specifically, when exposed to *Colec12* stripes, the initial NCCs to delaminate from the neural tube avoided the *Colec12*-positive stripes and appeared to form discrete streams (Suppl Fig. 1B; see Movie 1). Some NCCs that exited onto the *Colec12*-positive stripes rapidly reversed direction back towards the explanted neural tube (Suppl Fig. 1C). As NCCs moved within the corridors between the *Colec12*-positive stripes, cells were observed to move onto *Colec12*-positive stripes, but did so in a rapid manner to cross to a neighboring stream of NCCs on a *Colec12*-negative stripe (Suppl Fig. 1D; see Movie 1). In contrast, when cranial neural tubes were explanted onto *Trail* protein stripes, we observed the delaminating NCCs retract cell protrusions. NCCs that did delaminate successfully from the explanted neural tubes crowded onto the control stripes in clusters and immediately avoided the *Trail*-positive stripes (Fig. 2D). Any individual NCCs observed on the *Trail*-positive stripes migrated perpendicular to the stripes and onto the control stripes (data not shown). These data

clearly demonstrate that either Colec12 or Trail protein may confine NCC trajectories, with distinct cell behaviors in response to the protein stripes.

Colec12 or Trail protein introduced onto the NCC migratory pathway enhanced confinement of cell trajectories

To begin to test the *in vivo* effects of perturbing Colec12 and Trail signaling on cranial NCC migration, we over-expressed these factors by microinjection of the same human recombinant proteins as used in the stripe assays. Microinjections were directed into the paraxial mesoderm adjacent to r4, prior to NCC emigration from the dorsal neural tube (Fig. 3). After 16 hours of egg re-incubation, we harvested embryos and compared the NCC migration pattern on injected versus non-injected (control) sides, using HNK1 to fluorescently mark migrating NCCs (Fig. 3A-D). Introduction of either Colec12 or Trail led to enhanced confinement of NCC streams, as measured by the reduction in area covered by the typical migratory stream in comparison to the non-injected side of the embryo (Fig. 3E). Control PBS injections saw no significant changes (Fig. 3E). Combined microinjection of both Colec12 and Trail simultaneously into the r4 paraxial mesoderm led to a stronger phenotype of reduced cranial NCC confinement (Fig. 3E). These results show that introduction of Colec12 and/or Trail protein directly onto the cranial NCC migratory pathway prior to NCC emigration can significantly enhance the confinement of *in vivo* NCC trajectories.

Knockdown of Colec12 leads to precocious invasion of cells into the presumptive neural crest cell-free zones and disruption of collective cell migration

To determine whether blocking of Colec12 signaling would result in NCC invasion into typical NCC-free zones, a translation-blocking morpholino was used to reduce the expression of Colec12 in the typical NCC-free zone adjacent to r3. After 24 hours of egg re-incubation and embryo harvesting, we observed significant changes to migrating NCC morphologies adjacent to the NCC-free zone, increased numbers of migrating NCCs within the mesoderm lateral to r3, and an increased area of NCC migratory streams when compared to control sides in the same embryo or embryos transfected with a control morpholino (Fig. 4A-F). NCCs along the rostral border of the r4 NCC migratory stream had distinct cell protrusions towards the subregion lateral to r3 and a disruption in cell morphologies was observed in Colec12 MO injected vs control MO

embryos (Fig. 4A-D, insets). To quantify this phenotype during mid-migration of NCCs from the neural tube to the end of the branchial arches, we performed the same experiment but re-incubated eggs for 12 hours only. We found that the number of NCCs that invaded the typical r3 NCC-free zone was significantly higher when compared to control sides of the same embryo or embryos transfected with a control morpholino (Fig. 4E). Moreover, there was a significant difference in the typical area covered by the r4 NCC migratory stream in *Colec12* MO embryos, reducing collective cell migration over long distances (Fig. 4F).

In order to thoroughly investigate the dynamic responses of cranial NCCs to the inhibitory nature of *Colec12* *in vivo*, we transfected mesodermal cells with *Colec12* morpholino or control morpholino and performed time-lapse confocal imaging of intact chick embryos. When endogenous *Colec12* was knocked down in the typical r3 NCC-free zone, we observed two unique phenomena. First, the r4 NCC migratory stream widened towards the r3 NCC-free zone – this was not observed on the control side (Fig. 4G, first frame of sequence at t=0). Second, in a typical time-lapse imaging session we observed a secondary stream of NCCs (Fig. 4G, arrow at t=2h) that diverted from the r4 NCC migratory stream (Fig. 4G, asterisk) and continued to invade into the r3 NCC-free zone (Fig. 4G, arrow at t=4h). Together, these data clearly demonstrate an *in vivo* role for *Colec12* in confining cranial NCCs to the stereotypical r4 migratory pathway and promoting collective cell migration.

Combined knockdown of *Colec12* and *Trail* enhanced invasion of neural crest cells into the presumptive neural crest cell-free zones and further disrupted collective cell migration

In humans, TRAIL has five known receptors, including the death receptor 4 (DR4), DR5, decoy receptor 1 (DR1), DR2, and osteoprotegerin (Pan et al., 1997; Walczak et al., 1997; MacFarlane et al., 1997; Emery et al., 1998) and of these, the avian system has two homologs. *Tnfrsf10b* and *Tnfrsf11b* are the avian homologs of the human genes of the same names; *Tnfrsf10b* contains a cytoplasmic death domain, while *Tnfrsf11b* is a secreted decoy receptor. Both of these receptors are expressed by migrating r4 cranial NCCs at the RNA level (Morrison et al., 2017b). To inhibit cranial NCC interactions with Trail, we transfected premigratory NCCs with morpholinos designed against *Tnfrsf10B* and *Tnfrsf11B*. When either *Tnfrsf10b* only or both receptors are knocked down and embryos harvested and analyzed after 12 hours, we find

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3 invasion of NCCs into the typical r3 NCC-free zones (Fig. 4H,J). We then focused on inhibiting
4 both *Colec12* and *Trail* proteins by microinjection and transfection of the *Colec12* morpholino
5 directly into the mesoderm adjacent to r3 and either *Tnfrsf10B* morpholino only or both *Trail*
6 receptor morpholinos into premigratory NCCs. When both *Colec12* and *Trail* signaling were
7 inhibited, we observed a significant increase in the number of migrating NCCs and extensive
8 invasion of the r3 NCC-free zone at both 12 and 24 hours after embryo harvesting and imaging
9 (Fig. 4I,K). Specifically, both individual cells and NCC streams diverted from the r4 migratory
10 stream and invaded the r3 NCC-free zone; with a more striking phenotype at the 12 hr time
11 point (Fig. 4I,K). These data strongly support the roles of *Colec12* and *Trail* in confining cranial
12 NCC trajectories and signaling through mutually exclusive pathways to promote collective cell
13 migration.
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23 **Cranial neural crest cells exposed to *Colec12*, or *Trail* protein in the culture media show** 24 **significant and distinct changes in cell morphology and dynamic migratory** 25 **characteristics** 26 27

