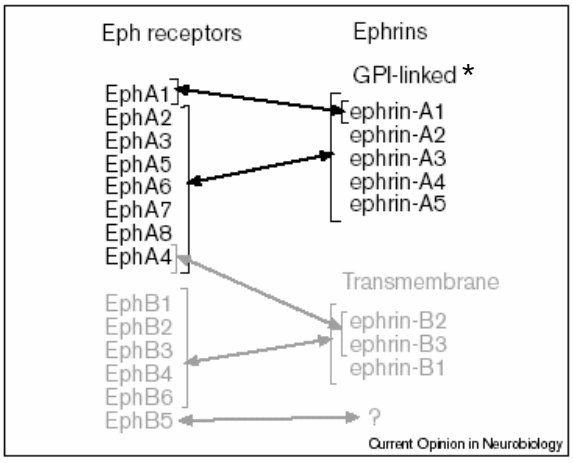
1- Eph- ephrin

Directional Guidance cues involved in CNS neuronal migration in vivo and in vitro

Ligands	Receptors	Defects in CNS neuronal migration in vivo	Neuronal migration in vitro
Slits	Robo	—	1. Slit repels postnatal SVZa cells[37] 2. Slit repels prenatal SVZ cells of GE[43]
Netrins	DCC	1. Abnormal pontine nuclei in DCC and netrin-1 mutants[46]	1. Netrin-1 attracts pontine nuclei[11]
	Unc-5h	2. Abnormal cerebellar development in unc-5h3[64]*	2. Netrin-1 repels postnatal cerebellar granule cells and prenatal SVZ cells[48,49] 3. Anti-DCC antibody blocks directed migration of postnatal SVZa cells[47]
Semaphorins	Neuropilin Plexin	1. Abnormal GABAergic interneurons in the striatum in neuropilin-2 mutants[50]	—
Ephrins	Eph	—	1. Disruption of Eph-B/Ephrin-B system affects the migration of postnatal SVZa cells[51]

*Unc-5h3/RCM mutant mice showed abnormal development of cerebellum. However, it is still unclear that the defect is primarily caused by migration abnormality or other reasons.

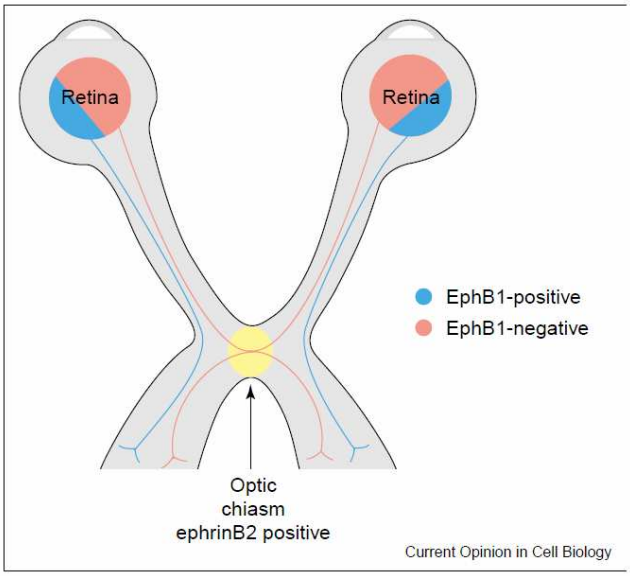
Eph receptors and ephrin ligands



*glycosylphosphatidyl-inositol membrane anchored

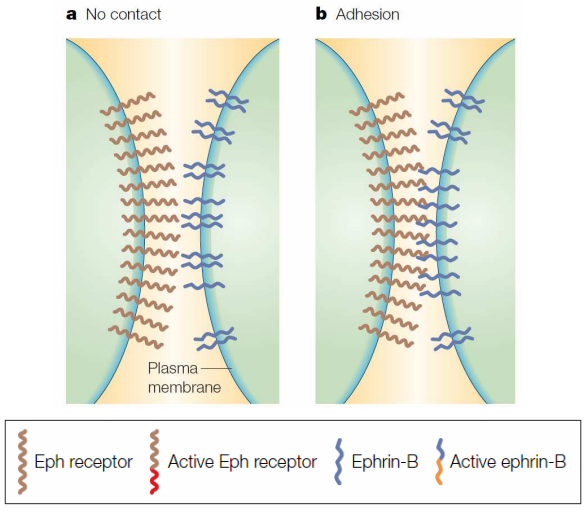
Binding specificities of Eph receptors and ephrins. Eph receptors and ephrins fall largely into two binding specificity classes, with the exception of EphA4, which interacts with ephrin-A and some ephrin-B proteins. Differences exist, however, in the relative affinity of a receptor for different ephrins that may be functionally important. Additional ephrins probably exist, because EphB5 does not bind to any known ephrin.

