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## Contact Inhibition of Locomotion *in vivo* controls neural crest directional migration

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### Abstract

Contact Inhibition of Locomotion was discovered by Abercrombie more than 50 years ago to describe the behaviour of fibroblast cells confronting each other *in vitro*, where they retract their protrusions and change direction upon contact<sup>1,2</sup>. Its failure was suggested to contribute to malignant invasion<sup>3-6</sup>. However, the molecular basis of Contact Inhibition of Locomotion and whether it also occurs *in vivo* are still unknown. Here we show that neural crest cells, a highly migratory and multipotent embryonic cell population, whose behaviour has been likened to malignant invasion<sup>6-8</sup>, exhibit Contact Inhibition of Locomotion both *in vivo* and *in vitro*, and that this accounts for their directional migration. When two migrating neural crest cells meet, they stop, collapse their protrusions and change direction. In contrast, when a neural crest cell meets another cell type, it fails to display Contact Inhibition of Locomotion; instead, it invades the other tissue, like metastatic cancer cells<sup>3,5,9</sup>. We show that inhibition of non-canonical Wnt signalling abolishes both Contact Inhibition of Locomotion and the directionality of neural crest migration. Wnt signalling members localise at the site of cell contact, leading to activation of RhoA in this region. These results provide the first example of Contact Inhibition of Locomotion *in vivo*, present an explanation for coherent directional migration of group of cells and establish a novel role for non-canonical Wnt signalling.

Neural crest (NC) cells cultured *in vitro* move away from each other, dispersing quickly<sup>10</sup>. *Xenopus* neural crest explants reveal that only the leading edge cells are polarised, having large lamellipodia at the front as shown by Scanning Electron Microscopy (SEM; arrowheads in Fig. 1a-c) or in live NC explant expressing membrane-localised GFP (Fig. 1d). Time-lapse analysis reveals that edge cells have a higher persistence in the direction of migration than cells in the interior of the explant (Fig. 1d'). To distinguish whether these differences in polarity and migration correspond to two different cell populations or are due to cell-cell contact, we dissociated NC explants into single cells and then re-aggregated them into small or large clusters. Peripheral cells in small or large clusters rapidly become polarised and migrate away from each other (Fig. 1e), while internal cells move randomly (Supplementary Fig 2 and Movie 1), similar to those in non-dissociated explants. In addition, the randomly migrating internal cells of these explants become polarised when

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**Author contributions.** C.C.-F. and R.M. designed the experiments. C.C.-F., H.K.M. and R.M. performed most of the experiments. C.C.-F. and R.M. did the movie analysis. C.C.-F., G.A.D. and R.M. planned and performed the statistical analysis. M.P., S.K., C.C.-F., H.K.M. and R.M. made the FRET analysis. M.M. made some of the constructs and the zebrafish transgenic. M.M., C.C.-F., H.K.M. and R.M. performed the PCP localization experiments. C.C.-F., C.S. and R.M. wrote the paper.

Supplementary Information is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature). A figure summarising the main result of this paper is also included as SI.

made to have a free edge by wounding or removal of their neighbours (Supplementary Fig. 3). These results suggest that the differential behaviour of leading *versus* internal cells in explants is not due to intrinsic cell differences, but rather to interactions between neighbouring cells. Furthermore, the average persistence of the leading cells (defined by their position at the border of an explant) in a non-dissociated cluster is much higher than that of individual cells; the latter often make little progress and move in circles, although their overall speed of migration is greater (Fig. 1f-i, Supplementary Movie 2). A similar phenomenon has been reported for cell types exhibiting Contact Inhibition of Locomotion<sup>1</sup>. Together, these results indicate that the directional migration of cultured NC cells is dependent on cell-cell contact, which is likely to inhibit the formation of cell protrusions leading to cell polarisation.

