# SUPPLEMENTARY MATERIAL

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Figure 1: Contact Inhibition of Locomotion controls Neural Crest (NC) directional migration *in vivo*. **a.** When two NC cells collide they change their respective direction of migration. We show here that this process is dependent on the PCP pathway (symbolised here with yellow tees). **b.** In the embryo, NC cells migrate with high directionality (green arrow) in streams of limited width (represented as a white area). We showed that cell-cell contact is essential for this directionality. **c.** Cell polarization is inhibited by cell contact. This inhibition is controlled by the same mechanism that the one shown in panel **a** (yellow tees). **d.** Only leading cells are polarised as they have a free edge. Cells can only migrate in this direction and thus directionality is achieved.
Figure 2. Directional cell migration in large re-aggregates.

*Xenopus* NC cells expressing nuclear-RFP and membrane-GFP were dissociated in low calcium MBS, re-aggregated by adding normal MBS or DFA, and then cultured on fibronectin as described in 14. Time-lapse analysis was performed and frames at the indicated times are shown. Last column shows the trajectory of migrating cells. (a,b) Migration of leading cells (b) versus internal cells (a); internal cells exhibit a much lower persistence (0.2±0.04, n= 20) than cells at the periphery (0.6±0.05, n=20; p<0.05). (c,d) If an internal cell with a typical low persistence (c) becomes located at the periphery of the explant, it becomes rapidly polarized (arrow) and its migration becomes persistent (d; 0.6±0.08, n=10) as a leading cell.
Xenopus NC cells were cultured in vitro and the trajectory of internal cells was analyzed as described in Supplementary Fig. 1. A low persistence in the migration of internal cells was observed (b, c; 0.3±0.07, n=10). Half of the NC explant was removed as indicated by the dashed red lines, and the trajectory of the cells at the new border was analyzed as shown in f. A much higher persistence of these initially internal cells was observed (e, g, h; 0.8±0.06, n=10).
Figure 4. NC cells invade mesodermal explants but not other neural crest explants

(a-c) Invasion Index (ι): Masks of thresholded explants (pink: neural crest; cyan: neural crest or mesoderm. The overlapping index (ι) was calculated by dividing the overlapping area (IA, in brown) by the initial area of the explant (Ri, in pink). Explants in (a) NC/NC; (b) NC/mesoderm. (c) boxplot of ι for a NC/NC (blue) and a NC/mesoderm (red) explants (p<<0.005, n=5). (d, e) Confocal section through the
top of the explants. (d) NC/NC explant. Green explant was labelled with GFP and red explant with membrane-RFP/nuclear-GFP. (e) NC/mesoderm explant. NCs were labelled with GFP. Note how NCs cover the mesoderm (unlabelled). Dashed lines in d and e show digital sections made to show the depth of the explant (shown in d’ and e’). (f) 3D reconstruction from a z-stack of the explant shown in e. (g, h) Confocal section through the bottom of the explants. (g) NC/NC explants. NC cells are labelled with nuclear –RFP or –GFP. A higher magnification of the box in g is shown in g’. (h) NC/mesoderm explant. NC cells are labelled with nuclear GFP and mesoderm is labelled with RLDX. White boxes indicate isolated NC nuclei, which are shown at higher magnifications in h’, h”, h’’. Note the elongated morphology of the nuclei that penetrate the mesodermal tissue. (i) Summary of mesoderm (red) invasion by NC cells (green).

Figure 5. Analysis of cell collisions in vivo using a membrane-GFP transgenic zebrafish line.

A transgenic zebrafish line was made by cloning membrane-GFP under the control of the Sox10 promoter (Sox10-mGFP) and time lapse analysis was performed. This new transgenic fish allows the visualisation of small and thin protrusions, which are highly dynamic (Supplementary Movie 7). (a) Dynamic cell protrusions, box is magnified in b, which shows how a thin protrusion growth and collapses (white arrows) after contacting (red arrowhead) a neighbouring cell. Time is indicated in minutes. Bar in a: 10µm, b: 7µm.
Figure 6. Inhibition of PCP signalling blocks Contact Inhibition of NC cells in vivo.

