Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells

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Contact inhibition of locomotion (CIL)—a process defined as "the stopping of the continual locomotion of a cell in the same direction after collision with another cell"

it was noted that many metastatic cancer cells demonstrate unimpeded migration when they come into contact with non-malignant cells.

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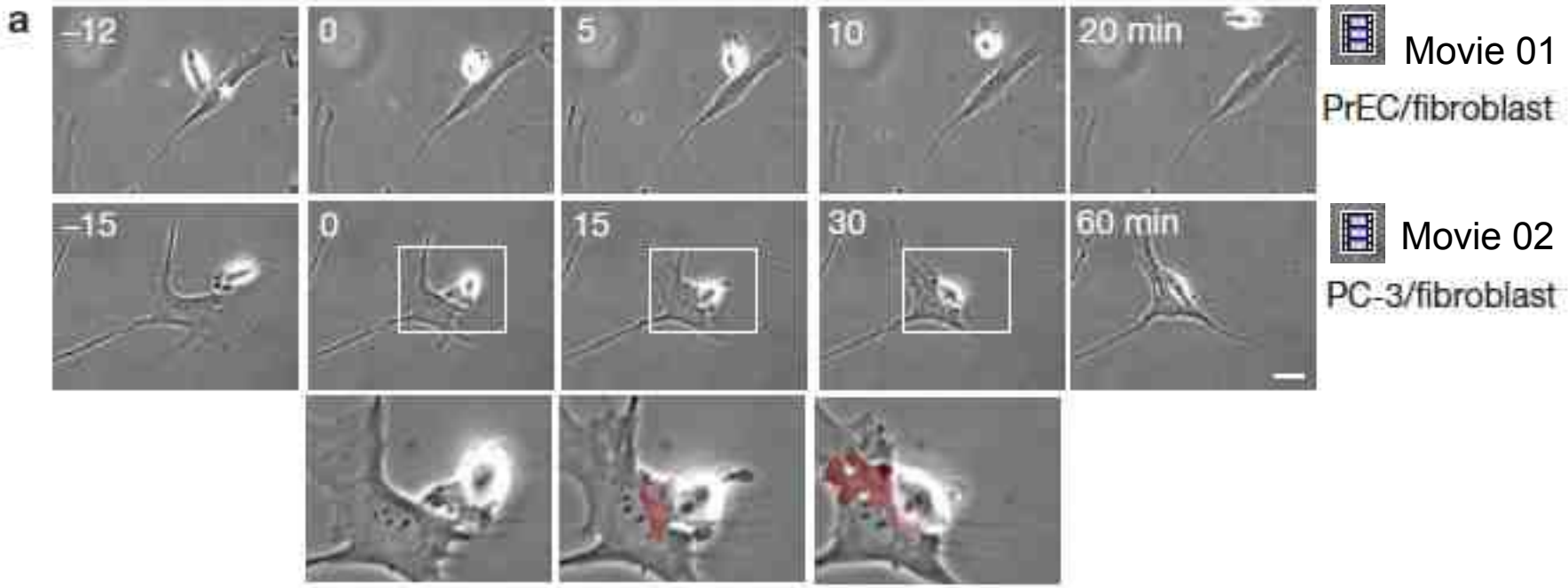
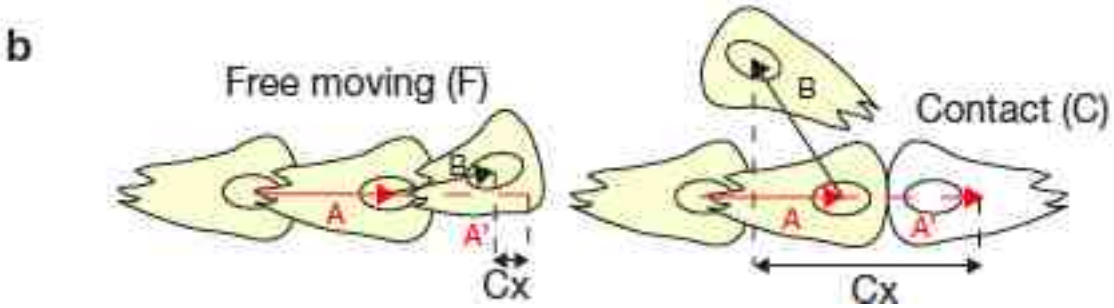
PC-3 cells do not display CIL on contact with fibroblasts and endothelial cells

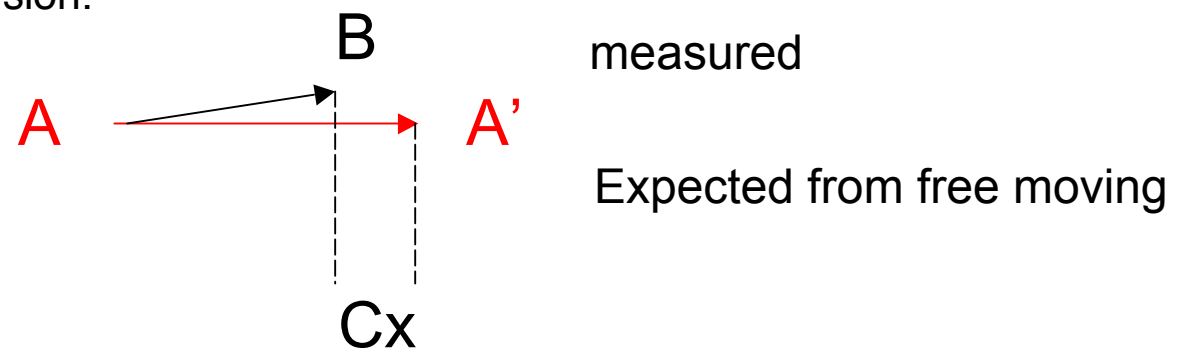
PC-3 cells = tumorigenic prostate cancer cell line that do form metastasis
DU-145 = tumorigenic prostate cancer cell line that di not form metastasis
PrEC cells = Non-tumorigenic primary prostatic cells

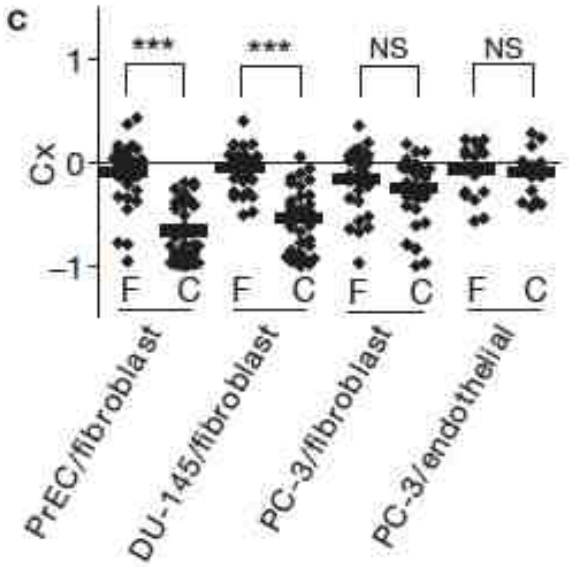


(a) Representative time-lapse microscopy images, at the indicated times, of a PrEC/fibroblast collision (top and Supplementary Information, Movie S1) and a PC-3/fibroblast collision (middle and Supplementary Information, Movie S2). Asterisk indicates fibroblast cell and insets at the bottom indicate magnification of boxed area; false-colour indicates region of PC-3 cell lamella extending beneath the fibroblast.

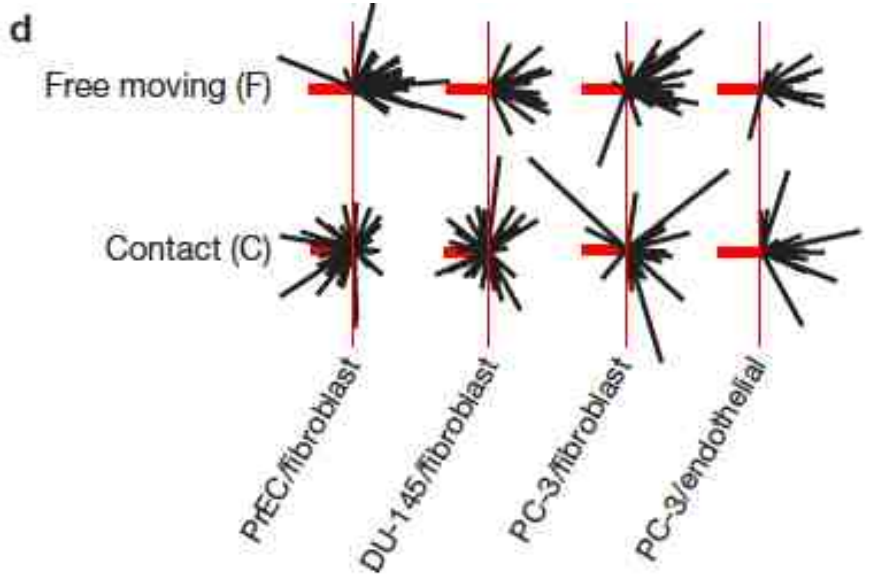


(b) CIL is quantified by comparing contact acceleration indices (Cx) of free-moving cells and colliding cells. Cells were tracked before (A) and after (B) a collision (free-moving cells were tracked for the same time periods). The component Cx of vector B–A represents the difference between how far the cell has progressed in the direction of A' and how far it would have gone had there been no collision.