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29 To better understand the function of *Colec12* and *Trail* in influencing NCC migratory behaviors
30 and to promote collective cell migration, we plated isolated cranial neural tubes in the presence
31 of *Colec12* or *Trail* protein added directly into the culture media (Fig. 5A). Changes in cell
32 morphology and cell behaviors were visualized using time-lapse confocal imaging and
33 quantified (Fig. 5B-E). We find that NCCs cultured in the presence of *Colec12* protein exhibited
34 persistent, lengthened protrusions with increased branching (Fig. 5B; compare Ctrl vs +*Colec12*
35 images; compare Movies 2 and 3). When individual NCCs were closely observed in the
36 presence of *Colec12* protein, we noticed that the tips of each extended filopodium appeared
37 unable to retract (see Movie 3). Static measurements after 8 hours in culture confirmed
38 significantly longer filopodial extensions (Fig. 5E); cell speed and displacement in the presence
39 of *Colec12* protein were unchanged (Fig. 5C-D).
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49 In striking contrast, when cranial NCCs were exposed to *Trail* protein in the culture media,
50 NCCs appeared to adhere to one another and move as cell clusters rather than individual cells
51 (Fig. 5B; compare Ctrl vs +*Trail* images, compare also Movies 2 and 4). Cell speed and
52 displacement were significantly reduced (Fig. 5C-D); filopodial lengths were unchanged (data
53 not shown). Closer observation of individual cells revealed changes in cell morphology to
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3 resemble a more-rounded phenotype and amoeboid-like motility prior to clustered migration
4 (Suppl. Fig. 1E). This phenotype was consistent with observations in the *in vitro* Trail protein
5 stripe experiments (see Fig. 2); the rounded-up cell morphology was more apparent in the
6 presence of Trail protein in comparison to Colec12 and control media (Suppl. Fig. 1F,G). In all
7 culture experiments with any of the aforementioned factors, there was no change in cell
8 directionality (data not shown). Thus, cranial NCC dynamic behaviors are dramatically affected
9 by the presence of Colec12 or Trail in the culture media and the distinct changes in cell
10 morphology and migratory characteristics suggest signaling through distinct downstream
11 pathways.
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20 **DISCUSSION**

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22 We have identified and tested the function of two previously undescribed embryonic neural crest
23 microenvironmental factors, Colec12 and Trail to confine chick cranial NCC trajectories and
24 promote collective cell migration. Computational model simulations of the interplay between cell
25 chemotaxis and cell communication predicted that changes to the cell confinement boundaries
26 within approximately the first one-third (0-400um) of the approximately 1000-1200 um
27 stereotypical cranial NCC proximal-to-distal (x-direction) migratory pathway would result in
28 diversion of NCC trajectories along the anterior-to-posterior axis (y-direction) and disruption of
29 collective cell migration. In agreement with model predictions, we confirmed enhanced
30 expression of Colec12 and Trail expression within the first one-third of the NCC-free zone
31 adjacent to r3, using multiplexed FISH analysis. Loss-of-function of Colec12 and/or Trail
32 resulted in diversion of NCC trajectories in the y-direction into the r3 NCC-free zone within the
33 first one-third of the migratory pathway and disrupted collective cell migration. By combining *in*
34 *vitro* Colec12 or Trail protein stripe and co-culture assays with confocal time-lapse microscopy,
35 we observed changes in NCC migratory characteristics, cell morphologies, and filopodial
36 dynamics. Together, these data provide unique insights into the spatial requirements to confine
37 cranial NCC trajectories to discrete streams and cell-microenvironmental interactions that
38 promote collective NCC migration.
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52 Computational model simulations predicted the unexpected result that signals confining cranial
53 NCC trajectories are not required along the entire stereotypical migratory pathway from the
54 dorsal neural tube to the branchial arches. Such predictions highlight the ability of model
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simulations to rapidly provide insights into the interplay and balance between cell chemotaxis, cell communication, and inhibitory signals to drive collective NCC migration. Our previous modeling efforts had determined the conditions under which a leader NCC responds to a gradient of VEGF chemical signal and invades a two-dimensional domain growing uniformly in time (McLennan et al., 2012, 2015). This provided a foundation to examine leader-to-follower cell communication in more detail to be either by leader-to-follower contact (promoting stream migration) or indirectly by NCCs following the path forged by a leader (McLennan et al., 2020). In this study, we were able to examine how relaxation of the 2D migratory domain boundaries along the anterior-to-posterior axis (y-direction) affected collective NCC migration along the proximal-to-distal axis (x-direction) (Figs. 1, 6). For computational convenience, we imposed zero flux boundary conditions at the internal boundary for VEGF but this, as we already have zero flux for cells at the boundary, did not significantly affect the qualitative outcome of our key model prediction. Namely, that full model confinement is not necessary for invasion of the branchial arches. Since otic vesicle formation occurs in the subregion adjacent to r5 during cranial NCC migration, it is experimentally challenging to validate the model prediction along the caudal border of the r4 migratory stream, but reasonable along the rostral r3/r4 boundary as performed in this study. To test this, the 2D model may be deployed to analyze trunk NCC migration, where NCCs are confined to discrete streams through neighboring caudal somite halves that offer access to molecular and surgical manipulation. Future modeling efforts may also explore how tissue-based expansion of the chick head mesoderm, recently shown to be heterogeneous in space and time (McKinney et al., 2020) affects the expansion of *Colec12* and *Trail* expression domains and this relationship to NCC discrete migratory streams.

Our expression analysis and *in vitro* protein stripe data support an inhibitory role for *Colec12* and *Trail* that may be mined for insights into how changes in dynamic cell behaviors promote collective NCC migration. Our integrated RNAscope, immunohistochemistry, and tissue clearing approach allowed us to visualize and confirm the spatial restriction of *Colec12* and *Trail* expression to the subregion lateral to the hindbrain at the axial level of r3, a typical NCC-free zone, with respect to the position of Sox-10 labeled migrating NCCs (Fig. 2). Further, we find that *Colec12* protein is expressed adjacent to r3 and r4, and in the tissue along the rostral border of the r4 NCC migratory stream (Suppl. Fig. 1A). This provided motivation to pursue *in vitro* protein stripe experiments that confirmed the restriction of uncontrolled NCC migration (Fig. 2). NCCs were confined to migrate in a very directed manner between *Colec12* and *Trail* protein stripes (Fig. 2) with cell morphologies aligned parallel to the protein stripes (Fig. 2). NCCs were

more confined between the Colec12 or Trail protein stripes the further cells migrated away from the neural tube explant (Fig. 2D,F). Knockdown of Colec12 led to changes in the morphology of r4 NCCs immediately adjacent to the neighboring NCC-free zone adjacent to r3; NCCs displayed enhanced protrusions in the rostral direction (Fig. 4) perpendicular to the direction of the target, ba2 (Fig. 4).

We observed distinct changes in NCC migratory characteristics and morphology depending on co-culture with either Colec12 or Trail, suggesting that NCCs use different signaling pathways in response to encountering these factors in the chick head mesoderm (Fig. 5). We were surprised to find that co-culture with Colec12 protein led to protracted filopodia, since we were expecting NCCs to collapse cell protrusions in its presence. However, this observation may represent its function to inhibit rather than repel cell movements (Fig. 5). We speculate that lengthened cell protrusions may provide a means for wayward NCCs in the anterior-to-posterior axis (y-direction) to re-contact cells moving along the proximal-to-distal stereotypical migratory pathway (x-direction). In support of this, we have previously observed *in vivo* NCCs rejoining a neighboring stream after migrating into cranial NCC-free zones (Kulesa and Fraser, 1998) and an inability to retract filopodial protrusions after blocking RhoA (Rupp and Kulesa, 2007). Future experiments may shed light on the signals downstream of Colec12 and Trail that regulate cell cytoskeletal properties.