The effect of cell-cell contact in vivo analyzed as in Fig 2j; each panel corresponds to the difference in two consecutive times (in minutes). Green: new area; red: collapsing area; red arrowhead: cell contact; white arrowhead: collapsing protrusion; arrow: direction of migration. (a) Control embryo. (b) DshDep+ embryo. (c) Embryo injected with a mixture of Stb/Pkk1 MO. Note that that as soon as the control cell (C1) contacts a neighbouring cell (C2) its protrusion collapses and the direction of migration changes (b); however no such collapse or change of direction is seen when PCP is inhibited by DshDep+ (b) or a combination of Stb/Pk1(c) MO injection. Similar results were obtained with the injection of a single MO against Stb or Pk1 (not shown). Controls to show specificity of Stb and Pk1 MO have been published in references 17 and 18 (see main text).
(a-d) Frames of time lapse movies of confrontational explants performed in vitro as in Fig 2a. Four different frames are shown at the indicated times (minutes) for a control experiment (a) and for three PCP+ experiments as indicated (b-d). Note that a clear collapse in the cell protrusion after cell contact (red arrowhead) and a change in the direction of migration is seen in control cell (a); but no collapse in cell protrusion (b,
c) is seen for the DshDep+ or the dnWnt11 cells. (d) NC cells expressing membrane-GFP/nuclear-RFP or DshDep+/membrane-RFP/nuclear-GFP were cultured in vitro and visualized at a high magnification to analyze contact inhibition. Contact Inhibition is observed for the control cells, but not for the DshDep+ cells. Supplementary Movie 11. (e-f) Vector analysis of Contact inhibition as described in Fig 2b. Left side of each panel shows velocity vector, right side shows acceleration vector. Red arrow: average initial velocity. (e) Embryos injected with 750pg of dominant negative Wnt11 mRNA. (f) Embryos injected with 15ng of Wnt11R MO. (f) Embryos were injected with 750pg of dominant negative Wnt11 mRNA and 15ng of Wnt11R MO. Note that the velocity of migration is not affected by the collision, with the acceleration being close to 0 and not significantly different to a random distribution (p>>0.05)
Figure 8. PCP elements are localized at the cell-cell contacts of NC migrating in vitro and in vivo.

(a-d) *Xenopus* embryos were injected with the indicated fusion constructs at the 8-cell stage, NC was dissected at the neurula stage, cultured in vitro and the fluorescence was analyzed. Yellow arrow: direction of migration; white arrowhead: accumulation of protein at the cell contact. mem-RFP: membrane-RFP. (a) NC was analyzed before migration, when most Dsh is localized in the cytoplasm. (b) NC was cultured on polylysine. Only a cytoplasmic localization of Dsh is observed. (c) Dsh is localized in the cell contact once the NC starts to migrate. Sometimes individual (leading) migrating NC cells exhibit Dsh localization at the back of the cell in a region that was previously in contact with other cells (arrowhead). (d) Leading cell showing co-localization of Wnt11 and Fz7 at the back of the cells in a region of contact with another cell (arrowhead). (e-g) in vivo localization of PCP components. Sox10-GFP zebrafish embryos were injected with the fusion constructs indicated and time-lapse analysis of NC migrating in vivo was performed. (e, f) Dsh localization in vivo. (e) Groups of migrating NC show Dsh localization at the cell contact (arrowhead). (f) Leader migrating NC exhibits Dsh in the cell contact at the back of the cell (arrowhead). (g) Groups of migrating NC show Wnt11 localization at the cell contact (arrowhead).
Figure 9. Effect of activation of Wnt signalling on Dsh localization.

Ectodermal *Xenopus* cells that lack Wnt signalling were used to analyze the effect of its activation. (a-c) Cells were injected with 75pg of Dsh-GFP mRNA and 200pg of membrane-RFP mRNA; no clear localization of Dsh is seen in the membrane. (d-e) Cells injected as in a-c were co-injected with 100pg of Fz7 mRNA and 100pg of Wnt11 mRNA. A clear accumulation of Dsh in the membrane was observed.
Figure 10. RhoA participates in Contact Inhibition of Locomotion in a PCP dependent manner.