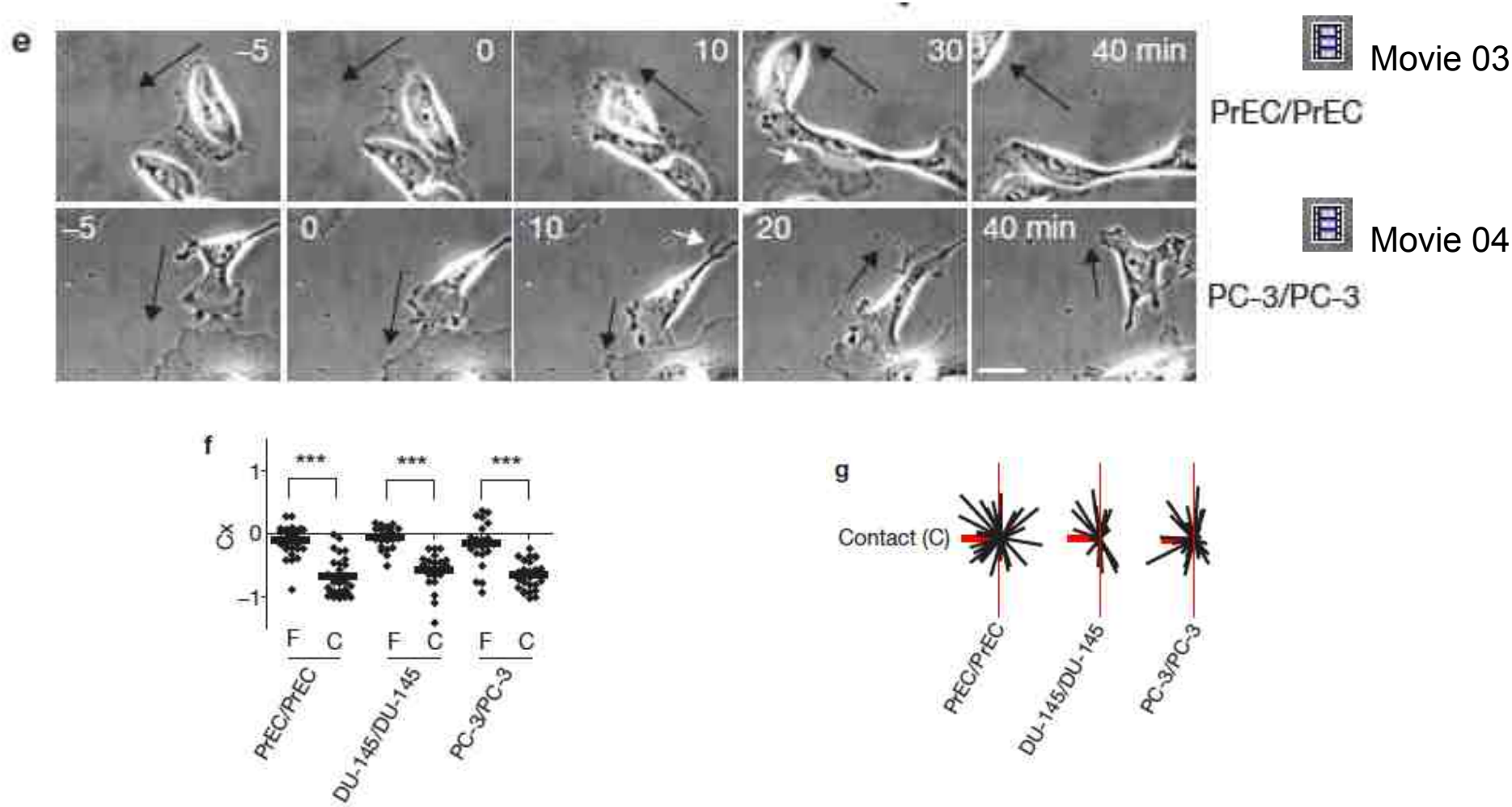




Contact acceleration indices (Cx) of free-moving cells (F) versus colliding cells (C); PrEC/fibroblast (*n* = 36), DU-145/fibroblast (*n* = 32), PC-3/fibroblast (*n* = 29), PC-3/endothelial cell (*n* = 15). Triple-asterisks indicate *P* < 0.001, NS; not significant, determined by a Mann-Whitney test(

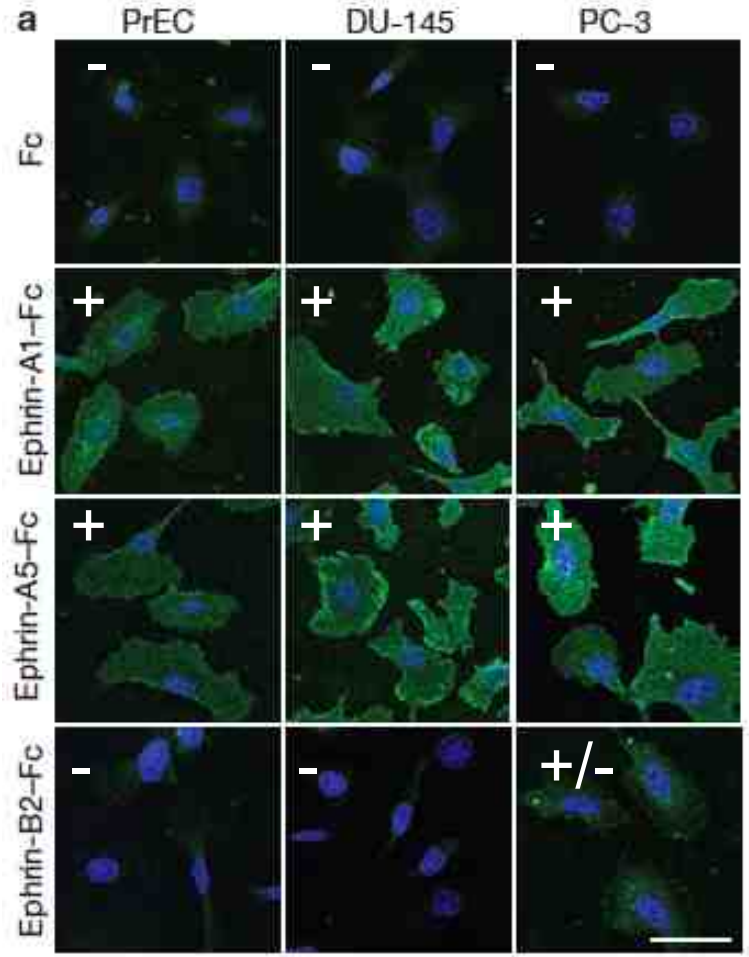


Scaled cell-displacement vector diagrams of free-moving cells and colliding cells, tracked during timelapse microscopy. Thick red line denotes the scaled displacement of all cells before contact and thin black lines are those of each cell following contact.



(e) Representative time-lapse microscopy images, at the indicated times, of collisions between two PrECs (top and Supplementary Information, Movie S3) and two PC-3 cells (bottom and Supplementary Information, Movie S4). Black arrows indicate direction of migration and white arrow indicates a new leading edge forming.

Ligand binding immunofluorescence



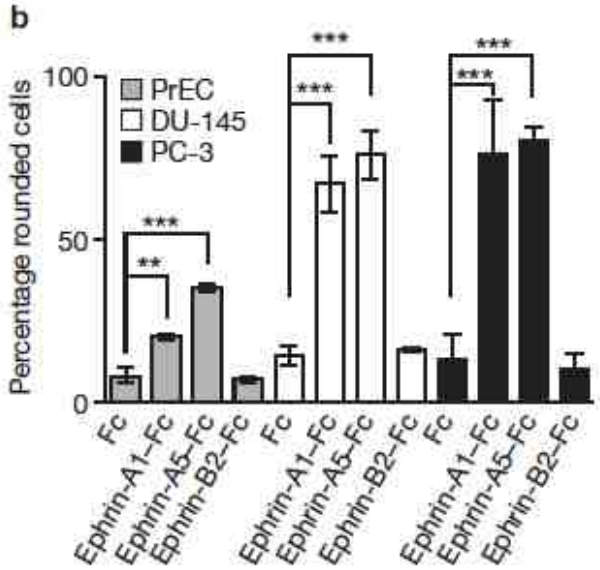
Immunofluorescence microscopy of cells treated with anti-Fc antibodies (green) to detect surface binding of ephrin-A1–Fc, ephrin-A5–Fc, ephrin-B2–Fc and control Fc, to PrEC, DU-145 and PC-3 cells. Hoechst (blue) stains nuclei. Scale bar, 50 µm.

Ephrin-A1 binding

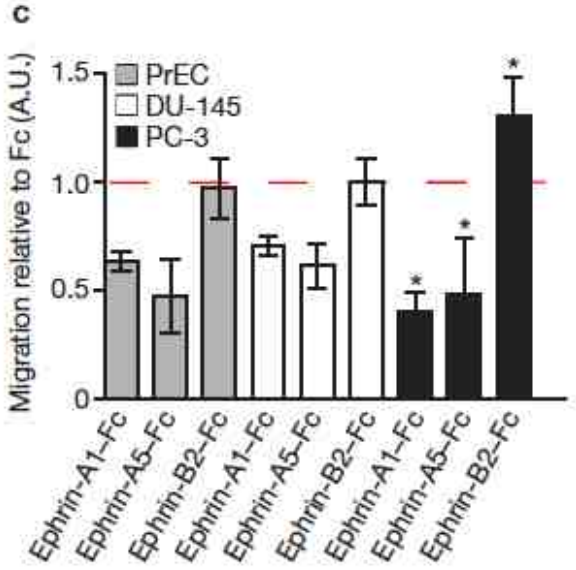
Ephrin-A5 binding

Ephrin-B2 binding

migration of PC-3 cells is inhibited by ephrin-A ligands, but enhanced by ephrin-B2

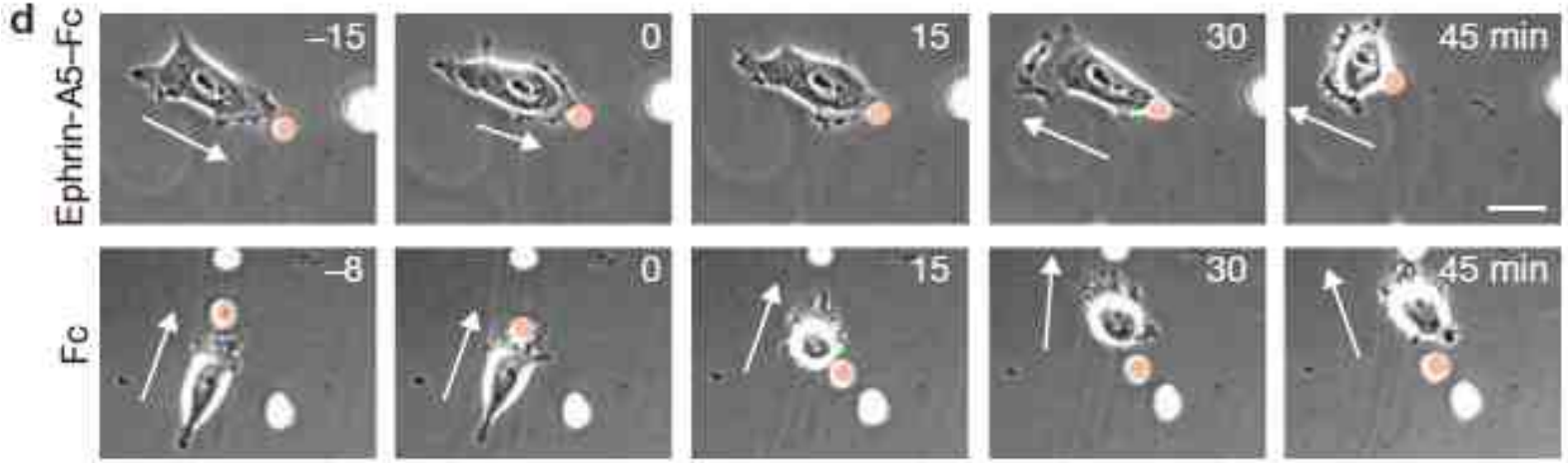


b) Prostate cells were treated with clustered ephrin–Fc proteins before fixation and phalloidin staining. Data are expressed as percentage of cells with retraction of the cell periphery (rounded cells) for each treatment as indicated. Data are means ± s.d. Triple asterisks indicate $P < 0.001$ and double asterisks indicate $P < 0.01$, as determined by an unpaired Student's t-test ($n = 4$; 100 cells counted per experiment).