In contrast to our Colec12 observations and the typical NCC migratory behavior as individuals, we observed NCCs adhering to one another and cluster migration in the presence of Trail protein (Fig. 5). This resulted in decreased average cell speed and displacement (Fig. 5). These features were observed *in vivo* in the Colec12/Trail double knockdown, as invasive NCCs formed discrete linear stream-like arrays of connected cells between the ba1/ba2 streams (Fig. 4). These stream-like arrays have previously been observed in mice carrying null mutations for either *Npn2 (neuropilin2)* or *Sema3F* and typically within the first 200um of the NCC migratory pathway (Gammill et al, 2006). The close proximity of these stream-like arrays in mice and not further downstream may be as a result of *Sema3F* expression in the hindbrain (r3 and r5) rather than paraxial mesoderm. In the presence of Trail protein in the culture media, we also observed several NCCs to adopt a rounded phenotype, but continue to move in an amoeboid-like manner (Suppl. Fig. 1E-G; see also Movie 4). Together, these quantitative data may now be integrated into our modeling framework to predict how changes in NCC migratory characteristics and cell morphology may influence collective NCC migration.

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5 The receptors for COLEC12 are currently unknown. Future mass spectrometry experiments
6 may help to determine candidates whose expression may then be verified *in vivo* on migrating
7 cranial NCCs and provide a foundation for loss-of-function studies. COLEC12 has also
8 previously been shown to have high binding specificity for glycans containing a terminal Lewis-X
9 structure (Coombs et al., 2005). It will be interesting to determine whether Lewis-X is expressed
10 in a similar spatial location as Colec12, adjacent to r3 in chick. Further investigation of the role
11 of Lewis-X in Colec12-mediated inhibition of NCC migration may include pre-incubated Colec12
12 with Lewis-X before preparing the protein stripe assays and a repeat of the experiments with
13 Colec12 protein stripes described above, including addition of Lewis-X antibodies (C3D-1 and
14 HI98) into the culture media that have previously been shown to block its function (Brazil et al.,
15 2016). This would allow the evaluation of whether inhibitory effects of Colec12 are enhanced by
16 Lewis-X. Together, these data would demonstrate the potential for investigation of signals
17 downstream of Colec12 that may regulate NCC migratory behaviors.
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28 The above findings may have important implications for human neural crest-derived cancers
29 and other aggressive cell phenomena. Interest in TRAIL gained momentum after the
30 observation that TRAIL could selectively kill cancer cells but not normal cells (reviewed in
31 Kimberley and Screaton, 2004). This was based on the expression of decoy receptors that
32 could sequester Trail and divert it away from death receptors initially found to be restricted to
33 normal cells (Pan et al., 1997). However, the presence or absence of decoy receptors may be
34 an unreliable indicator of sensitivity, and in some tumors Trail expression has been implicated
35 as an immune evasion mechanism (Koyama et al., 2002). In treatment of melanoma, emerging
36 therapeutic strategies are combining known clinically approved kinase inhibitors with TRAIL-
37 induced apoptosis, using second generation TRAIL receptor agonists (Fleten et al., 2016).
38 However, TRAIL resistance in metastatic melanoma remains a problem (Eberle et al., 2019).
39 Future studies that examine the downstream signals in embryonic NCCs that escape TRAIL-
40 induced apoptosis may shed light on the interplay of downstream signals that prevent the
41 activation of *Caspase-3* as a no return step in apoptosis and inform the design of synthetic
42 TRAIL receptor agonists. Furthermore, since human metastatic melanoma cell lines may be
43 readily transplanted onto the vascularized chick chorioallantoic membrane (CAM) (Bailey and
44 Kulesa, 2014) or into the chick embryonic NCC microenvironment (Kulesa et al., 2006;
45 Kasemeier-Kulesa et al., 2018), these *in vivo* models may offer a more rapid means than typical
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xenografts to test the efficiency of emerging TRAIL sensitizing agents. Also, COLEC12 has been studied in the innate immune system and implicated in brain development and muscular dystrophy, respectively (Ohtani et al., 2001). However, its functional role in cancer remains largely unexplored (Koudelkova et al., 2017). Identification of the receptor(s) for Colec12 ligand and the study of the role of Lewis-X mentioned above may provide the foundation to investigate the potential for COLEC12 to mitigate human neural crest-derived cancer cell invasion.

EXPERIMENTAL PROCEDURES

Computational model

In the computational model, we assume that there are two types of cells, namely “leaders” and “followers”, with a fixed number of leader cells. The leaders undertake a biased random walk with volume exclusion up a cell-induced gradient of chemoattractant. We use a reaction-diffusion equation to model the dynamics of the chemoattractant VEGF. The leaders perform this biased random walk by extending three filopodia in random directions per time step. These filopodia can sense the concentration of chemoattractant at their tip, and the cell moves in the direction of the highest concentration sensed, provided it is higher by a threshold value than that at the position of the center of the cell. If there is no measured difference, then the cell moves in a random direction. On the other hand, followers are either in a stream, tunnel or move randomly. A stream consists of a group of followers that are close to each other with at least one of the cells close to a leader. All the followers in a stream move in the same direction as the leader that is at the front of the stream. If a follower is close to more than one leader, so that it may be part of more than one stream, then the cell randomly chooses which leader to follow. The tunnelling mechanism, which approximates the extracellular matrix (ECM) degradation induced by the leader cells (McLennan et al., 2020), is modelled by recording the history of leader positions, which we define as a “tunnel”. If a follower is sufficiently close to a tunnel, then it starts moving along that tunnel towards the front of the stream. We include a simplified version of phenotype switching between leaders and followers based on the position of a cell within a migratory stream.

We assumed a two-dimensional rectangular growing domain to describe the region between the neural tube and the branchial arches. We assumed that the growth of the domain is uniform in

space and logistic in time. The boundary conditions are modelled as follows (Fig. 6E): new cells enter the domain at a constant rate at the left-hand (proximal) boundary (the neural tube). There is an attempt to insert a new cell at every time step with a center at a random position along the y-axis with the coordinate " $x = \text{cell radius}$ ", but volume exclusion impedes constant influx, that is a new cell may not be inserted if by entering the domain it will overlap with another cell which is already in the domain. The influx of neural crest cells is only allowed at the left-hand boundary of the rectangular domain, while zero-flux boundary conditions for cells are set at the other three boundaries of the rectangular domain. Zero-flux boundary conditions for the cells are defined as follows: a cell cannot move outside the domain but it may extend its filopodia outside the domain. If a subset of its filopodia are extended outside the domain, then the direction of cell movement is determined by the filopodia inside the domain. If all of its filopodia are extended outside the domain, then the cell does not move. We assume zero flux boundary conditions everywhere for the chemoattractant. When the extent of the internal confinement boundary assumption is relaxed, the boundary conditions are applied to a larger domain specified by the confinement restrictions.