(a-c) FRET analysis of RhoA activity was performed in NC cells as described in 14. (a) FRET efficiency of RhoA. Black bar: control cells (b); Grey bar: cells in which PCP has been activated by expression of DshΔN (c). A significant increase in RhoA activity was observed after PCP activation (***: p<0.005, n=15). (D) NC cells cultured in vitro in confrontational explants were treated with the 10µM of the Rock inhibitor Y27632. A clear inhibition of Contact Inhibition of Locomotion was observed, as the NC cells migrated on the top of each other, which was never observed for control cells. Four different frames are shown at the indicated times. The outline of a migrating cell is indicated. Arrow: direction of migration. Note that the migrating outlined cell seems to ignore the adjacent cells, does not collapse its protrusion after cell contact and does not change the direction of migration.
Methods

General Methods

*Xenopus* embryo manipulation: microinjections and cell culture were performed as previously described\(^\text{14}\). Briefly, all injections to target the NC were performed into lateral animal blastomeres at the 16-cell stage (blastomers V1.2 and D1.2). General nuclear and membrane labels were injected into both blastomers at the two-cell stage. To analyse NC migration *in vitro*, NC explants were cultured in plastic or glass dishes coated with fibronectin and filled with DFA. Time-lapse analysis *in vitro* was performed using DIC microscopy or fluorescent microscopy of cells injected with nuclear-RFP/membrane-GFP or membrane-RFP/nuclear-GFP\(^{13,14,30}\), using a DM5500 Leica compound or a Leica Confocal Microscope. Zebrafish strains were maintained and bred according to standard procedures\(^\text{31}\). Zebrafish manipulation, microinjection and time-lapse analysis was performed as described\(^\text{14}\). For time-lapse analysis zebrafish embryos were orientated laterally to have an orthogonal view to the migration plane. SEM was performed as described in\(^\text{13}\). Cell tracking was performed with the ImageJ software using the manual tracker plug-in as previously described\(^\text{14}\). Persistence is defined as the ratio between the linear distance, between the initial and the final point, and the total length of the migratory path. Imaris software (Bitplane) was used to generate 3D-reconstructions (isosurface) form a xyz-confocal image stack.
Quantifications and Statistical Analysis

Contact Inhibition of locomotion is defined as a change in velocity in a migrating cell as a product of a collision with another cell. Acceleration, by definition, is the rate of change of velocity over time and thus, an ideal parameter to estimate contact inhibition\textsuperscript{11}.

To quantify Contact Inhibition of Locomotion (the acceleration produced by the collision) \textit{in vitro}, colliding cells were treated as particles centered on its own nuclei and then tracked. A similar thing was done for the \textit{in vivo} analysis but as their nuclei is difficult to visualize, a different location method was used. In order to have an unbiased location method, consecutive images were blended. In the resulting color-coded image, it is possible to distinguish the areas in both frames (null areas) from the areas in the first frame only (negative area) and from the areas in the second frame only (positive areas). Negative areas are considered retractions and positive areas are considered protrusions. Tracks were made from sequences of these combined areas.

Colliding cells were tracked and their positions were imported to Matlab (R14b, Mathworks). Mean velocities were measured before and after the collision and the corresponding acceleration was calculated according previous works\textsuperscript{11}. The resulting vector was orientated in relation with the initial velocity as described in the same work\textsuperscript{11}. We also used the collision plane as a reference, and this did not change our results (data not shown).

Statistical Analysis was done by custom-made functions and scripts in Matlab to implement the modifications by Moore to the Rayleigh’s test\textsuperscript{32}. This approach was specifically developed to test non-random distribution of vectors and thus, it is ideal for our analysis. As recommended, we used a two-tailed version of this test\textsuperscript{32}. The
graphic outcome of this analysis was processed either in Matlab itself or exported as a post-script file and edited in Adobe Illustrator (v.13.0, Adobe).

To quantify Invasion we devised an invasion index, similar to what has been previously used (5; see below). To do this colliding cell populations were labeled with different fluorescent colors. Red and green channels (each one corresponding to different population) were imported to ImageJ, thresholded and masked. Both masks were pseudocolored (different colors) and then blended. The resulting blended image has a different color for the overlapping area.