To study the behaviour of NC cells when confronted to other cells, we developed an explant-confrontation assay<sup>1,11</sup>. Two NC explants were cultured in close proximity such that their leading cells would encounter cells from the other explant migrating in the opposite direction (Fig. 2a). We found that cells from NC explants aggressively invade mesodermal (Fig. 2e) and ectodermal (not shown) explants, but never invade other NC explants (Fig. 2c; Supplementary Fig. 4a-c and Movie 3). Confocal microscopic analyses show that while NC stop migrating when they are confronted with other NC cells, they engulf the mesoderm explant with some cells penetrating deep layers of this tissue (Fig. 2d, f; Supplementary Fig. 4d-i). These observations suggest that Contact Inhibition of Locomotion occurs between NC cells (homotypic), but not between NC cells and other cell types (heterotypic), as is the case with some malignant cells<sup>4,5,9</sup>. It is tempting to speculate that such homotypic Contact Inhibition of Locomotion could play a role in guiding the migration of NC cells in normal development. To determine whether this is indeed the case we developed a confrontation assay *in vivo*, showing that NC cells can invade the tissue of an adjacent *Xenopus* embryo lacking NC (Fig. 2o), but this invasion is blocked when the host NC is present (Fig. 2n). This result is compatible with Contact Inhibition of Locomotion having this role, but does not directly demonstrate that NC cells exhibit Contact Inhibition of Locomotion. To this end, we performed time-lapse analysis in the explant-confrontation assay focusing on individual cells. When a NC cell comes into contact with a cell from the opposite group, its lamellipodium collapses and the direction of its migration changes (Fig. 2g, Supplementary Movie 4). This is described precisely by Abercrombie's original definition of Contact Inhibition of Locomotion as "the phenomenon of a cell ceasing to continue moving in the same direction after contact with another cell"<sup>5</sup>. To date, Contact Inhibition of Locomotion has only been described *in vitro*. To address whether it also occurs *in vivo*, we used two Sox10-GFP transgenic zebrafish lines, one that expresses GFP in the cytoplasm<sup>12</sup> and the other in the membrane of NC cells, and analysed their migration using live-imaging techniques. Similar to the observations *in vitro*, whenever two NC cells make contact, they change their direction of migration and their protrusions collapse (Fig. 2j; Supplementary Fig 5 and Supplementary Movies 5-7). Contact Inhibition of Locomotion can be measured by the change in velocity after cell-cell contact<sup>4,11</sup>. Large changes in the direction of cell migration are seen after each cell collision (Fig. 2b, Supplementary Methods) both *in vitro* and *in vivo* (velocity: Fig. 2h, k; acceleration: Fig. 2i, l). The changes are not stochastic but are strongly biased in the opposite direction to the collision ( $p < 0.005$ ), as predicted by Abercrombie<sup>5,11</sup>. Likewise, in *Xenopus*, grafted NC cells integrate into the endogenous migratory pathway and migrate directionally (Fig 2p). However, grafted NC lose their directional migratory behaviour when the host NC is removed (Fig 2q), suggesting that the directionality of NC migration depends on interactions with other NC cells and supporting our conclusion that Contact Inhibition of Locomotion is required for normal NC migration *in vivo*.

We next explored the molecular mechanisms underlying Contact Inhibition of Locomotion in NC cells. Previous studies have shown that the Wnt Planar Cell Polarity (PCP, or non-canonical) pathway is required for NC migration in *Xenopus* and zebrafish embryos, whereas canonical Wnt signalling is not<sup>13,14</sup>. To determine whether PCP signalling is involved in Contact Inhibition of Locomotion, we analysed cells expressing a dominant negative form of Dishevelled (DshDep+), which specifically inhibits the PCP pathway<sup>15</sup>. In control explants, only the leading cells are highly polarised, extending cell protrusions at the front, while trailing cells are not (Fig. 1a-c). In contrast, DshDep+ cells are not polarised but extend large protrusions in all directions (Fig. 3a-c). Live imaging shows that cells expressing DshDep+ crawl on top of one another extending protrusions between the neighbour cells, a characteristic of some metastatic cancer cells<sup>3,5,9</sup> (Fig. 3d-e; Supplementary Movie 8). All DshDep+ (leading and trailing) cells behave similarly to trailing control cells based on their low persistence of migration and the angles at which they change direction (Fig. 3f-i). No difference in persistence and speed of migration are observed between dissociated control and DshDep+ cells, both behaving like trailing cells (Fig. 3j-m, Supplementary Movie 9). This indicates that the effect of PCP signalling on NC migration requires cell-cell contact.