To investigate the effect of the proximal-to-distal length of an internal confinement boundary we defined the following different cases: a full internal confinement boundary, no internal confinement and a partial internal confinement boundary (of varying proximal-to-distal length). In the full internal confinement case, the migratory domain is defined as a rectangular domain of width 120um with the zero-flux boundary conditions for chemoattractant and NCCs imposed along the top and bottom bounding edges, *ceteris paribus* (Fig. 1A,6E). This domain corresponds to the migratory domain previously used in the models of chick NCC migration (McLennan et al. 2012, 2015, 2017). To model the case with no internal confinement, we simply use a domain of width 360um with zero-flux boundary conditions for chemoattractant and NCCs imposed along the top and bottom bounding edges (Fig. 1B). In the partial internal confinement case, part of the domain is modelled as in the full internal confinement case, and part of the domain as in the no internal confinement case (Fig. 1F). In the no internal confinement case, we still assume that the cells only enter the domain in a confined region (width 120um). We assume that the length of the internal confinement boundary does not elongate as the domain grows. We fix the value of the confinement boundary length. This assumption is used to clearly distinguish the effect of a fixed internal confinement boundary length but in reality, the internal confinement boundary could be elongating as the domain grows and, in that scenario, the

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fraction of the confined domain may not remain constant, due to our previous observation domain growth is spatially non-uniform (McKinney et al., 2020). Boundary conditions for the chemoattractant (VEGF), NCCs and internal confinement are shown in Fig. 6E).

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Fertilized White Leghorn chicken eggs (Centurion Poultry, Inc., Lexington, GA) were incubated in a humidified incubator at 38 deg C to the desired developmental stage (HH; Hamburger and Hamilton, 1951).

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Identification of Colec12 and Trail in the Chick Embryonic Neural Crest

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As previously described in McLennan, et al. (2017) a microarray screen was performed to identify genes enriched in the neural crest cell exclusion zone adjacent to r3. Briefly, at stages HH11, HH13, and HH15, tissue was isolated in triplicate from the subregion adjacent to r3 and from leader NCCs from the r4 migratory stream and then analyzed by microarray analysis. Model simulations helped to guide us to compare gene expression in the two sets of samples and generate a short list of candidate genes with elevated expression in the subregion adjacent to r3.

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Multiplexed Fluorescence *In Situ* Hybridization by RNAscope

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RNAscope on whole chick embryos was performed as previously described (Morrison et al., 2017a). Briefly, embryos were harvested and fixed in 4% paraformaldehyde for two hours at room temperature. Following a dehydration gradient in methanol, embryos were stored overnight at -20 deg C. After rehydration, embryos were digested in diluted protease solution from Advanced Cell Diagnostics (Newark, CA). Sox10, Colec12, and Trail RNAscope probes were designed by Advanced Cell Diagnostics against GenBank accession numbers NM_204792, NM_001039599, and NM_204379, respectively. Probes were hybridized with embryos overnight at 40 deg C and were amplified and labeled the following day. The embryos were then optically cleared by the FRUIT method (Hou et al., 2015) prior to imaging. Probes were ordered from IDT (Coralville, IA), pooled, and extended as described in Kishi et al., 2019. Embryos were harvested, fixed, dehydrated in methanol, and rehydrated as described above for

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3 RNAscope. Embryos were then incubated in 5 ug/mL proteinase K (V3021, Promega, Madison
4 WI) in PBST (PBS + 0.1% Tween-20) for 5 minutes at room temperature. Embryos were
5 washed three times with PBST, then twice with hybridization wash buffer (2× SSC, 1% Tween-
6 20, 40% formamide). Extended probes were diluted in hybridization buffer (2× SSC, 1% Tween-
7 20, 40% formamide, 10% dextran sulfate), 2 ug in 200 uL total volume, and hybridized with
8 embryos overnight at 43 deg C. Embryos were washed three times with hybridization wash
9 buffer at 43 deg C, washed twice with 2× SSC + 0.1% Tween-20 at 43 deg C, then returned to
10 room temperature and washed twice with PBST. Complementary imager probes ordered from
11 IDT were diluted to 0.2 uM in 200 uL hybridization buffer (1× PBS, 0.2% Tween-20, 10%
12 dextran sulfate) and hybridized with embryos at 37 deg C for 30 minutes. Embryos were
13 washed three times with PBST at 37 deg C, then returned to room temperature for imaging.
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23 ***In Vitro Neural Crest Cell Assays***

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25 Stripe assays using cranial neural tubes (r3-r5) were performed as previously described (Krull et
26 al., 1997). Stripe assays were generally performed using 40 µm stripe matrices (Karlsruher Inst
27 Fur Technologie, Karlsruhe, Germany). In order to visualize the stripes, Texas Red-BSA
28 (A23017, ThermoFisher Scientific, Waltham, MA) was added to all stripe solutions at 20 ug/mL.
29 Control stripes contained no added protein. Experimental stripes contained recombinant human
30 Colec12 or Trail proteins (2690-CL-050, 375-TL-010; R&D Systems, Minneapolis, MN) at the
31 concentrations listed in the results section of this paper. Cranial neural tubes were adhered to
32 the prepared stripe assay plates and incubated in 5 ug/mL Hoechst in media for five minutes,
33 which was then removed and replaced with fresh media. The plates were transferred to an LSM
34 800 (Zeiss, Oberkochen, Germany) with an incubator box set to 37 deg C. After at least 1 hr of
35 equilibration time, the neural tubes and surrounding area were imaged overnight in 5-minute
36 time intervals. Cells were automatically detected using spot detection in Imaris (Bitplane) and
37 counted at the 12 hr time point as on or off stripes and normalized to stripe area as calculated
38 by Imaris. P-values were calculated using a standard Student's *t*-test. Data distribution was
39 assumed to be normal, but this was not formally tested. The number of explanted neural tubes
40 analyzed were (Negative Ctrl=5, Colec12=4, Trail=6) and the number of cells counted were
41 (Ctrl=629, Colec12=349, Trail=428). For neural crest cultures with protein added to the media,
42 cranial neural tubes (r3-r5) were isolated and cultured as previously described (McLennan et al,
43 2010). Colec12 or Trail protein was added to media at 20 ug/mL, or 2 ug/mL, respectively.
44 Cultures were imaged as described above for neural crest stripe assays. Cells were
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3 automatically detected and tracked using spot detection in Imaris, which generated speed and
4 straightness calculations for each track. P-values were calculated using a standard Student's *t*-
5 test.
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11 ***In Vivo* Perturbations**
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13 All morpholinos were designed and synthesized by Gene Tools, LLC (Philomath, OR, USA).
14 Electroporations of morpholino into the dorsal neural tube were performed as previously
15 described (McLennan and Kulesa, 2007). To electroporate morpholino into the paraxial
16 mesoderm, fluorescein-labeled morpholino was first combined with a carrier plasmid (pMES-
17 h2b-mCherry) at 1:1 ratio, giving a morpholino concentration of 0.5 mM and carrier
18 concentration of 2.5 ug/uL. The morpholino/carrier mixture was injected at multiple sites in the
19 mesoderm on one side of the neural tube near the hindbrain in HH9 embryos, and electrodes
20 were placed above and below injections for electroporation. After electroporation, sterile
21 Ringer's solution was pipetted onto the embryo, and the opening in the eggshell was sealed
22 with tape. For over-expression experiments, Colec12 or Trail protein was microinjected into the
23 mesoderm adjacent to r4 at a concentration of 500ug/ml and volume of approximately 2-4
24 picoliters. Eggs were then re-incubated for 16 hours, harvested, and fixed in 4%
25 paraformaldehyde at room temperature for 2 hours. Embryos (at least n=12 for each
26 experiment) were then processed for HNK1 staining to visualize migrating NCCs. Morpholinos
27 were either injected and electroporated with Dil before being re-incubated for 12 hours or
28 injected and electroporated without Dil before being re-incubated for 16 hours. Time-lapse
29 imaging was performed as previously described in McKinney et al., 2013.
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43 **Image Analysis and Measurement of Projected Area of NCC Migration**
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45 We calculated the projected area covered using the "Surfaces" function of Imaris (Bitplane) to
46 create a surface mask. We then calculated the area of the fluorescence signal (HNK1) using the
47 masked arch surface. We set a consistent intensity threshold to the same value for each
48 dataset, a surface grain size of 1 um was set, the diameter of the largest sphere was set to 1
49 um, and then the automatic "Surfaces" function was applied. The box plots were generated by
50 using the values from each dataset indicated. X indicates outliers, and the box plots and
51 whiskers indicate the quartiles and range, respectively, of each dataset. P-values were
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3 calculated using a standard Student's *t* test or paired *t* test. Data distribution was assumed to be
4 normal, but this was not formally tested.
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8 9 **Time-Lapse Imaging of Whole Chick Embryos on EC Culture**