Midline guidance in the visual system



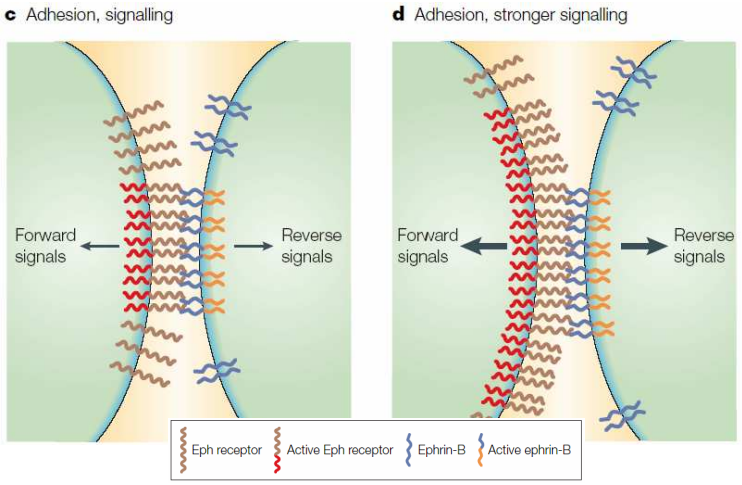
In animals with binocular vision, most retinal axons (red) cross to the contralateral side of the brain, while a smaller subset of retinal axons (blue) project to the ipsilateral side. Retinal axons expressing EphB1 are repelled from the optic chiasm by ephrinB2 and directed to an ipsilateral pathway. Contralaterally projecting axons do not express EphB receptors and therefore are not repelled by ephrinB2.

Steps in cell-contact-dependent Eph bidirectional signalling



a,**b -** Eph receptors and ephrins on opposed cell surfaces mediate cell adhesion on cell contact.

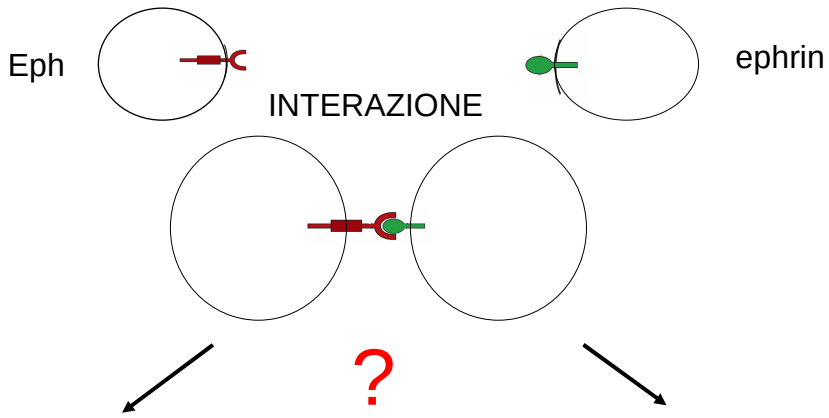
Steps in cell-contact-dependent Eph bidirectional signalling



c - tetramerization leads to tyrosine phosphorylation and signalling.
d - the tetrameric complexes can further grow into larger clusters that, in the Eph receptor-expressing cells, can extend beyond the region of contact through homophilic interactions between Eph receptors. The degree of clustering might regulate signal intensity and the nature of the signals.

Paradoxes of Eph signalling

- **Paradox 1.** The interaction between Eph receptors and ephrins requires cell–cell contact and mediates strong cell adhesion, but often the ensuing signals induce the separation of the two cells.



Come fanno le due cellule a separarsi dopo l'interazione fra recettore e ligando?