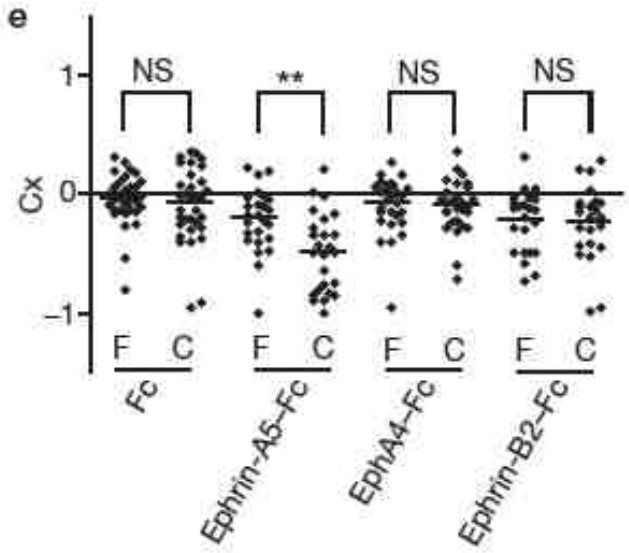
(c) The underside of a transwell chamber was coated with ephrin–Fc proteins, as indicated, and the numbers of cells migrating through were scored. Data are expressed as fold-change with respect to control Fc-coated chambers (red dotted line). Data are means ± s.d. Asterisk indicates$P < 0.05$, as determined by a paired Student's t-test ($n = 5$). A.U.; arbitrary units.

Ephrin-A ligands are sufficient to induce CIL between PC-3 cells.



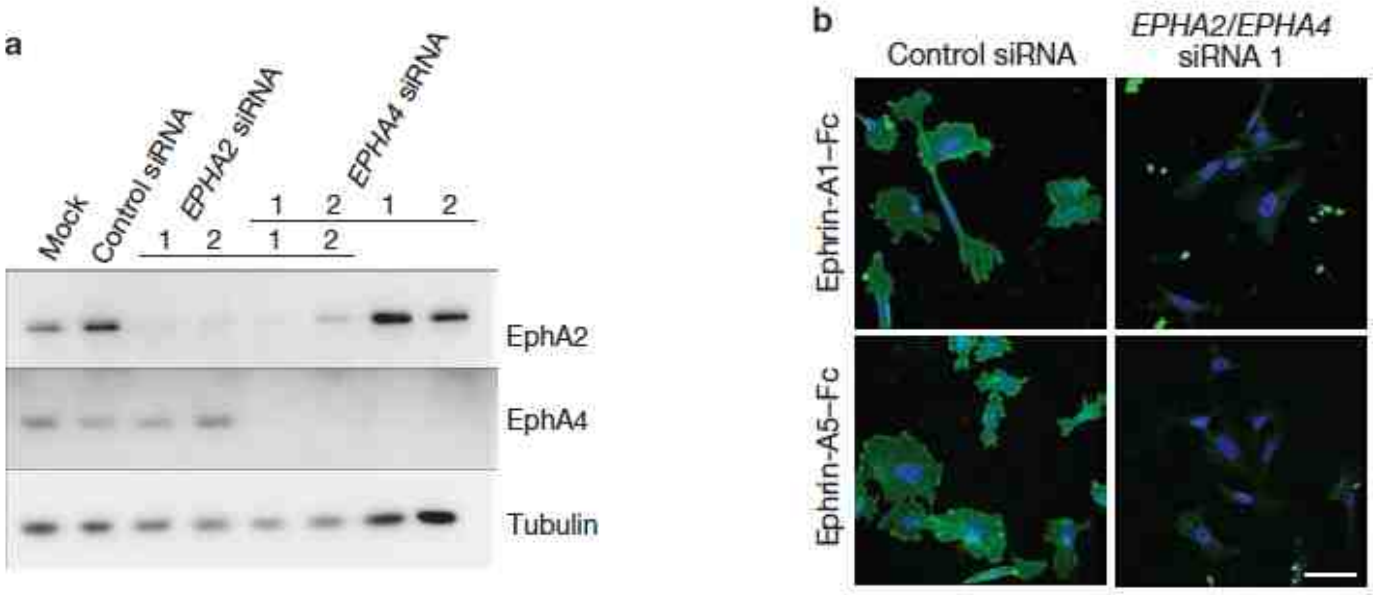
Movie 05

Movie 06

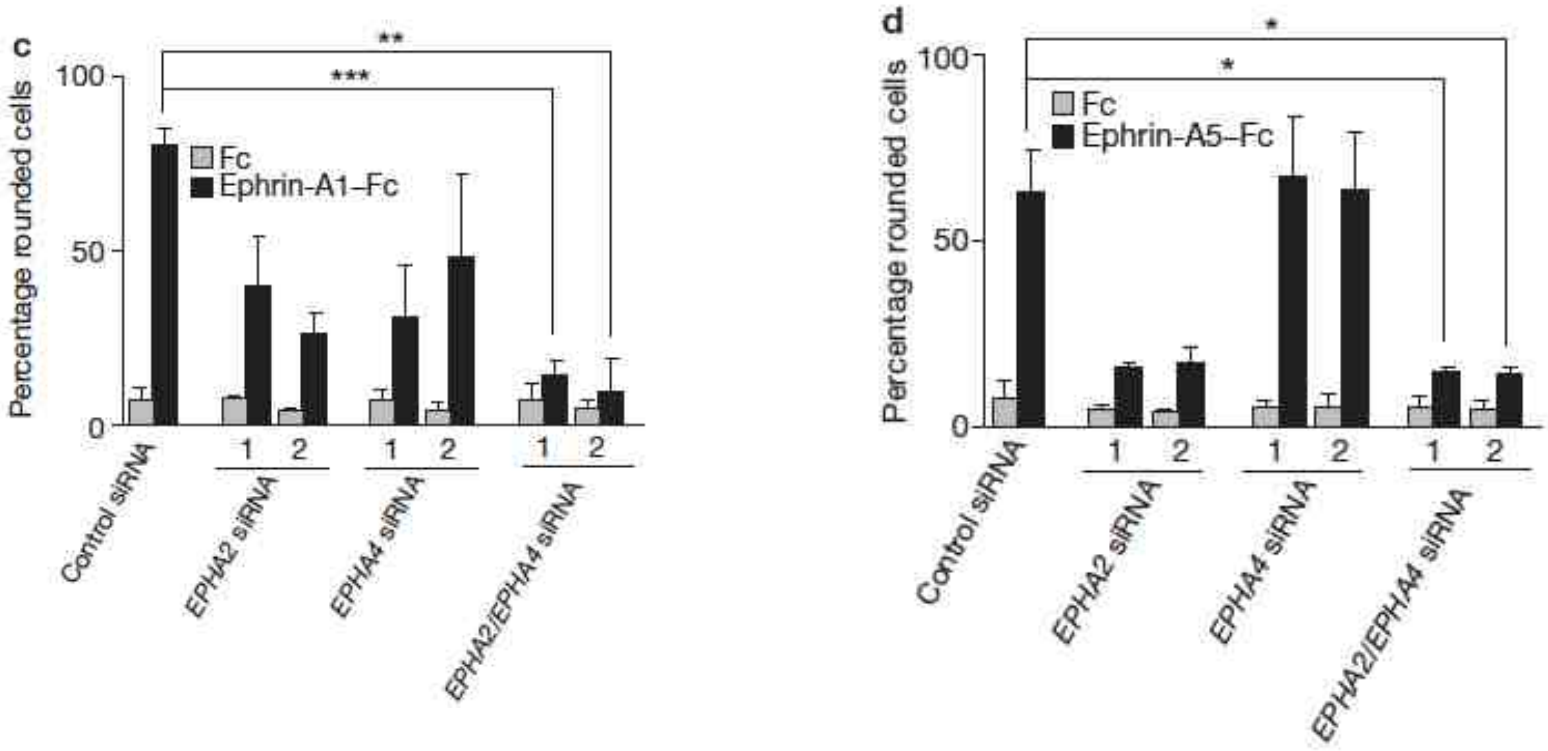


(d) Representative time-lapse microscopy images, at the indicated times, of a PC-3 cell colliding with a silica protein-A bead (red pseudocolour) coated with ephrin-A5–Fc (top and Supplementary Information, Movie S5) or with Fc (bottom and Supplementary Information, Movie S6). Arrows indicate direction of migration. Scale bar, 25 μm. (e) Contact acceleration indices (Cx) of free-moving cells (F) versus cells colliding with beads (C), coated as indicated; Fc (n = 33), ephrin-A5–Fc (n = 25), EphA4–Fc (n = 28), ephrin- B2–Fc (n = 23). Double asterisks indicate $P < 0.01$, as determined by a Mann-Whitney test.

Eph receptor interfering

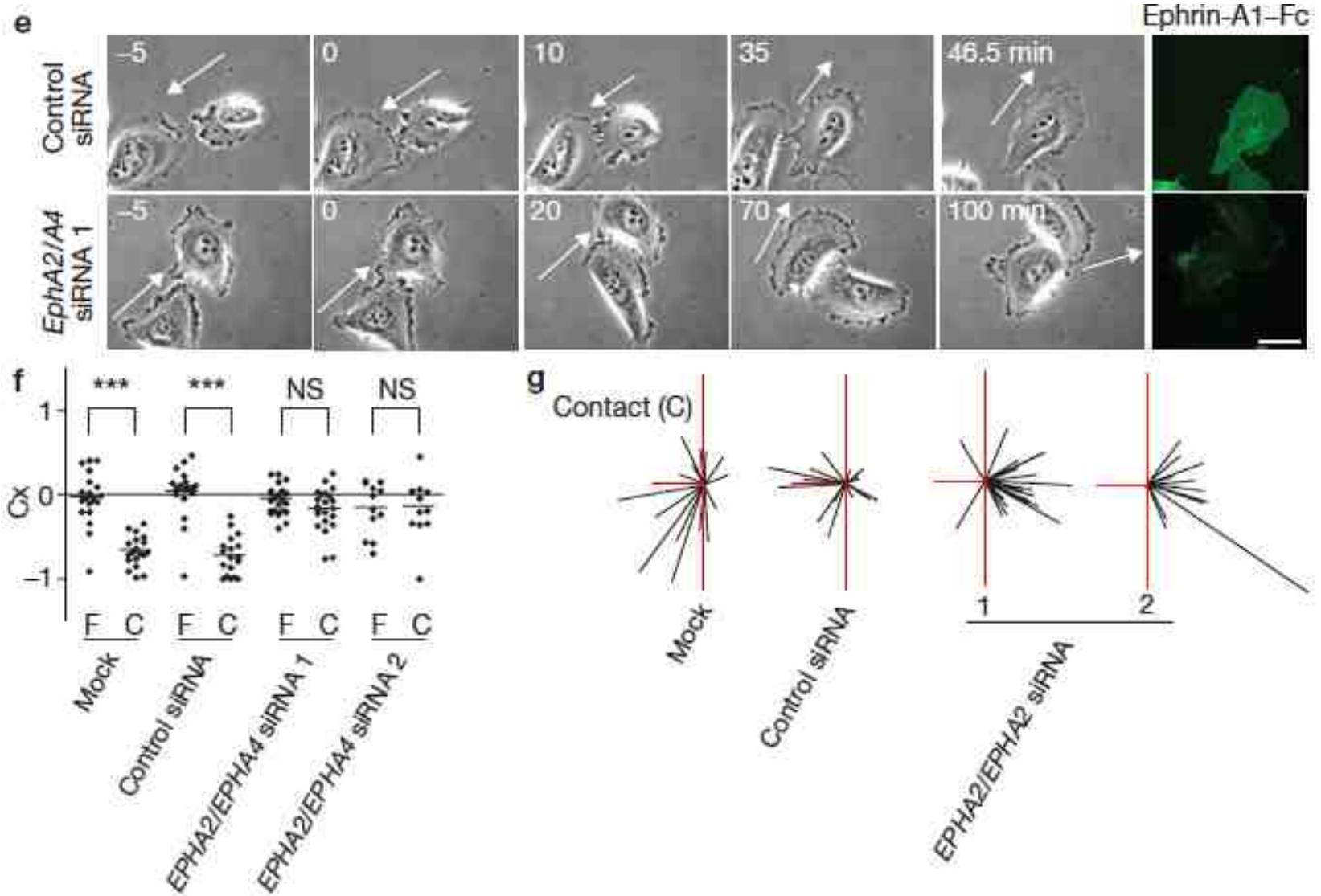


PC-3 cells mock transfected, transfected with a non-targeting siRNA oligonucleotide (control siRNA) or transfected with siRNA oligonucleotides specific to *EPHA2* and *EPHA4* (two different oligonucleotides for each) were a) immunoblotted using antibodies against the indicated proteins. Tubulin was used as a loading control b) analysed for Ephrin-fc binding