To test whether Contact Inhibition of Locomotion is dependent on PCP signalling *in vivo*, we measured the speed of cell migration before and after cell collision in control embryos and in embryos in which PCP signalling has been disrupted (either by expressing DshDep+, a dominant negative of the PCP ligand Wnt11 or antisense morpholinos against the PCP pathway members *strabismus*, *Stb16* or *prickle1*, *Pk117*). The lamellipodia of PCP-inhibited cells fail to collapse when they collide with each other *in vivo*, even after 1h of contact (Supplementary Fig. 6a: control, b-c PCP disrupted, Supplementary Movie 10). In contrast to control cells (Fig. 4a, g), these cells do not significantly change the direction of migration (Fig. 4b-d), and as shown by the small acceleration vectors, cell velocity is hardly affected by cell-cell contact ( $p \gg 0.05$ , Fig. 4h-j). Rather, PCP inhibited cells migrate on top of one another, remaining in close contact (Supplementary Movie 10). Similar observations were made *in vitro* after inhibition of PCP signalling by expression of DshDep+, a dominant negative of Wnt11 (dnWnt11)<sup>15</sup>, a morpholino against a protein closely related to mammalian Wnt11 (Wnt11R)<sup>18,19</sup>, or a mixture dnWnt11/Wnt11R MO (Fig. 4e, f, k, l; Supplementary Fig. 7e, f), where PCP inhibited cells did not collapse their protrusions after the collision (Supplementary Fig. 7a-c). Moreover, when control cells and DshDep+ expressing cells are confronted, only control cells collapse their lamellipodia and move away from the DshDep+ cells (Supplementary Fig. 7d, and Movie 11), indicating that PCP/Dsh signalling is required only in the responding cell.

Activation of the PCP pathway is typically accompanied by membrane localization of Dishevelled (Dsh)<sup>20</sup>. We therefore analysed the subcellular localization of Dsh during NC migration *in vitro* and *in vivo*. In both, premigratory NC cells and NC cells cultured on poly-L-lysine (a non-permissive substrate for NC migration<sup>21</sup>), Dsh is seen in cytoplasmic dots (Supplementary Fig. 8a, b). In contrast, clear membrane localization of Dsh is observed at cell-cell contacts of NC cells grown on a permissive fibronectin substrate (Fig. 4o). For the leading cell, the region of cell-cell contact, and therefore the region of Dsh accumulation, is at the trailing end of the cell *in vitro* (Supplementary Fig. 8c). When two leading cells collide, Dsh becomes re-localised at the point of cell-cell contact, with a subsequent change in the direction of migration (Fig. 4p, Supplementary Movie 12). Similar observations on Dsh localisation at cell-cell contacts between NC cells and at the trailing end of a leading cell were made *in vivo* (Supplementary Fig. 8e, f). Lack of PCP signalling leads to loss of Dsh accumulation in the cell contact (Supplementary Fig. 9). This membrane-localised Dsh is reminiscent of the foci described after activation of Dsh in mesodermal cells<sup>22</sup>. We also observed redistribution of Wnt11 and the receptor Fz7 at cell-cell contacts *in vitro* (Fig 4m,

n) and *in vivo* (Supplementary Fig 8g; not shown); both become co-localised at the trailing end of the leading cell (Supplementary Fig. 8d). In summary, our results suggest that cell-cell contacts polarise the cell by regulating the accumulation of ligands, receptors and intracellular element of the PCP signalling pathways.

It is well established that small GTPases play an important role in cell polarity and cell migration. One of them, RhoA is a known downstream effector of PCP/Dsh during NC migration<sup>14</sup>. To examine a possible role for RhoA in Contact Inhibition of Locomotion, we analysed the levels of RhoA activity in isolated and colliding cells by FRET. A significant increase in RhoA activity was detected during cell collision, with highest activity in regions of cell-cell contact (Fig. 4q-s). When PCP signalling was activated in individual cells by expressing Dsh $\Delta$ N<sup>13,15</sup> a similar increase in RhoA activity was observed (Fig. 4s, Supplementary Fig. 10a-c). Finally, inhibition of Rock, a downstream target of RhoA, leads to a complete lost of Contact Inhibition of Locomotion (Fig. 4t, u; Supplementary Fig. 10d and Movie 13). These results implicate RhoA as downstream effector of the PCP in Contact Inhibition of Locomotion.