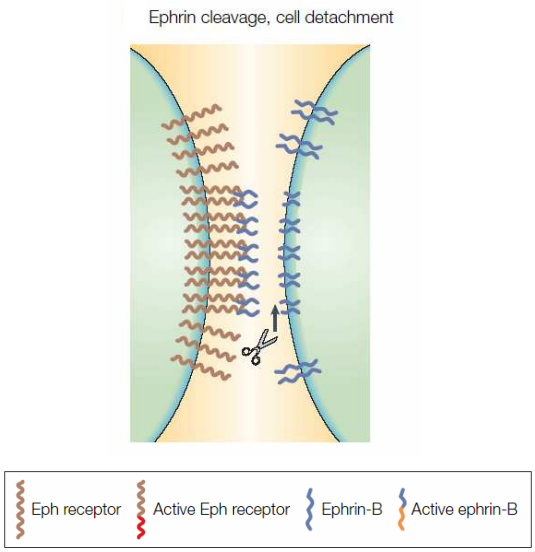
• many axon guidance molecules, including ephrins, netrins, semaphorins and slits, elicit repulsive responses when bound to their receptors; some of these factors are diffusible and growth cones respond to concentration gradients, whereas others, including the ephrins, are membrane-bound and repulsion happens after cell–cell contact

• interactions between repellent guidance cues and their receptors are high affinity, contrasting with the rapid process of contact-mediated repulsion

• this results in a paradox: although the formation of a complex between ligand and receptor is an adhesive event, it results in detachment and retraction of cells and their cellular processes

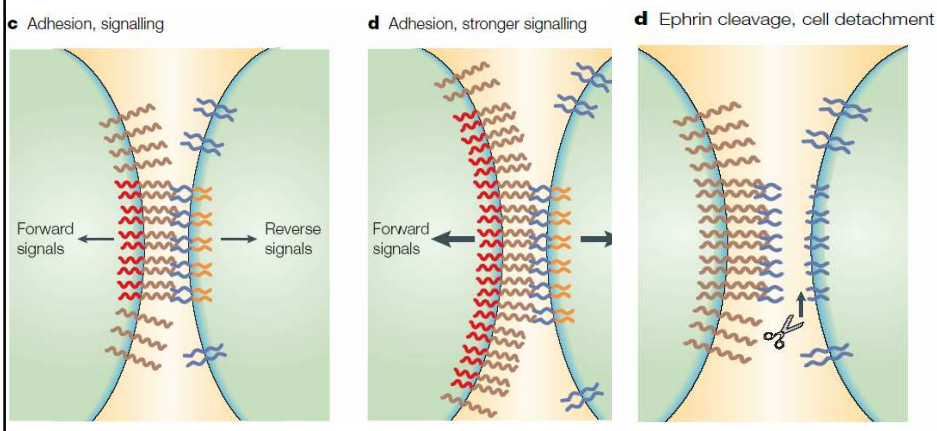
→ one mechanism that may remove ligand–receptor complexes from the cell surface is **PROTEOLYTIC CLEAVAGE**

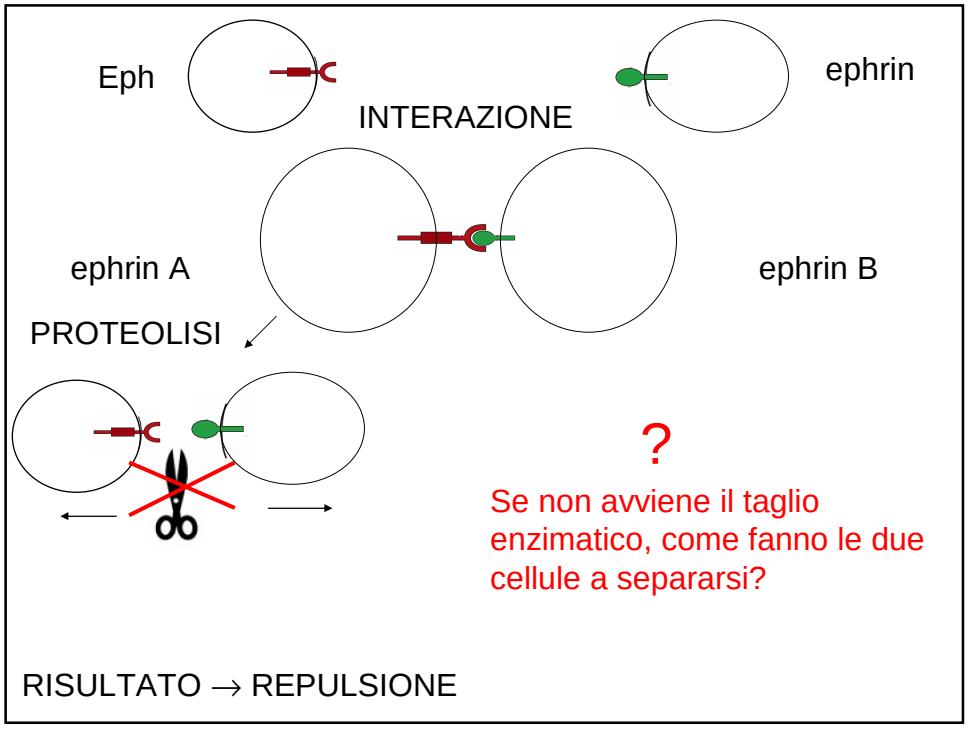
Mechanisms of Eph signal attenuation and termination.