(c, d) PC-3 cells, transfected with siRNA oligonucleotides as indicated, were treated with clustered ephrin-A1–Fc, ephrin-A5–Fc or Fc, and rounded cells were counted after fixation and staining with phalloidin. Data are means ± s.d. (siRNA 1, n = 4; siRNA 2 n = 3; 100 cells counted per experiment). Triple asterisks indicate $P < 0.001$, double asterisks indicate $P < 0.01$ and asterisk indicates $P < 0.05$, as determined by an unpaired Student's t-test.t

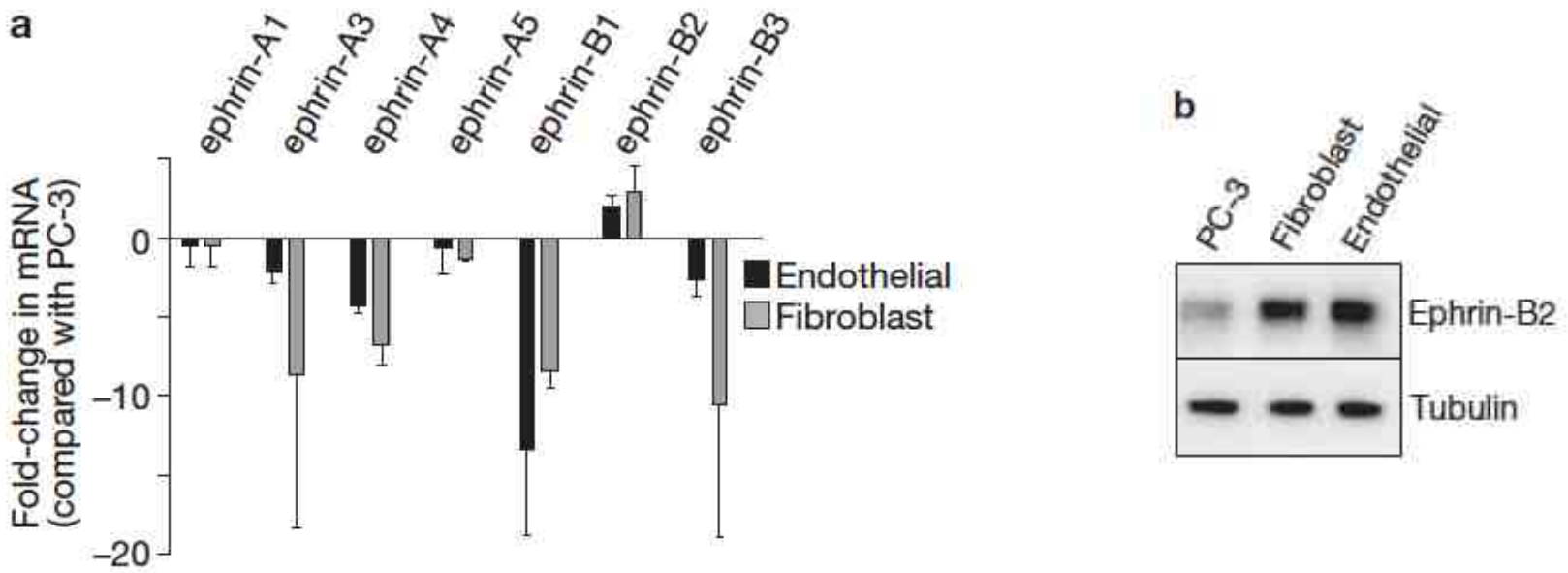
EphA2 and EphA4 are required for CIL between PC-3 cells



Movie 07

Movie 08

Ephrin mRNA in endothelial cells and fibroblasts / PC-3 cells

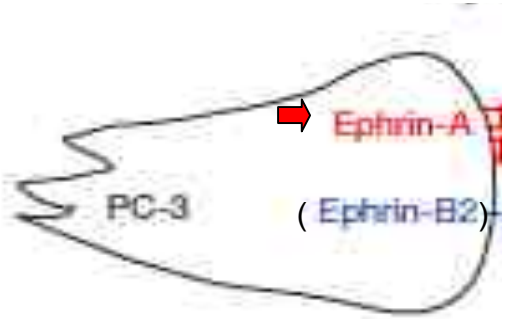


(a) Relative expression profiles of ephrin ligands by fibroblasts and endothelial cells versus PC-3 cells. Data are expressed as fold-change in mRNA with respect to PC-3 cell mRNA levels, as determined by real-time RT–PCR. Data are means ± s.d. (n = 3). (b) PC-3, fibroblast and endothelial cell lysates were immunoblotted with antibodies against the indicated proteins.

ephrin-A1, ephrin-A3, ephrin-A4 and ephrin-A5 were expressed by fibroblasts and endothelial cells, but all at lower levels than PC-3 cells (a). Interestingly, ephrin-B2 expression is higher in fibroblasts and endothelial cells, compared with PC-3 cells (a and b).

Ephrin mRNA in endothelial cells and fibroblasts / PC-3 cells

PC3 cells: Ephrin A



ephrin-A1, ephrin-A3, ephrin-A4 and ephrin-A5 were expressed by fibroblasts and endothelial cells, but all at lower levels than PC-3 cells

Fibroblasts: Ephrin B2

Interestingly, ephrin-B2 expression is higher in fibroblasts and endothelial cells, compared with PC-3 cells (a and b).

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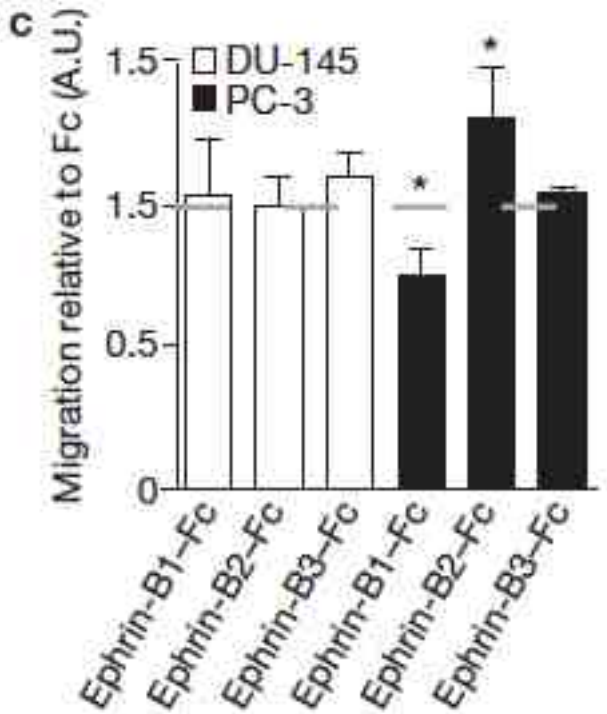
only ephrin-B2–Fc promoted migration of PC-3 cells in transwell assays

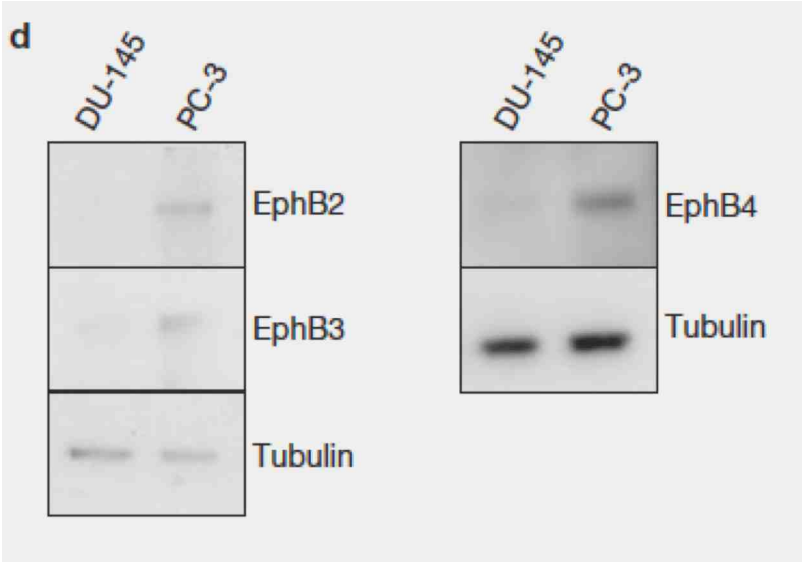


(c) The underside of a transwell chamber was coated with Fc-coated ephrin-B-ligands as indicated, and numbers of cells migrating through the chamber were scored. Data are expressed as fold-change with respect to control Fc-coated chambers (grey dotted line). Data are means ± s.d. ($n = 5$). Asterisk indicates $P < 0.05$, as determined by a paired Student's t-*t*est.

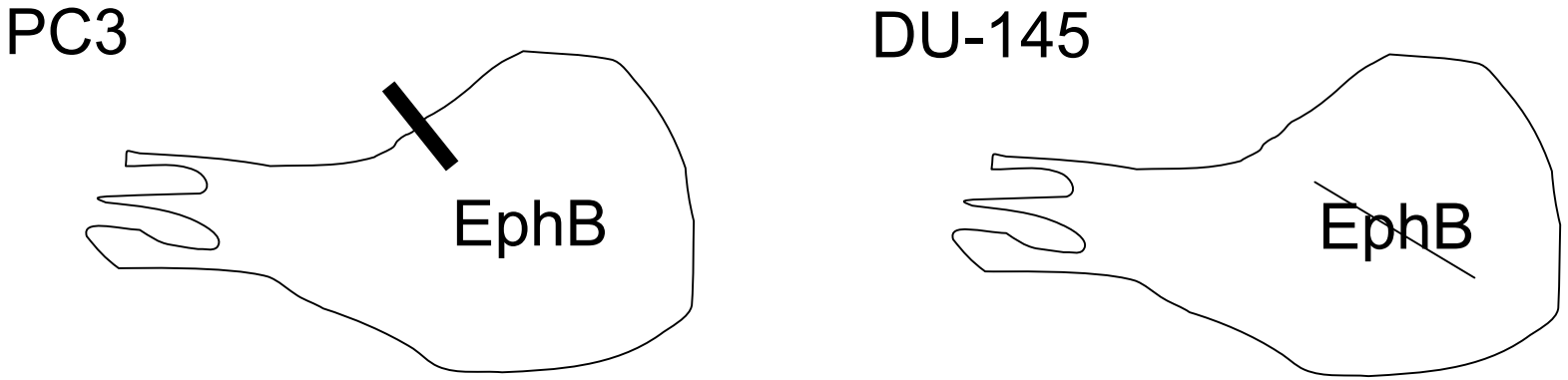
Movie 09

ephrin-B2 can stimulate migration specifically in PC-3 cells, but not in DU-145 cells, and the defective CIL demonstrated by PC-3 cells on contact with fibroblasts and endothelial cells may be because of ephrin-B2 expression by these cells