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11 Live embryos for time-lapse imaging were mounted on EC culture dorsal-side down beginning
12 at HH9 or 10 modifying the protocols (Chapman et al., 2001; McKinney et al., 2013), such that
13 the EC culture was plated with only 500uL of liquid to reduce light scattering. Confocal z-stacks
14 were collected every 5 minutes for up to 12 hours with a 10x 0.45 objective.
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18 19 **Immunostaining**

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21 Immunohistochemistry was performed on fixed whole embryos or heads only by first
22 permeabilizing in PBS + 0.5% Triton X-100 at room temperature for 1 hour. Embryos were
23 incubated in blocking buffer (PBS + 0.1% Triton X-100 + 4% BSA) for 2-4 hours at room
24 temperature, then incubated in primary antibody diluted in blocking buffer overnight at 4 deg C.
25 Embryos were washed three times in blocking buffer for >1 hour per wash at 4 deg C, then
26 incubated in secondary antibody diluted in blocking buffer overnight at 4 deg C. Embryos were
27 washed three times in PBS for >1 hour per wash at 4 deg C, then either kept whole or bisected
28 down the midline as described previously and mounted in PBS for imaging.
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41
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45 facilities.
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MOVIE LEGENDS

Movie 1: Neural crest cell (NCC) dynamics in response to Colec12-protein stripes. A typical neural tube explant placed in culture in the presence of Colec12-protein stripes (red). Notice how the NCCs migrate on the stripes without Colec12-protein. Some NCCs may cross between streams and do so by rapidly moving across the Colec12-protein stripes. Typical time-lapse movies lasted approximately 12 hrs with a 5 min interval between frames.

Movie 2: Neural crest cells (NCCs) rapidly migrate throughout the in vitro culture dish. A typical neural tube explant placed in culture. Notice how the NCCs extend and retract filopodia as cells spread out. A typical time-lapse movie lasted up to 10 hrs with a 5 min interval between frames.

Movie 3: Neural crest cells in the presence of Colec12-protein in the media fail to retract extended filopodia. A typical neural tube explant placed in culture in the presence of Colec12-protein. Notice how the NCCs spread out and extend long filopodia, but fail to rapidly retract the processes, resulting in large gaps in between migrating cells. A typical time-lapse movie lasted over 12 hrs with a 5 min interval between frames.

Movie 4: Neural crest cells in the presence of Trail-protein in the media fail to retract extended filopodia. A typical neural tube explant placed in culture in the presence of Trail-protein. Notice how the NCCs spread out slower than compared to controls and tend to adhere to each other, resulting in small sized clusters of moving cells. A typical time-lapse movie lasted over 12 hrs with a 5 min interval between frames.

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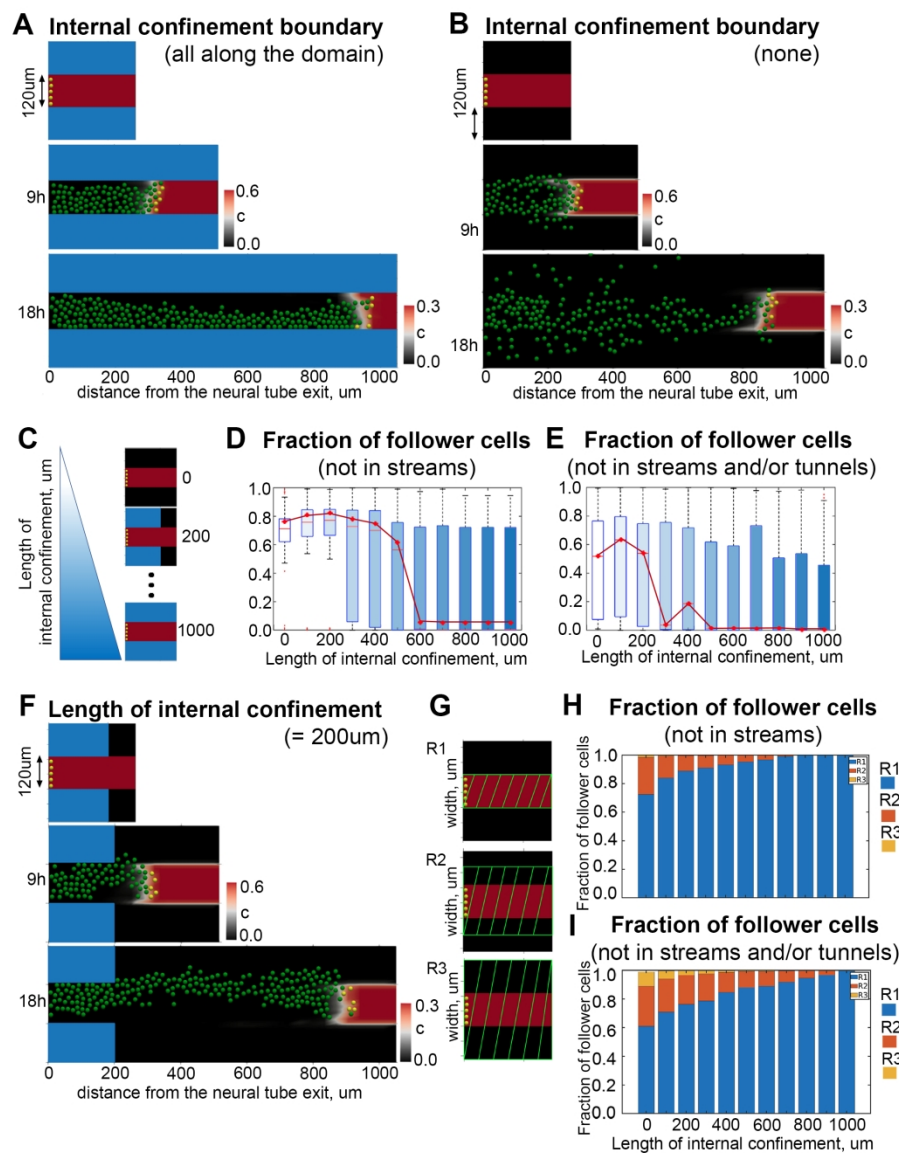
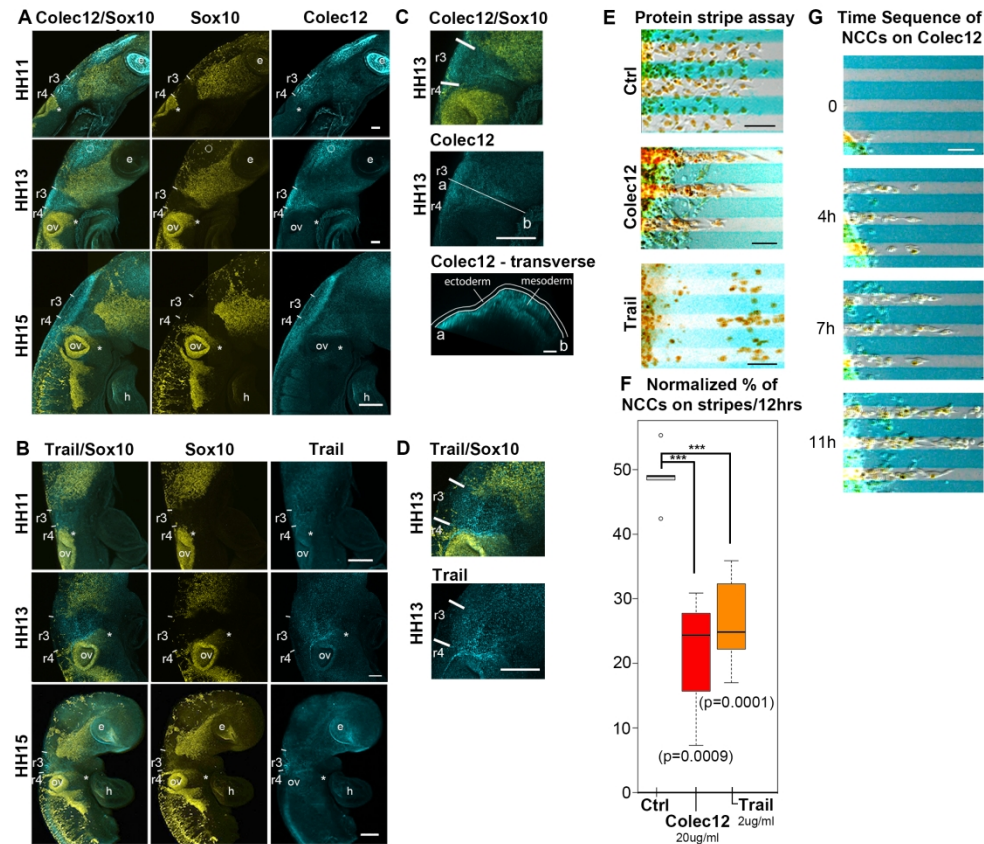