Although Contact Inhibition of Locomotion was described for cultured cells more than 50 years ago<sup>1,2</sup>, we present the first evidence that it occurs *in vivo*, and has a key role in the directional migration of NC cells. We show that the PCP (non-canonical) Wnt pathway is involved in the process. The data are consistent with a model (Fig. 4v) in which cell-cell contact leads to the localised activation of the PCP signalling in the region of cell contact, which is required for the activation of RhoA. The localization of RhoA at the cell contact directs collapse of cell protrusions and change in cell polarity. It is commonly believed that directional cell migration during embryogenesis involves localised production of molecules that attract migrating cells (chemotaxis)<sup>23-25</sup>. While not ruling out chemoattraction, we suggest that Contact Inhibition of Locomotion could be sufficient for NC directional migration. This mechanism could also direct coherent migration of groups of cells (e.g. mesoderm<sup>26</sup>, lateral line primordium<sup>27</sup>) and the efficient occupation by one cell population of another's territory during metastasis or development (including NC, angioblasts and neurons<sup>28,29</sup>). Most cells migrating *in vivo* maintain close proximity and move in groups. Accordingly, inhibition of cell protrusions between these clustered cells is equivalent to the process of Contact Inhibition of Locomotion. Their typical coherent directional migration is accomplished through a “tip-toe” movement where front cells can only move towards the NC-free zone, i.e. forward. This opens a little space where trailing cells move, and so on (Supplementary Fig1).

NC cells behave similarly to some cancer cells in that they display Contact Inhibition of Locomotion towards like but not unlike cell types<sup>3,5-9</sup>. We propose that homotypic Contact Inhibition of Locomotion confers cells with directionality during migration and that the lack of heterotypic contact inhibition allows them to invade other tissues.

## Methods Summary

*Xenopus* neural crest was labelled with nuclear-RFP/membrane-GFP or membrane-RFP/nuclear-GFP. *In vitro* analysis of neural crest migration was performed using *Xenopus* neural crest cultured on fibronectin-coated plates. For *in vivo* studies we used *Xenopus* embryos grafted with labelled neural crest or zebrafish transgenic lines embryos that express cytoplasm or membrane-GFP under the neural crest promoter *sox10*. Time-lapse was carried out using DIC or fluorescent/confocal microscopy. FRET analysis was performed as described in<sup>14</sup>. For full methods, see Supplementary Material.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