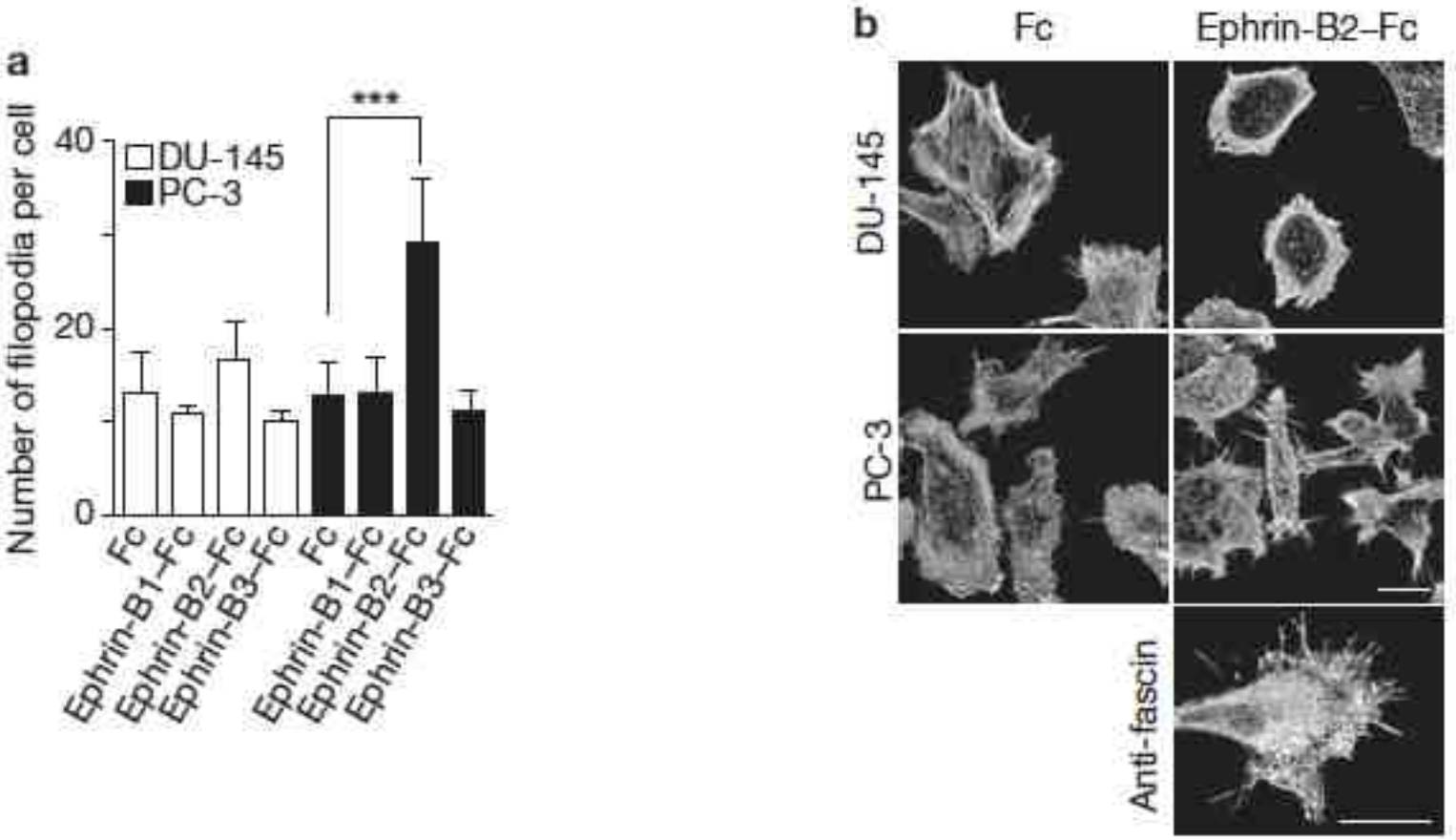
PC-3 cells express higher levels of EphB receptors than DU-145 cells



(d) Left: EphB2 and EphB3 were immunoprecipitated from lysates of DU-145 or PC-3 cells and detected by immunoblotting. Right: lysates of DU-145 or PC-3 cells were immunoblotted with antibodies against EphB4. Tubulin was used as a loading control in both cases. Uncropped images of blots are shown in Supplementary Information, Fig. S8.



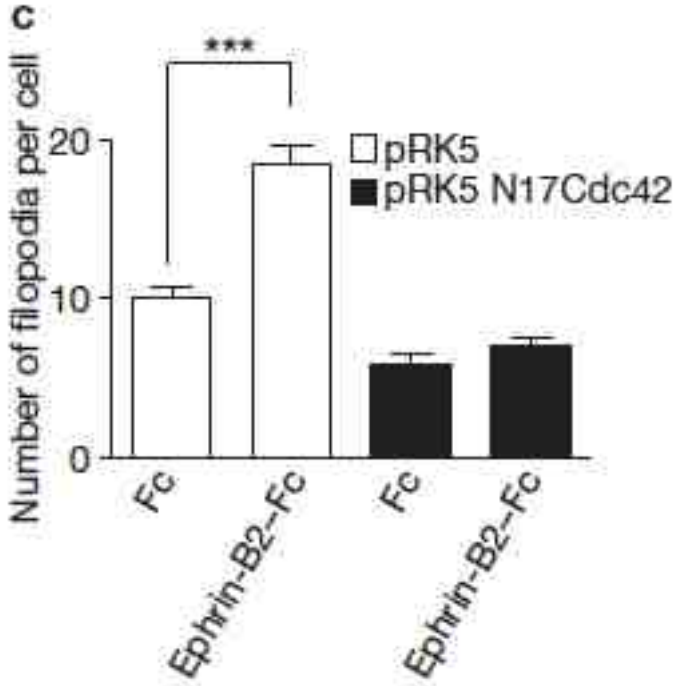
Ephrin-B2 induces filopodia



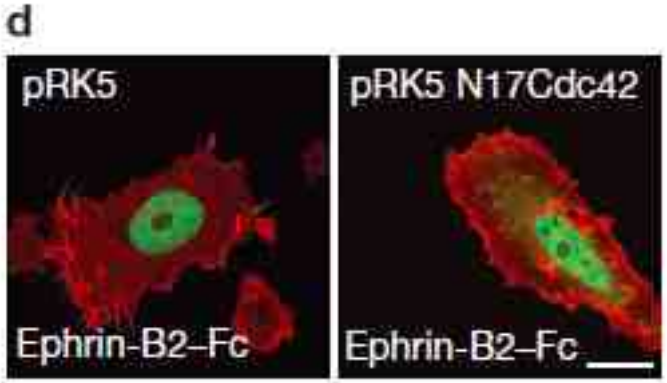
DU-145 or PC-3 cells were treated with clustered ephrin-B–Fc or Fc and analysed for the formation of filopodia. Data are expressed as mean number of filopodia per cell ± s.d. (n = 3, 50 cells counted per experiment). Triple asterisks indicate $P < 0.001$, as determined by an unpaired Students t-test. (b) Confocal microscopy images of DU-145 and PC-3 cells treated with clustered ephrin-B2–Fc or Fc and fixed and stained with phalloidin or with antibodies against fascin (PC-3 cell, bottom).3

Increased migration, coupled with the induction of filopodia, suggested that Cdc42 was being activated downstream of the ephrin-B2 cue

Ephrin-B2 induces filopodia by activating Cdc42 in PC-3 cells.



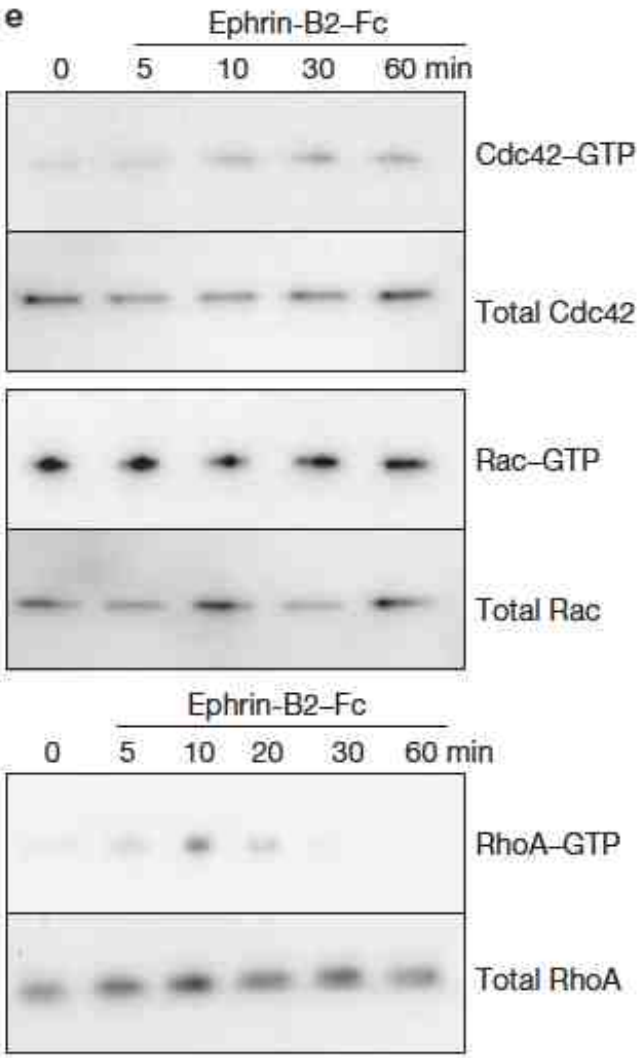
N17Cdc42 (dominant negative) expression => no increase in filipodia formation after Ephrin-B2-fc treatment

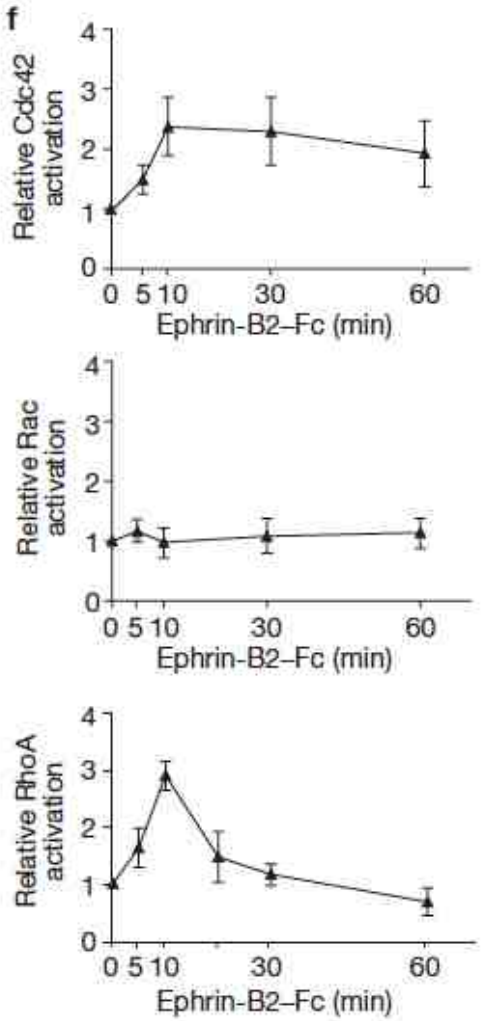


(c) PC-3 cells were microinjected with expression vectors as indicated, and then treated with ephrin-B2–Fc or Fc, and analysed for the formation of filopodia. Data are expressed as mean number of filopodia per cell ± s.d. (pRK5 and Fc, n = 92, pRK5 and ephrin-B2–Fc, n = 82; N17Cdc42 and Fc, n = 87; N17Cdc42 and ephrin-B2–Fc, n = 101). Triple asterisks indicate $P < 0.001$, as determined by an unpaired Student's t-test. (d) Confocal microscopy images of phalloidin-stained (red) PC-3 cells after microinjection of indicated expression constructs, followed by treatment with ephrin-B2–Fc; injection marker (green).