Figure 1: Computer Model Simulations Predict That Neural Crest Cell Confinement Along the Entire Length of the Migratory Domain is Not Required to Maintain Discrete NCC Streams Over Long Distances. Different confinement possibilities for the initial set up: (A) full internal confinement; (B) no internal confinement. The red rectangle corresponds to the region with uniform initial chemoattractant concentration, $c = 1$. The chemoattractant concentration in the black region is zero, $c = 0$. The blue rectangles represent internal confinement subregions adjacent to rhombomeres 3 (r3) and r5. Yellow circles represent NCCs. (C) Shades of blue corresponding to different extents of internal confinement. (D) Boxplots of fractions of follower cells not in streams and/or tunnels (E) at $t = 18$ hours. This statistic corresponds to the likelihood of breakdown of collective migration. For each model, the red line indicates the median, and the bottom and top edges of the boxes indicate the 25th and 75th percentiles, respectively. The dotted lines extend to the most extreme data points not considered outliers, and the outliers are plotted individually as red dots. The breakdown of collective migration is most likely when there is a short internal confinement length. For the same internal confinement length, the stream is less likely to break for streams/tunnels model (right) than for streams only model (left). 1020µm corresponds to a full internal confinement boundary. (F) partial (200µm) internal

1
2
3 confinement. (G) Different subregions (indicated by the green diagonal lines) that are used to quantify how
4 widely the cells are spread out. (H) Fraction of cells in different subregions (1, 2, or 3) for the model
5 scenario of streams and/or tunnels (I). In (D), (E), (H), (I) Results are averaged over one hundred
6 simulations.

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Colec12 and Trail Expression are Enhanced in Neural Crest Cell Free Zones and Confine Neural Crest Cell Trajectories in Protein Stripe Assays. (A) Colec12 expression (light blue) in the chick head at HH11, HH13 and HH15 with Sox10 (gold) marking the migrating NCCs; the migratory front of the r4 stream is marked by an asterisk. The rhombomeres r3 and r4 are labeled with short white lines marking the rhombomere boundaries in the neural tube. Colec12 expression is also in the region rostral to r1 and adjacent to the midbrain (marked by an open circle at HH13). (B) Trail expression (light blue) in the chick head at HH11, HH13, and HH15 adjacent to r3 with Sox10 (gold) marking the migrating NCCs; migratory front of the r4 stream marked by an asterisk. The rhombomeres r3 and r4 are labeled with short white lines marking the rhombomere boundaries in the neural tube. (C) Same embryo image as in (A; HH13) with individual images of Colec12 staining. The line marked a to b is the plane of the yz image collected. (D) Same embryo image as in (B; HH13) with individual images of Trail staining. (E) Typical protein stripe assays show confinement of NCCs with Ctrl, Colec12 and Trail on the protein stripes (light blue) versus fibronectin only in the control. (F) Boxplot graph of the normalized percentage of NCCs on stripes. (G) Sequence of images from a typical time-lapse imaging session show the cranial neural tube explant (left) and migrating NCCs confined to the lanes without Colec12 protein (light blue). In (A-B), the eye (e), otic vesicle (ov), and heart (h) are labeled where appropriate. The scalebars in (A) are 50um, in (B) are 100um (HH11), 50um (HH13), and 100um (HH15), in (C-F) are 50um.