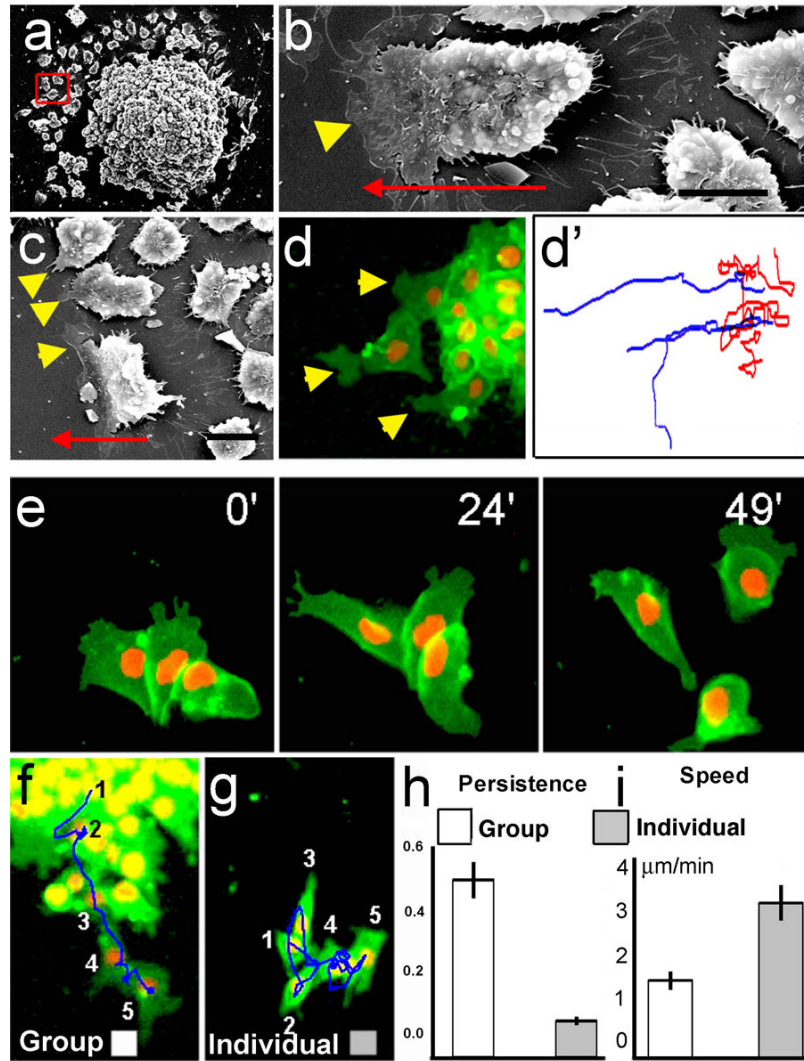
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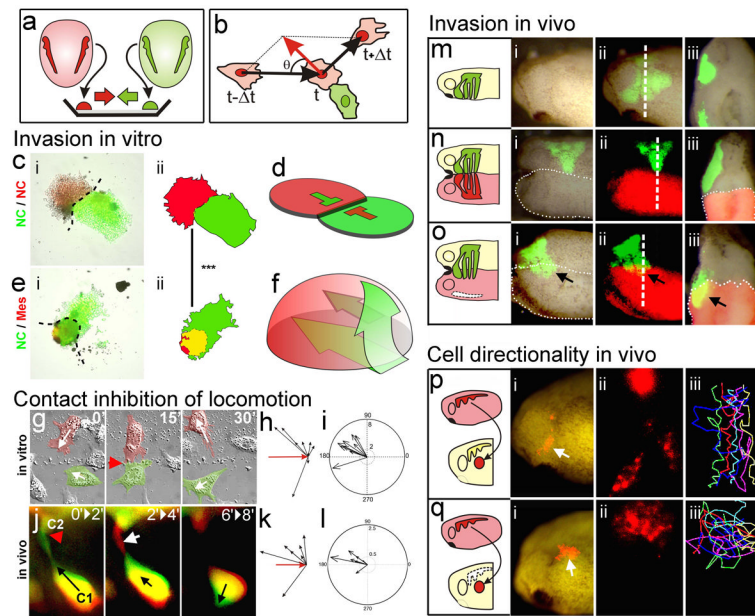
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**Figure 1. Cell-cell contacts polarise migrating NC cells *in vitro***

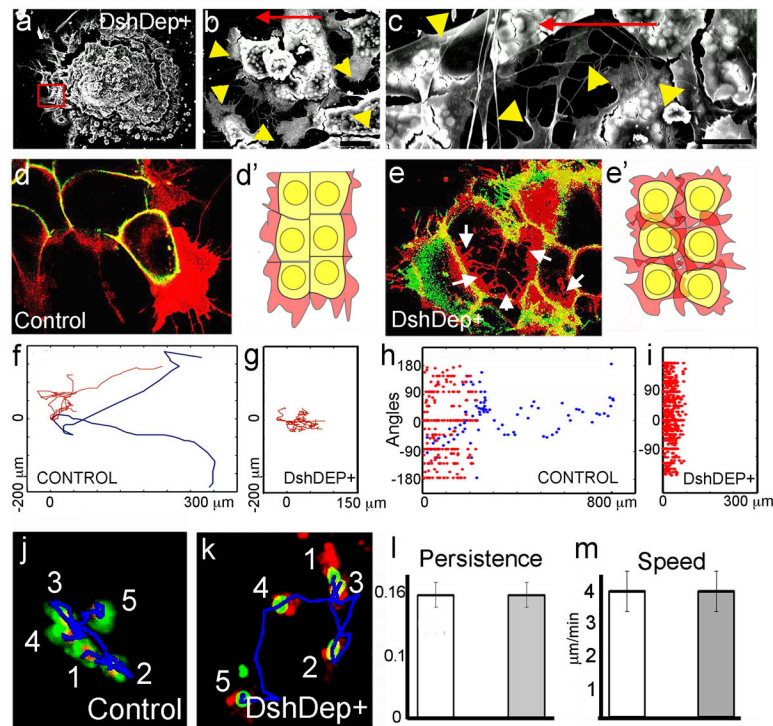
NC were cultured *in vitro* and analysed by SEM (a-c) or time-lapse microscopy of cells expressing membrane-GFP and Nuclear-RFP (d-g). Red square in a indicates leading cells, (defined by its position at the edge of migration, higher magnifications of other leading cells are shown in b and c). Arrowheads: lamellipodia (note their presence only in the leading cells, either by SEM, c; or fluorescence, d). Arrow: direction of migration. Bars in b,c:  $50\mu\text{m}$ . (d') Tracks of leading (blue) and trailing (red) cells shown in d. (e) Three frames of a time-lapse for dissociated and re-aggregated NC cells. (f, g) Temporal projection to compare the migration of a group of NC cells *versus* individual cells. Numbers indicate the position of the same cell at different time frames 10 minutes apart. Track in a blue line. Persistence (h) and Speed (i) of migration for the migration as a group (white bar) or as an individual cell (grey bar) ( $p < 0.005$ ,  $n = 60$ ).