Ephrin-B2 induces filopodia by activating Cdc42 in PC-3 cells.

pulldown studies: sustained activation of Cdc42 activation in pc3 cells with transient activation of Rho

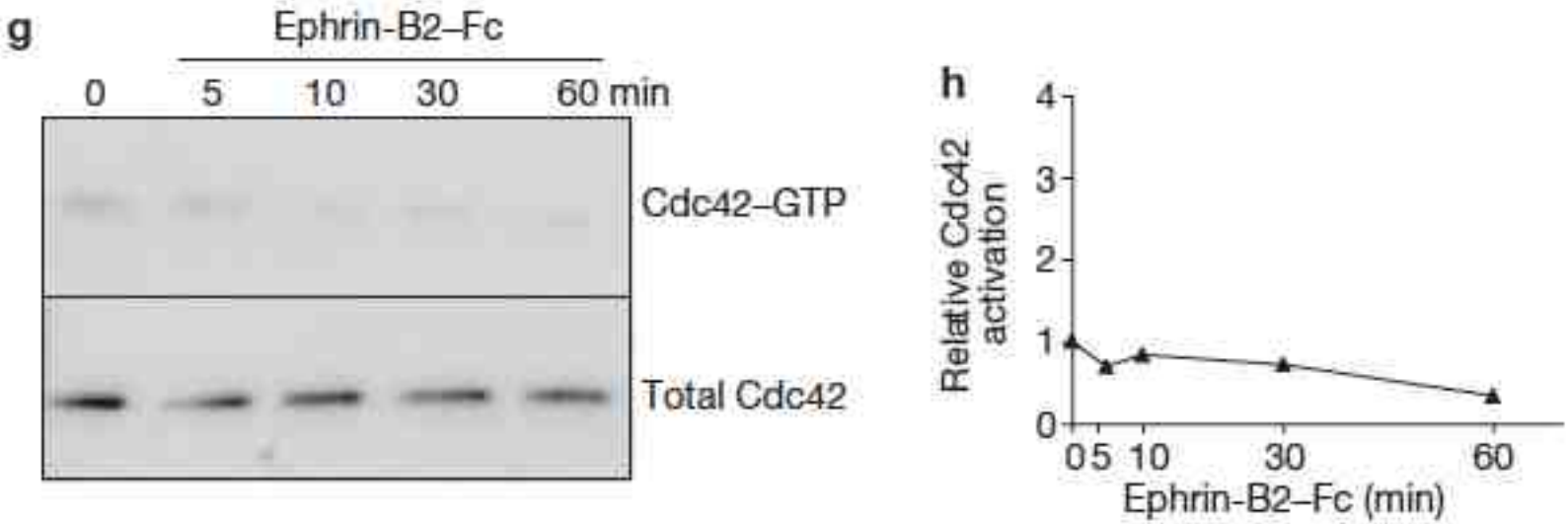




(e) PC-3 cells were treated with ephrin-B2–Fc. At the indicated times, cells were lysed, followed by pulldown of Cdc42–GTP, Rac–GTP (using PAK1-CRIB beads) and RhoA–GTP (using Rhotekin Rho binding domain beads). Proteins were resolved by SDS–PAGE and detected by immunoblotting.

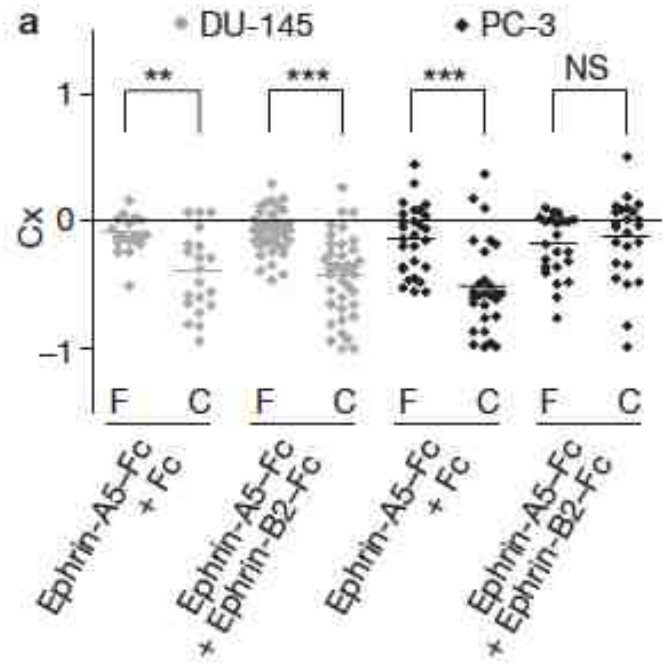
(f) Quantification of Rho-GTPase activation in PC-3 cells following the addition of ephrin-B2–Fc. Experiments were carried out as in e, band intensities were quantified, and normalised intensities were calculated relative to control untreated cells at 0 min. Data are means ± s.d. (n = 3).

No Cdc42 activation was observed in DU-145 cells following addition of ephrin-B2–Fc

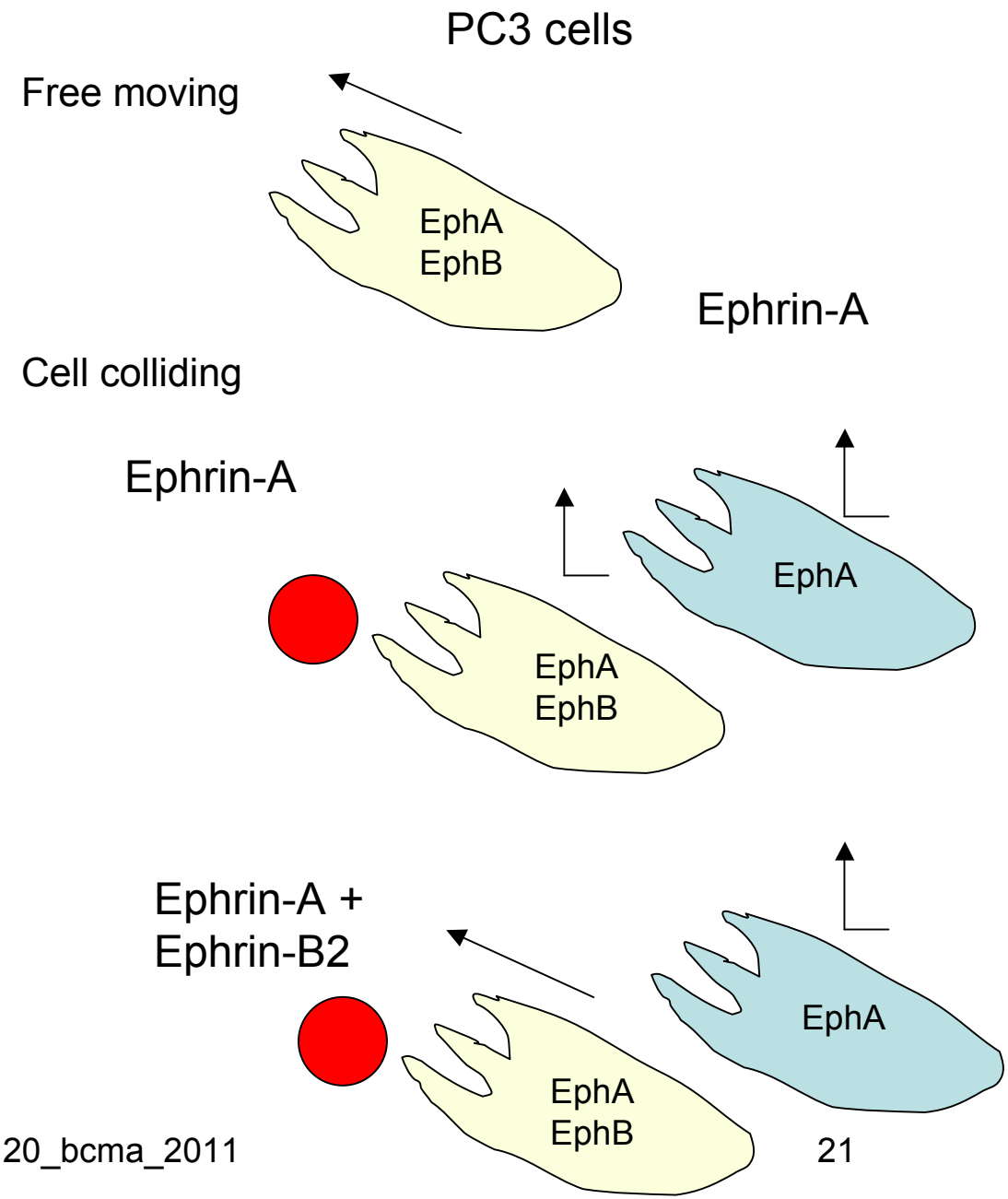


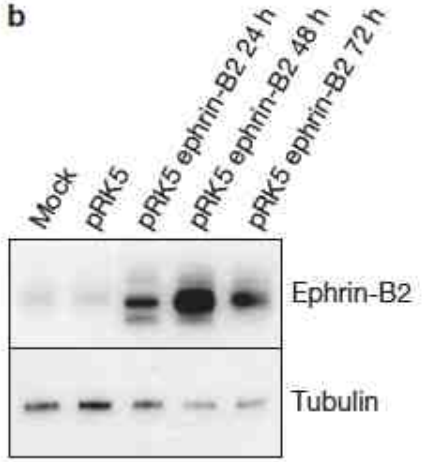
(g) DU-145 cells were treated with ephrin-B2–Fc. At the indicated times, cells were lysed, followed by pulldown of Cdc42–GTP using PAK1-CRIB beads. Proteins were resolved by SDS–PAGE and detected by immunoblotting.

(h) Quantification of Cdc42 activation in DU-145 cells following the addition of ephrin-B2–Fc. Experiments were carried out as in g, band intensities were quantified, and normalized intensities were calculated relative to control untreated cells at 0 min. Data are means (n = 2).