Invasion Index ($\iota$) was estimated by measuring the overlapping area (IA) between the two different explants standardised by the initial surface of the invaded explant (Ri). The initial area was used for standardisation instead of the more intuitive final one because NC explants grows notoriously while other cells populations do not change significantly their size. Therefore, NC final areas would not be comparable with mesodermal or ectodermal ones. Ri did not show any significant variation neither among nor between groups. Areas were normally distributed and thus One-way ANOVA was used to compare the resulting $\iota$ values.
Grafts of Xenopus NC cells (Figure 2m-o).

(a) Xenopus embryos were injected at the 1-cell stage with green (FLDX) or red (RLDX) fluorescent molecules. At the early neurula stage NC were taken from a FLDX injected embryo and grafted into an uninjected embryo. At the same stage, in some of the RLDX embryos the NC were removed. In situ hybridization against the NC marker snail2 was used to check that all the dissections were correct. The ventral region of the embryo was removed and two embryos were “sandwiched” together facing each other along these ventral wound according to the following combinations. (b) Control embryo with FLDX labelled NC. (c) Sandwich of FLDX NC embryos with a normal RLDX embryo. (d) Sandwich of FLDX NC embryos with a RLDX embryo in which the NC has been removed. In vivo migration of the neural crest was analyzed and shown in Fig. 2m-o.

Two-plane confocal image (Fig 3d, e)

For the analysis of cell protrusions shown in Fig 3d, e, we constructed a two plane confocal image, in which the plane of the FN-coated dish was shown in red, which corresponded to the protrusions, while a second plane through the center of the cell was shown in green and corresponds to the shape of the cell. Both planes were overlapped to generate the images shown in Fig 3d and e.
Sox10-membrane-GFP transgenic fish

A Sox10 reporter construct was generated containing membrane GFP driven by the Sox10 promoter. For this a 7 kb DNA fragment corresponding to the zebrafish Sox10 promoter, obtained from the p-7.2sox10:egfp plasmid (kindly donated by R Kelsh) was cloned upstream of the GFP gene. 40 to 80 pg of DNA linearised with NotI was injected into 1-cell zebrafish embryo to generate the transgenic fishes.

zebrafish Wnt11 dominant-negative and zWnt11-RFP generation

Xenopus and zebrafish Wnt11 proteins are highly conserved. Thus, a C-terminally truncated dominant-negative zebrafish wnt11 construct was generated according to the Xenopus version. PCR amplification was performed using the primers 5’ggtccaggatccatgacagaatacaggaact3’ and 5’ggtccagatctcgaatctgagactgtga3’ zWnt1. The PCR fragment was then cloned into the BamHI and EcoRI sites of pCS2+ vector. This construct comprises aminoacids 1-281 lacking the cysteine-rich region (panel a, b). mRNA injection of this dominant negative of Wnt11 produces the expected phenotypes for the loss of function of Wnt11: convergent extension defects (arrows in c, d); NC migration defects (GFP+ cells in c, d); positioning of the eye analge defects (yellow bar in e, f) and shortening of the body length (g, h).
The Wnt11-RFP fusion protein was constructed as a C-terminal fusion of zebrafish wnt11 cDNA and RFP. The YFP region of Wnt11-YFP\textsuperscript{22} was swapped with a modified version of RFP (cherry-RFP). A linker sequence of nine amino acids (GSSEFSIDG) was introduced between Wnt11 and RFP. The Wnt11-RFP fusion cDNA was cloned into pCS2+ between the BamHI and XbaI polylinker sites.

**Measurement of RhoA activity**

RhoA activity was measured using FRET analysis. The general protocol used is similar to the one described in (Matthews, 2008) but to improve the efficiency of the probe expression it was subcloned into a vector more suitable for amphibian system.

The original plasmid containing the RhoA biosensor (pTriEx/RhoA, Pertz, 2006), was digested with NcoI and XhoI, and subcloned in StuI-XhoI sites of pCS2+ vector. A combination of 75 pg of the new RhoA biosensor and 300 pg of mCherry mRNA as a lineage tracer, was injected at the 16-cell stage to target the NC cells. NC was dissected as previously described, cultured *in vitro* and time-lapse analysis was performed to identify colliding cells.
Supplementary Notes

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