**Figure 2. Contact Inhibition of Locomotion in NC cells *in vitro* and *in vivo***

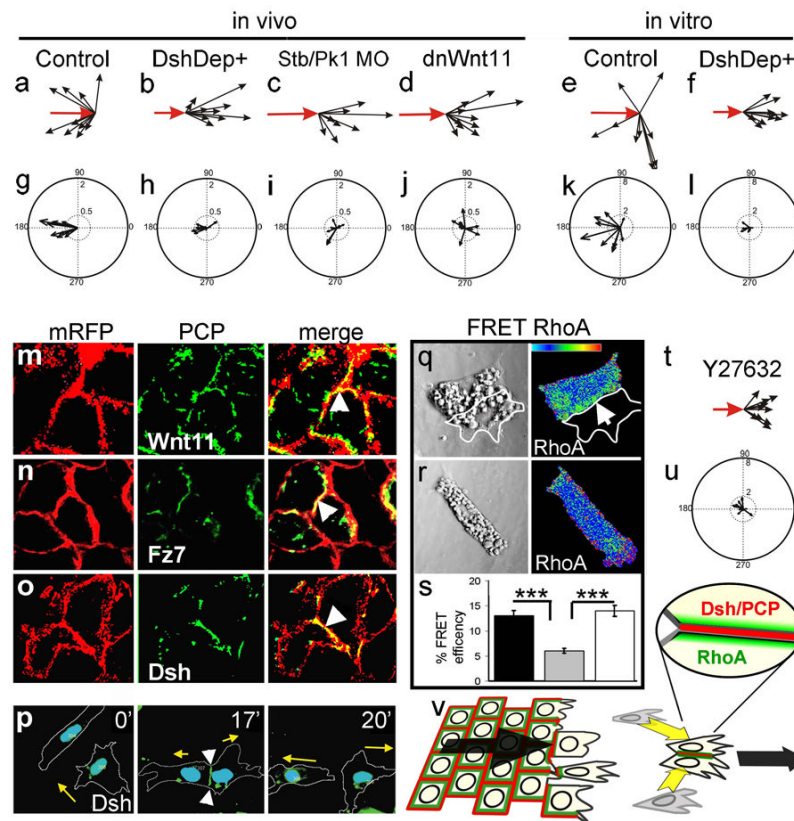
(a) Experimental design. (b) Analysis of Contact Inhibition of Locomotion. Mean velocities were measured  $\Delta t$  minutes before and after the collision. Acceleration (red) was calculated for each cell. Angle of collision calculated after initial trajectory alignment ( $\theta$ ). (c-f) Invasion of confronted explants. (c) There is no invasion in NC/NC confrontations (i), outlines in (ii), overlapping area in yellow; schematised in (d). (e) NC explants completely invade and cover mesodermal explants (i), outlines in (ii), overlapping area in yellow; schematised in (f). Green arrows in f: NC path of invasion (see Supplementary Fig. 3 for supporting confocal images). (g-l) Contact Inhibition of Locomotion. (g) Collision between two pseudocoloured NC cells *in vitro*. Time in minutes. White arrows: direction of migration; red arrowhead: collision. (h) Velocity vectors for NC *in vitro*, initial velocity vector (red arrow). (i) Acceleration vectors for NC collisions *in vitro*. They are clustered after the collision ( $p < 0.005$ ,  $n = 10$ ). (j) Collision of two NC cells (C1, C2) *in vivo* shown as the difference between two consecutive two-minute frames. Green: new area; red: collapsing area; black arrow: direction of migration; red arrowhead: cell contact; white arrow: collapsing protrusion. (k) *In vivo* velocity vectors. (l) *In vivo* acceleration. They are clustered after the collision ( $p < 0.01$ ,  $n = 10$ ). (m-o) NC invasion *in vivo*. i, ii: lateral view; iii: transverse section along the dashed line showed in ii. NC cells are not able to invade an adjacent embryo that has NC (n; 0% of invasion,  $n = 15$ ), but they can to invade an embryo without NC (o; arrow, 80% of invasion,  $n = 10$ ). (p, q) Cell directionality *in vivo*. A small group of Nuclear-RFP-labelled NC cells were grafted into a normal embryo (p) or in an embryo in which the NC were previously removed (q). Note that grafted cells migrate directionally in the intact embryo (persistence:  $0.6 \pm 0.04$ ,  $n = 30$ ), but not when the host NC were removed (persistence:  $0.2 \pm 0.02$ ,  $n = 20$ ).