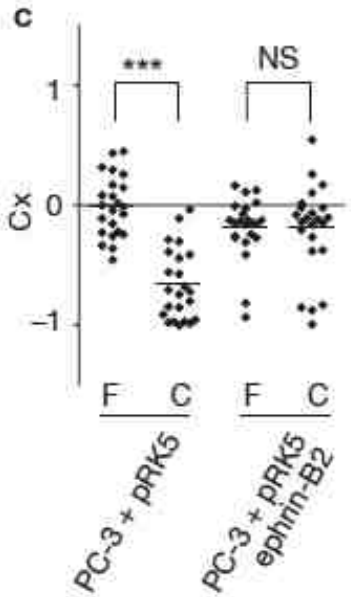


(a) Contact acceleration indices (Cx) of free-moving cells (F) versus cells colliding (C) with beads coated with the indicated ephrin–Fc (1:1 molar ratios). Triple asterisks indicate $P < 0.001$, double asterisks indicate $P < 0.01$, NS; not significant, as determined by a Mann-Whitney test.

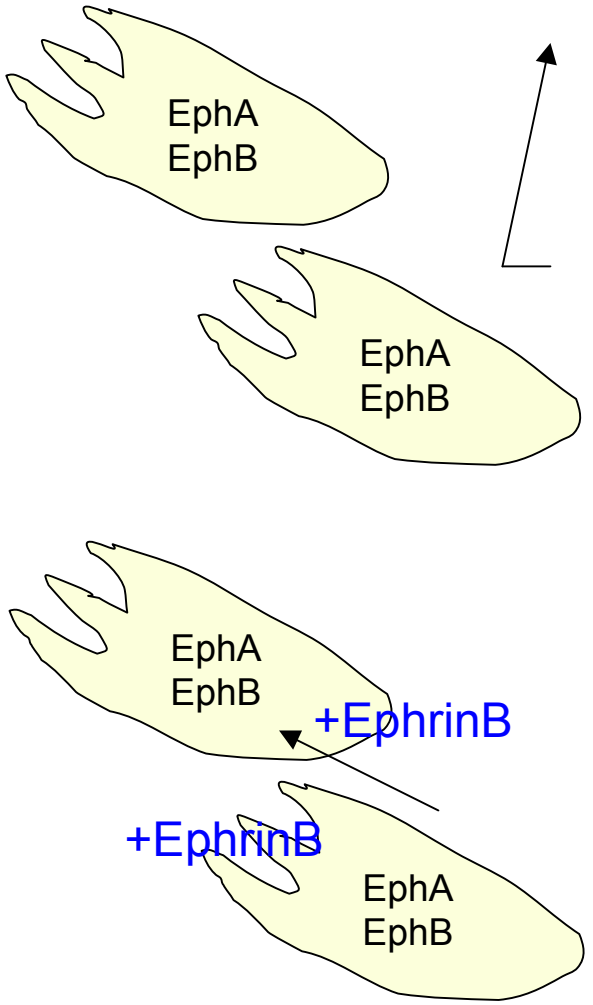




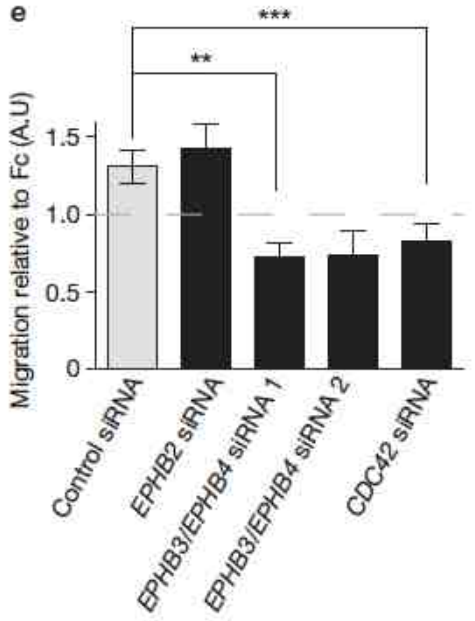
(b) Immunoblot of lysates from PC-3 cells transfected with no vector (mock), pRK5 or pRK5 containing ephrin-B2 (at the indicated times after transfection), as indicated. Tubulin was used as a loading control.



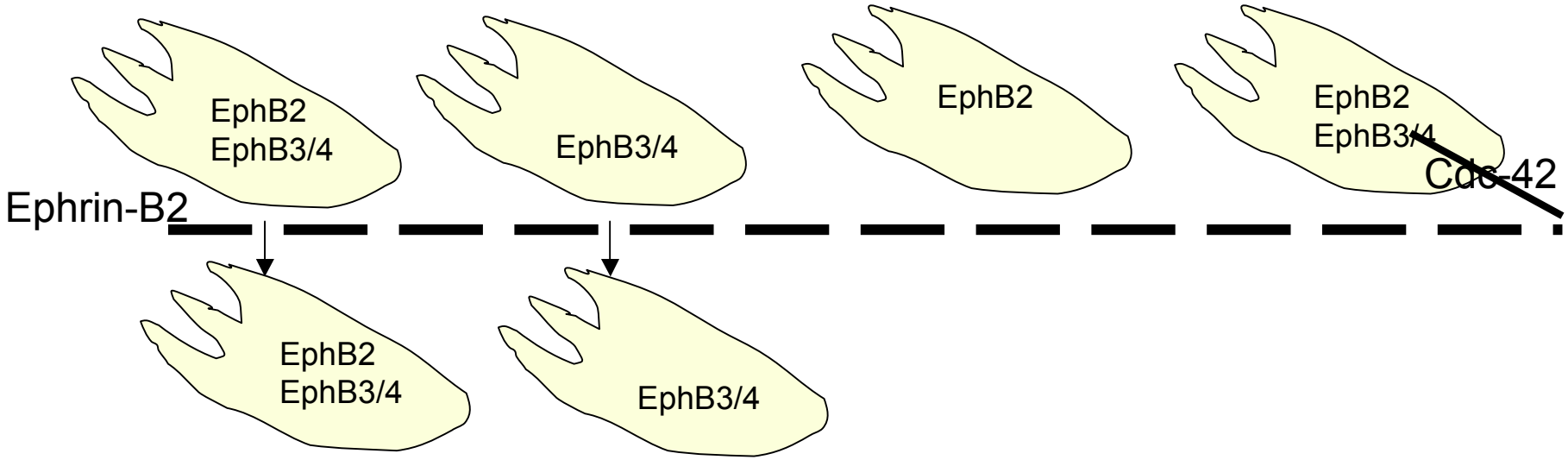
(c) Contact acceleration indices (Cx) of free-moving cells (F) versus collisions (C) between PC-3 cells transfected with the indicated plasmids. Cells were imaged between 48–72 h posttransfection. Triple asterisks indicate $P < 0.001$, NS; not significant, as determined by a Mann-Whitney test.



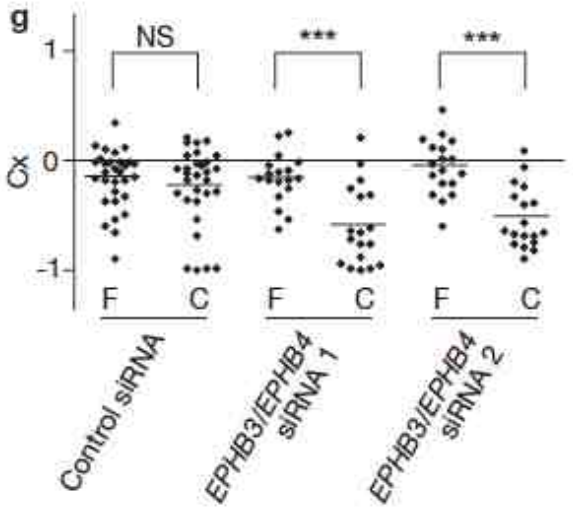
PC-3 cells transfected with the indicated siRNA oligonucleotides were added to transwell chambers coated with ephrin-B2–Fc, and numbers of cells migrating through were scored.



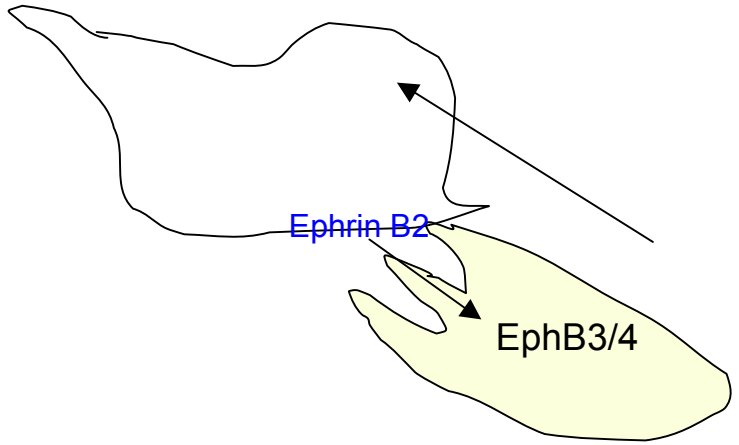
Transwell assay

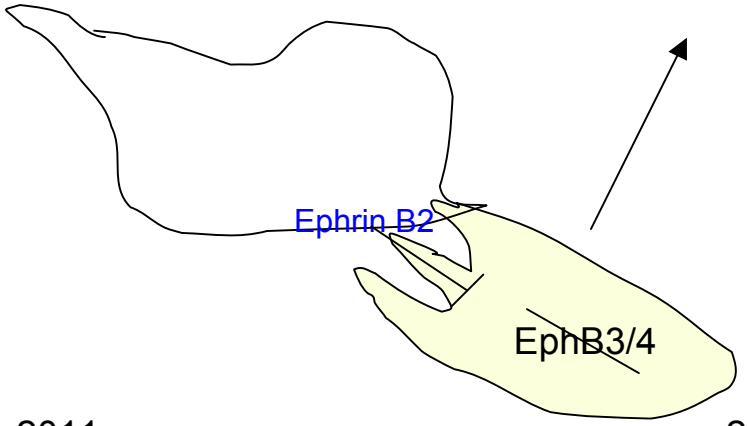


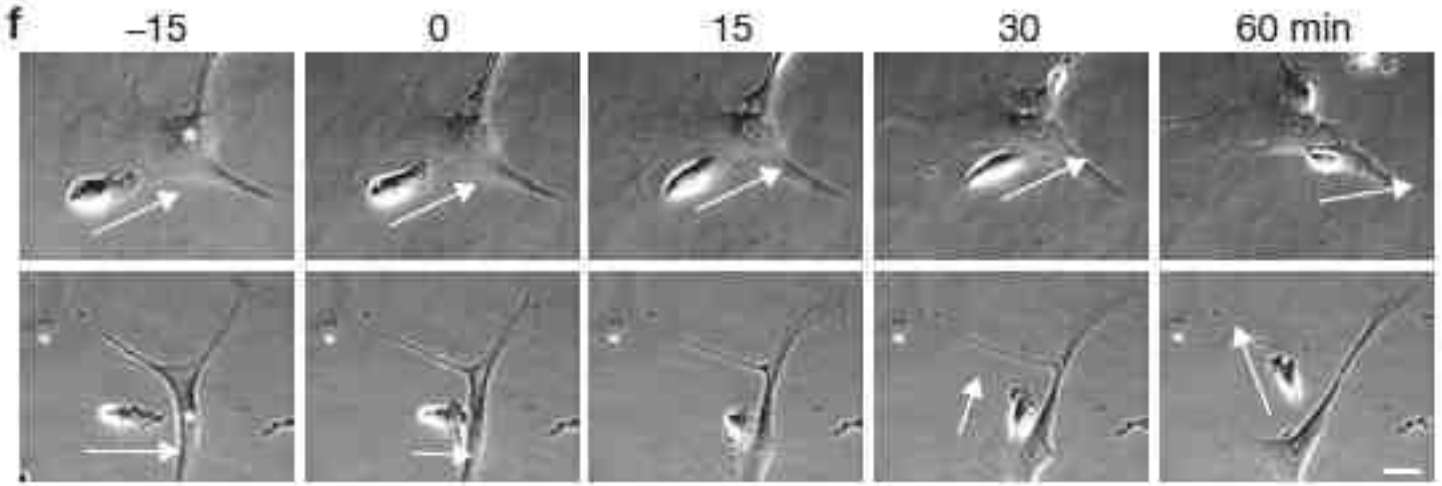
EphB3 and EphB4 knockdown restores CIL of PC-3 cells on contact with fibroblasts.