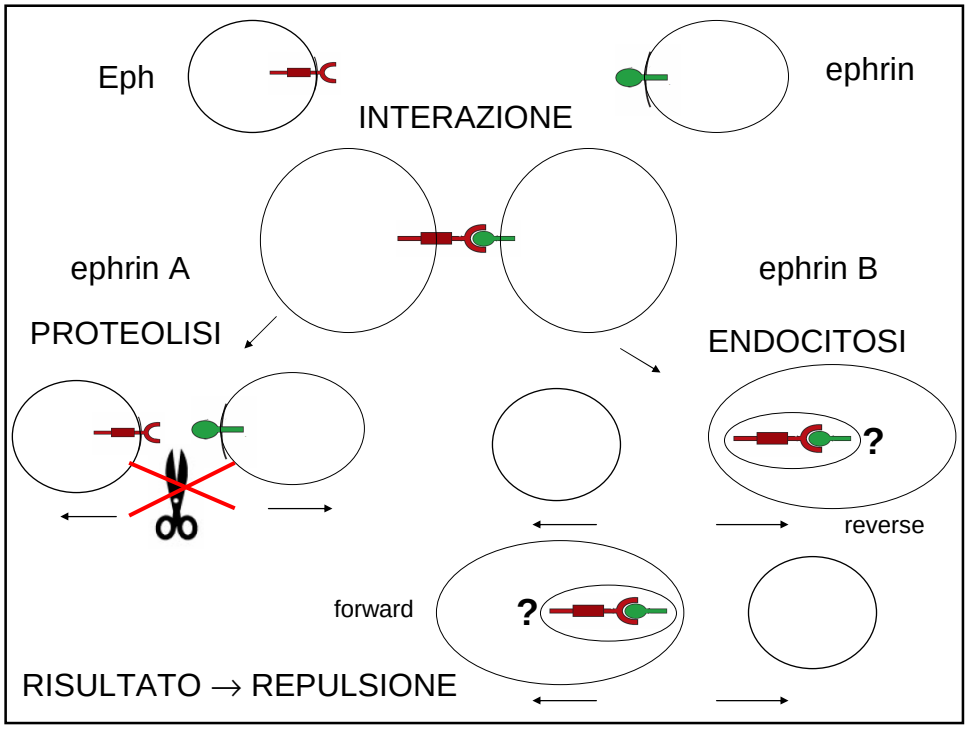


Cleavage of the ephrin by a protease also allows cell separation following Eph–ephrin engagement.

PROTEOLYTIC CLEAVAGE




Eph
ephrin
INTERAZIONE
ephrin A
ephrin B
PROTEOLISI
?
Se non avviene il taglio enzimatico, come fanno le due cellule a separarsi?
RISULTATO → REPULSIONE


Eph
ephrin
INTERAZIONE
ephrin A
ephrin B
PROTEOLISI
ENDOCITOSI
?
reverse
forward
?
RISULTATO → REPULSIONE

Attraction or Repulsion? Ligand or Receptor?

Repulsion by ephrin A ligands requires **CLEAVAGE**

- growth cone contact
- ectodomain shedding
- collapse and withdrawal

Repulsion by ephrin B ligands requires **TRANS-ENDOCYTOSIS** of ephrinB/EphB complexes

- growth cone contact
- trans-endocytosis
- collapse and withdrawal

- endocytosis of protein complexes involving the intercellular (trans) interaction of two transmembrane proteins is unusual and rarely documented in the literature

- in *Drosophila melanogaster* the seven transmembrane ligand, Boss, is internalized into the R7 photo-receptor precursor cell after trans interaction with the sevenless (sev) tyrosine kinase receptor
-the entire Boss protein enters the sev-expressing cell and endocytosis occurs only in forward direction

- the receptor patched-1 (Ptc-1) is able to retrieve membrane-bound forms of sonic hedgehog (Shh) from adjacent cells, a process that is uni-directional