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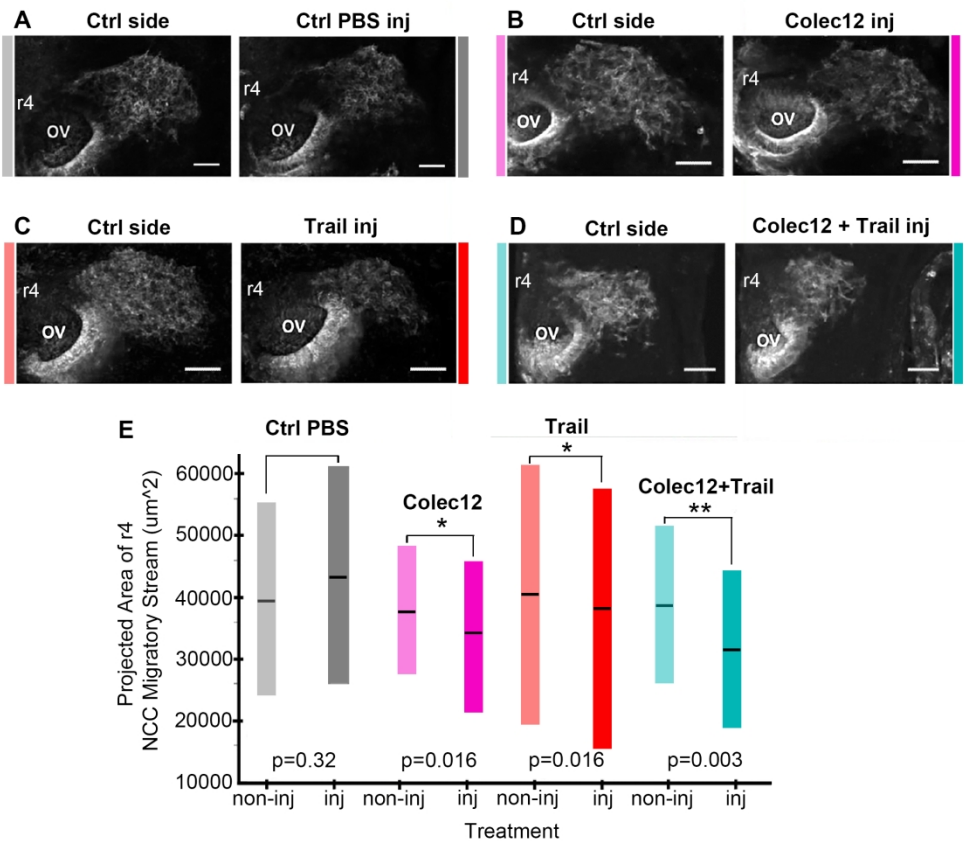
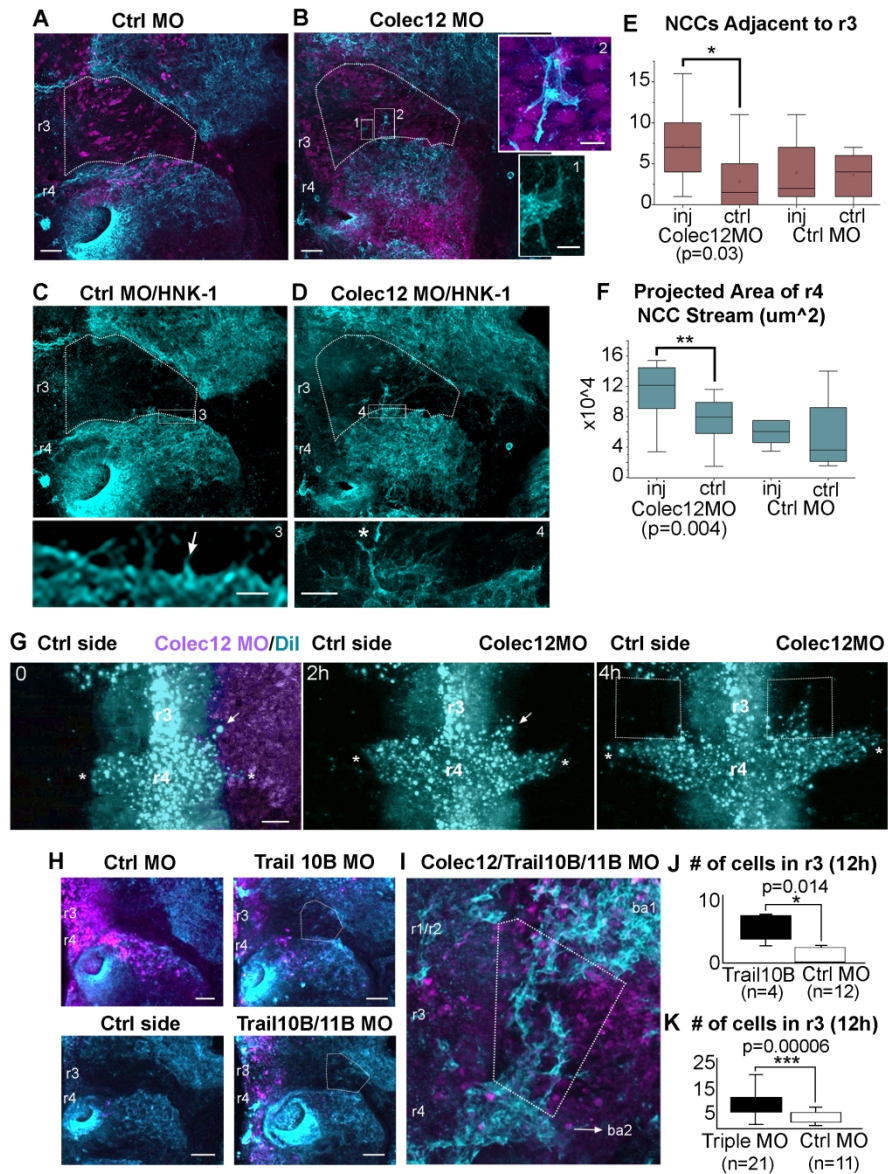


Figure 3: Microinjection of Colec12 or Trail Protein or in Combination Onto the Presumptive R4 NCC Pathway Enhances Confinement of NCCs. (A) Control (Ctrl) PBS injection into the right-hand side of the chick embryo and control non-injected side (left) showing the r4 NCC migratory stream and otic vesicle (ov). (B) Colec12, (C) Trail, (D) Colec12+Trail. (E) Measurement of the projected area of coverage of the r4 NCC migratory stream in each of the control and perturbation scenarios. The scalebars are 50um in all images.

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Knockdown of Colec12 and/or Trail Leads to Diversion of NCCs Into the Presumptive NCC-Free Zones. (A) Control (Ctrl) and (B) Colec12 morpholino (MO) in purple showing the migrating NCCs (HNK1; blue). The subregion adjacent to r3 (typical NCC-free zone) is enclosed by a dotted line. In the Colec12 MO image, note the diversion of NCCs into this subregion with individual diverted NCCs (boxed, 1 and 2; insets). (C-D) HNK-1 staining only of the (C) Ctrl MO and (D) Colec12 MO. Note the differences in the rostral border of the r4 NCC migratory stream (boxed, 3 and 4; insets) showing the short NCC protrusions and coherent border (box 3; arrow) in contrast to lengthy NCC protrusions and disrupted border (box 4; asterisk). (E-F) Measurements of the number of diverted NCCs into the subregion adjacent to r3 and projected area of the r4 NCC migratory stream in control versus Colec12 morpholino embryos (n=12 embryos in each experiment). (G) Sequence of images (0,2h,4h) from a typical time-lapse imaging session with DiI-labeling of NCCs (blue) showing the diversion of NCCs into the subregion adjacent to r3 (marked by the arrowhead (0,2h) and dotted box (4h)) in comparison to the migratory front (asterisk). (n was at least 12 embryos/experiment). (H) Control versus Trail10B and/or Trail11B morpholino knockdown showing the NCC-free zone adjacent to r3 outlined by a dotted box and diversion of NCCs into this subregion. (I) Triple

combination knockdown of Colec12/Trail10B/Trail11B with the subregion adjacent to r3 outlined by the dotted box and showing extensive invasion of HNK1-labeled NCCs and diversion from the targets ba1 and ba2. (J-K) Measurements of diverted NCCs into the subregion adjacent to r3 in (J) Trail10B and (K) triple combination knockdown. The scalebars are 50um in all images.