Contact acceleration indices (Cx) of free-moving cells (F) versus collisions between PC-3 cells and fibroblasts (C).







Movie 10

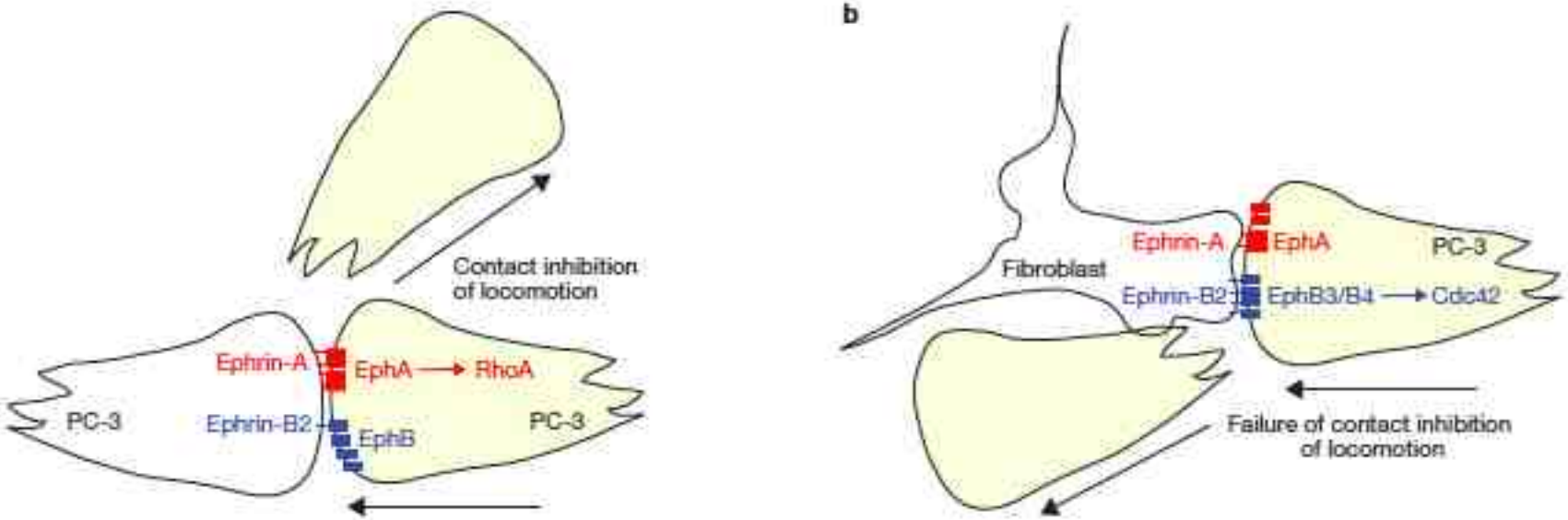
Control siRNA
PC-3/Fibroblast

EPHB3/EPHB4 siRNA 1
PC-3/fibroblast

Movie 11

Model of CIL regulation in PC-3 cells.

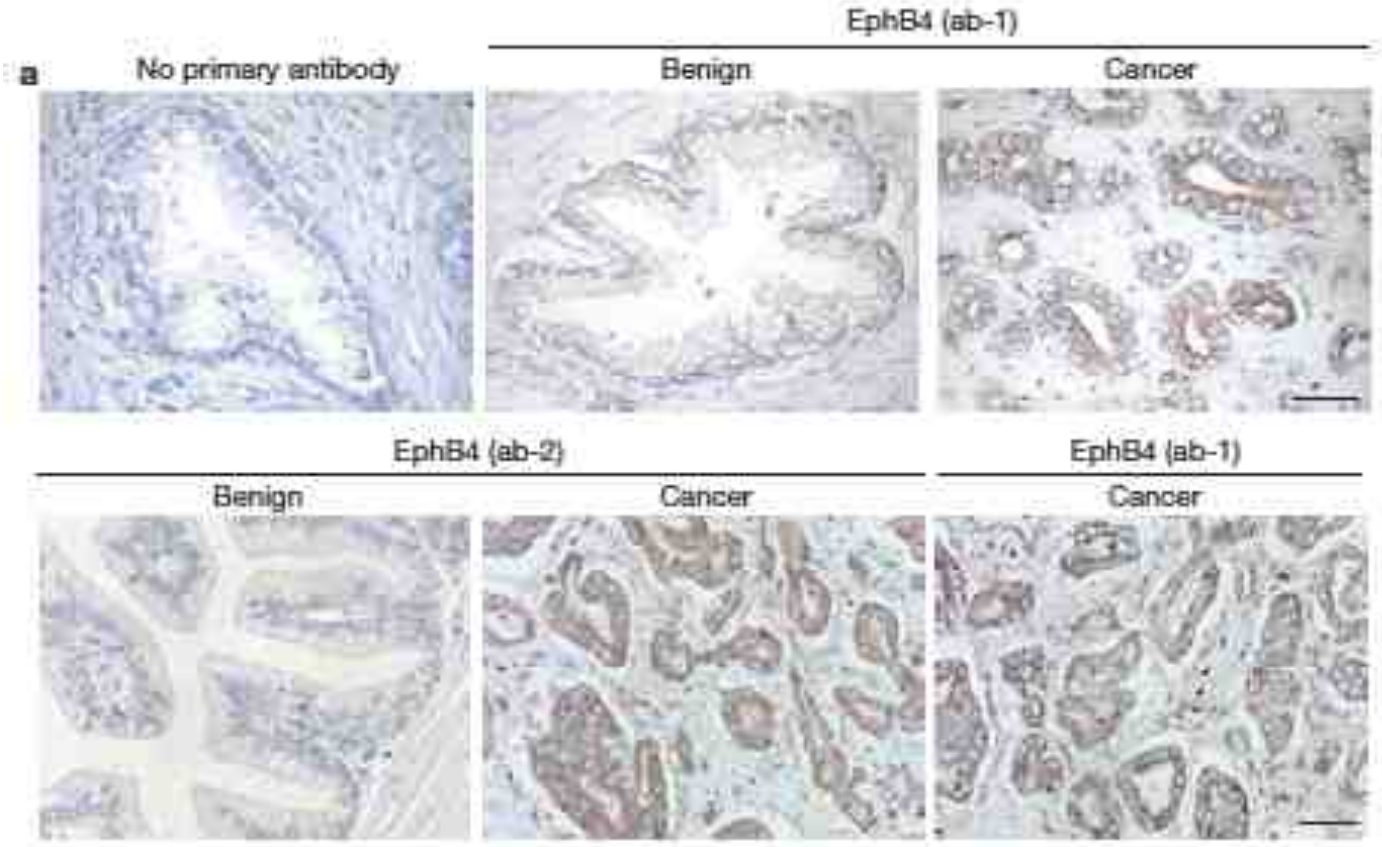
There are two competing pathways that regulate CIL in PC-3 cells:
---> repulsive EphA–RhoA signalling triggered by ephrin-A ligands
---> attractive EphB3/EphB4–Cdc42 signalling triggered by ephrin-B2 ligand.
Thus, the ratio of ephrin-A/ephrin-B2 on a cell will dictate whether the PC-3 cell colliding with it will display CIL or not.



(a) PC-3 cells have a high ephrin-A/ephrin-B2 ratio and therefore CIL is induced between PC-3 cells by EphA forward signalling, possibly by activation of RhoA.

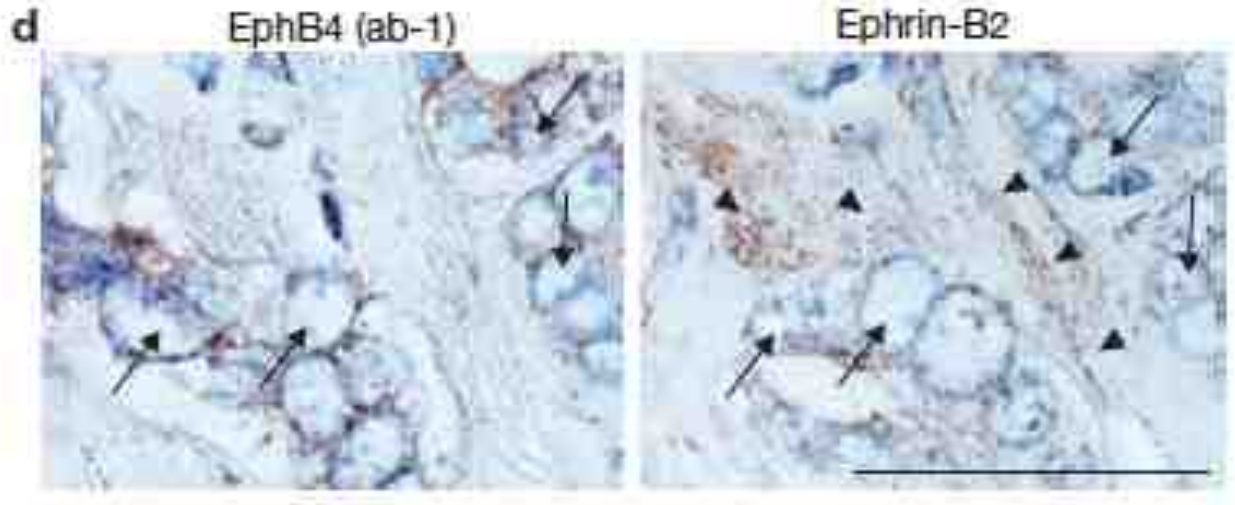
(b) Fibroblasts have a high ephrin-B2/ephrin-A ratio which activates EphB3/EphB4–Cdc42 signalling in PC-3 cells, stimulates migration and causes defective CIL.

Immunohistochemical staining of EphB4 and ephrin-B2 in prostate cancer.



(a) EphB4 expression in either benign prostate epithelium or in areas of prostate cancer (Gleason pattern 3+). Top and bottom panels represent sections from two different patients. ab-1 indicates staining with an antibody specific to the EphB4 C-terminal sequence and ab-2 indicates staining with an antibody specific to the EphB4 N-terminal sequence. The increased EphB4 staining was observed in 11 out of 15 cases.

Complementary staining of EphB4 in cancer cells (nuclei indicated by arrows) and ephrin-B2 staining in the surrounding stromal cells (arrowheads) from serial sections.



Similar complementary staining was observed in 6 out of 6 cases. Scale bar, 50 µm.

Malignant cells did undergo CIL when contacting one another.

It was proposed that these alternate responses might increase metastasis because cancer cell dissemination would be enhanced by repulsive interactions between cancer cells, but local invasion would not be impeded, and might even be facilitated, by interactions with stromal cells within and beyond the tumour.