**Figure 3. Effect of PCP signalling on cell contacts**

(a-c). SEM of *Xenopus* cultured NC expressing DshDep+. Red square indicates leading cells. Higher magnification views of other leading cells are shown in b and c. Arrowheads: cell protrusions; arrow: direction of migration. Bars: 25 $\mu$ m in b, 50 $\mu$ m in c. (d, e) Two-Plane Confocal image to show cell protrusions (red) and cell shape (green). Cell protrusions are produced only at the border of the control explant (d), while they are observed between the DshDep+ cells (arrows in e). (d', e') Schematic representation of d and e. (f-i) Analysis of tracks of migrating NC cells. Blue: leading cells; red: trailing cells. Tracks of control (f) or DshDep+ (g) cells. Distribution of angles of migration for leading (blue) and trailing (red) control (h) or DshDep+ (i) cells *versus* the distance from the origin. (j-m) Analysis of migration of dissociated NC cells. (j, k) Five frames taken every 10 min were overlapped for a control cell (j) and a DshDep+ cell (k). Numbers: consecutive position of the cell. Blue line: track. Persistence (l) and Speed (m) were calculated for control (white bar) and DshDep+ (grey bar) cells. ( $p < 0.05$ ;  $n = 62$ ).



**Figure 4. Contact Inhibition of Locomotion: requirement of PCP and RhoA activities**  
 Cell collisions were analysed *in vivo* (a-d, g-j) and *in vitro* (e, f, k, l). Velocities (a-f) and accelerations (g-l) were measured after the indicated treatments. Scale is the same for all panels. The change of velocity is significantly and clustered in the controls ( $p < 0.005$ ,  $n = 10$ ). No significant change is observed in any of the PCP treatments ( $p \gg 0.05$ ,  $n = 10$  for all cases) (m-p). Different PCP components are localised at the cell-cell contact (arrowheads). mRFP: membrane-RFP; (m) Wnt11-YFP. (n) Fz7-YFP. (o) Dsh-GFP. (p) Cells expressing Dsh-GFP were analysed during a cell collision. The outline of the cell is taken from the DIC images, time in minutes; arrow: direction of migration; arrowhead: cell contact showing Dsh localization. (q-u) Role of RhoA. (q-s) FRET analysis of RhoA activity. (q) Two NC cells in contact showing RhoA activity localised at the cell contact (arrow). (r) Single NC cell. (s) RhoA FRET efficiency. Black bar: cells in contact; grey bar: single cell; white bar: single cell in which PCP has been activated by expression of Dsh $\Delta$ N. \*\*\*:  $p < 0.005$ ;  $n = 12$  each condition. (t, u) Cell collisions were analysed in the presence of the Rock inhibitor Y27632. (t) Velocity vectors; (u) acceleration vectors. No significant change in the velocity ( $p \gg 0.05$ ,  $n = 10$ ) (v) Contact Inhibition of Locomotion is controlled by localization of PCP elements (red) and RhoA activity (green) at the cell contact leading to directional migration (arrows).