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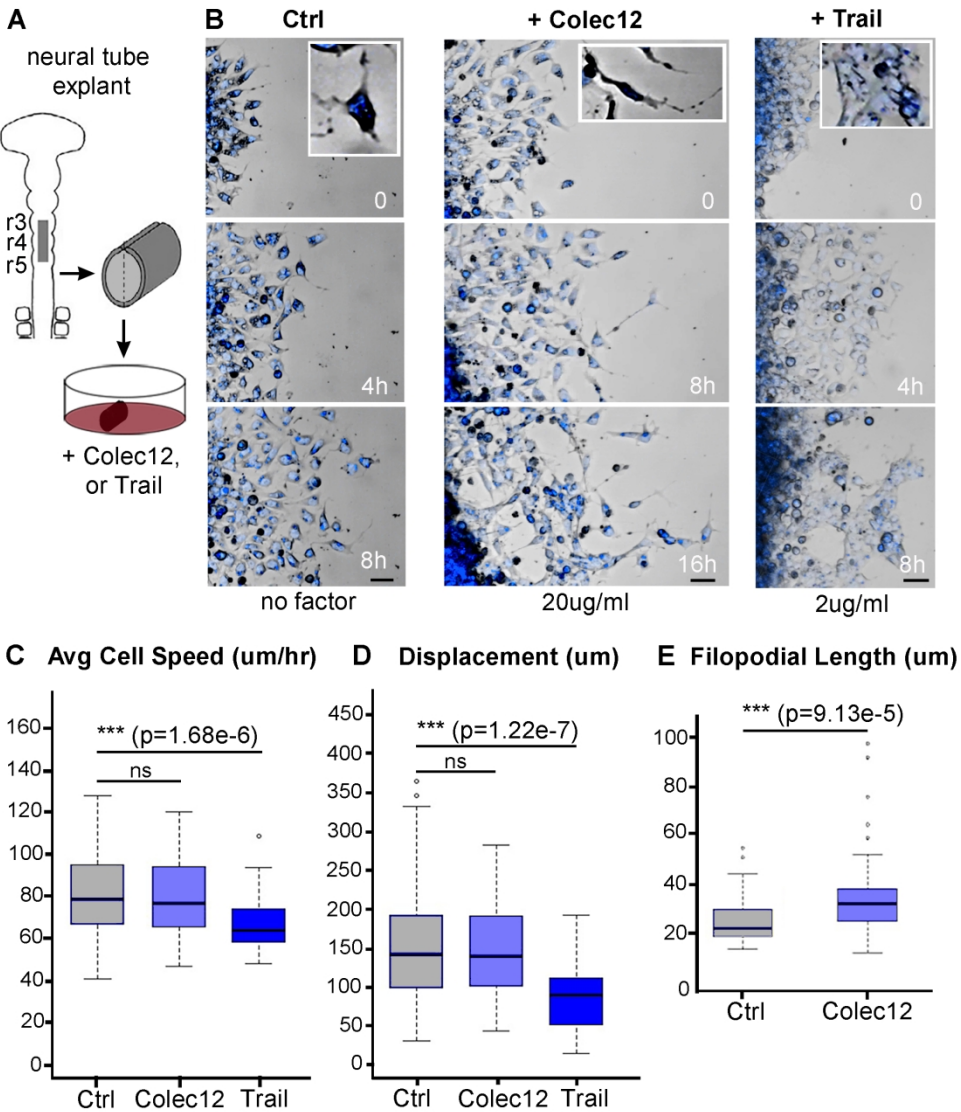
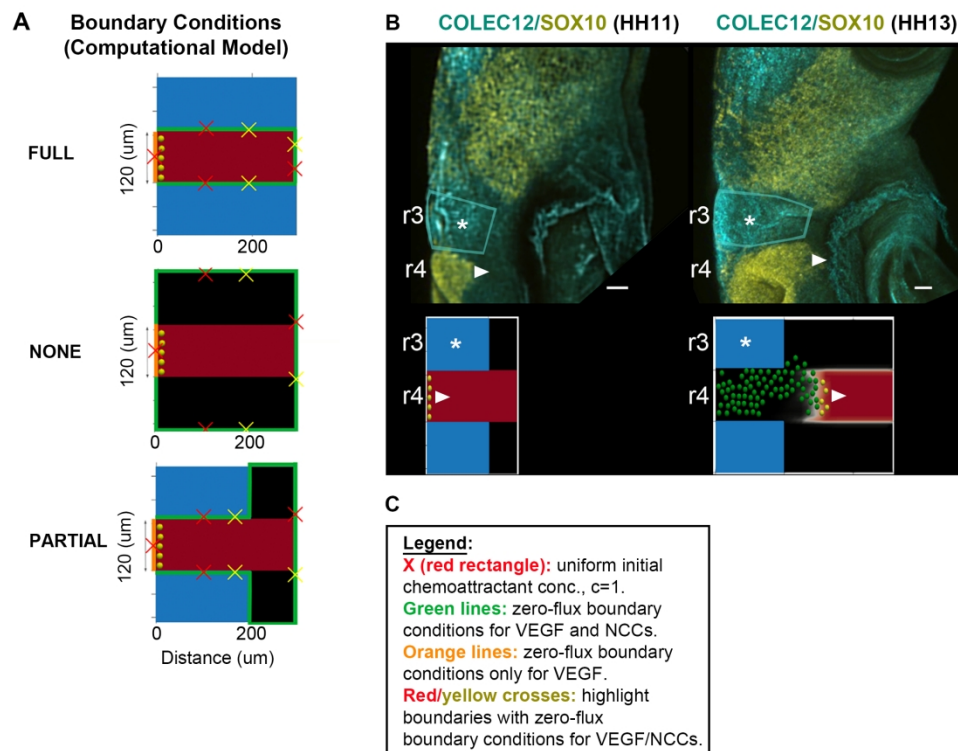


Figure 5: Co-Culture of NCCs with Colec12 or Trail Protein in the Media Significantly Affects NCC Morphology and Migratory Characteristics. (A) Schematic of cranial neural tube explant culture with Colec12 or Trail protein added to the culture media. (B) Static images of changes in migrating NCC morphologies extracted from typical time-lapse imaging sessions with insets showing individual NCCs (16 hours each, n=3 neural tube explant cultures for each experiment and >180 cells tracked and analyzed in each experiment). (C-D) Changes in NCC migratory characteristics measured for average cell speed and displacement. (E) Filopodial length measurements from co-culture with Colec12 protein experiments. The scalebars are 20um in each image in (B).

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Boundary Conditions for the Computational Model and Relationship to Colec12 expression. (A) Different confinement possibilities for the initial set up: (far left) full internal confinement; (middle) no internal confinement; (right) partial internal confinement. The red rectangle corresponds to the region with uniform initial chemoattractant concentration, $c = 1$. The green wide lines correspond to zero-flux boundary conditions for VEGF and NCCs. The orange wide lines correspond to zero-flux boundary conditions only for VEGF. Red and yellow crosses highlight boundaries with zero-flux boundary conditions for VEGF and NCCs, respectively. On the left the chemoattractant concentration in the black region is zero, $c = 0$. The blue rectangles represent internal confinement, as before. Yellow circles represent NCCs. (B) Comparison of the experimental data of Colec12/Sox10 expression in HH11 and HH13 embryos (same images as shown in Fig. 2A, rotated slightly counter-clockwise) with the Colec12 expression in the presumptive NCC-free zone adjacent to r3 surrounded by a light blue highlighted line (with asterisk in center) and front of r4 NCC migratory stream (arrowhead), corresponding to the model confinement of NCCs adjacent to the rostral border of the NCC migratory domain at time $t = 0$ and $t = 9$ hours of simulation time (marked by an asterisk) and simulated representation of NCCs (leader in yellow and followers in green; front marked by arrow). (C) Legend for (A). The scalebars are (B) 50um.

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