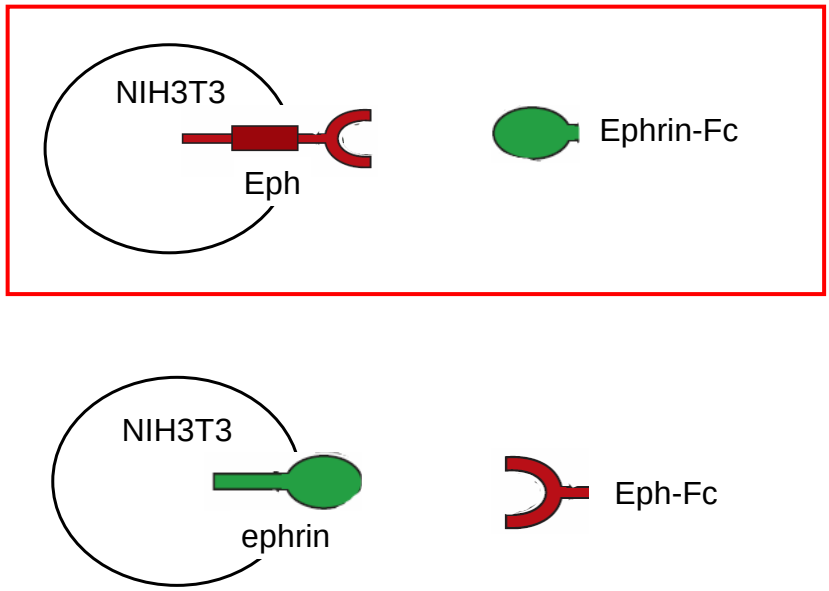
- Notch receptor binding to its membrane-anchored ligand, Delta, triggers proteolytic shedding of the Notch ectodomain and endocytosis of the Notch-Delta protein complex into the Delta-expressing cell. Notch endocytosis into the Notch-expressing cell also occurs but after a second cleavage event. In this case endocytosis is bi-directional, but involves proteolytic cleavage of one of the proteins.

EphB–ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion

Manuel Zimmer[1], Amparo Palmer[1], Jenny Köhler[1] and Rüdiger Klein[1,2]

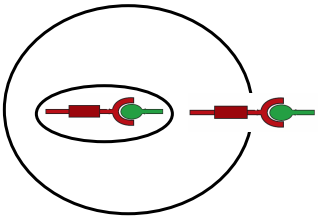
NATURE CELL BIOLOGY VOLUME 5 | NUMBER 10 | OCTOBER 2003

- Eph receptors and their membrane-associated ephrin ligands mediate cell–cell repulsion to guide migrating cells and axons
- repulsion requires that the ligand–receptor complex be removed from the cell surface, for example by PROTEOLYTIC PROCESSING of the ephrin ectodomain
- cell contact-induced EphB–ephrinB complexes are rapidly ENDOCYTOSED during the retraction of cells and neuronal growth cones
- ENDOCYTOSIS occurs in a bi-directional manner that comprises of full-length receptor and ligand complexes
- ENDOCYTOSIS is sufficient to promote cell detachment and seems necessary for axon withdrawal during growth cone collapse
- this is a mechanism for the termination of adhesion and the promotion of cell repulsion after intercellular (trans) interaction between two transmembrane proteins



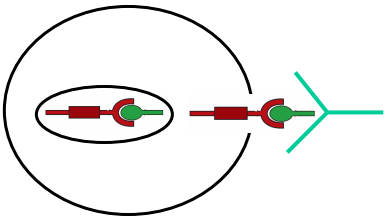
?

Ipotizzo che il complesso recettore-ligando possa essere internalizzato



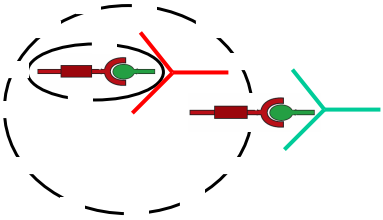
-ho a disposizione anticorpi primari in grado di riconoscere il recettore Eph

-come posso distinguere la componente sulla membrana cellulare, dalla componente internalizzata in seguito a stimolazione?



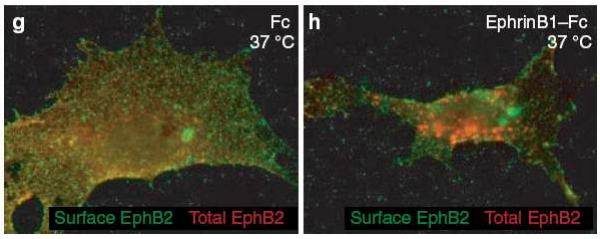
Cells were fixed in the absence of detergents and immunolabelled for **Eph (or ephrin) on the cell surface**.

PERMEABILIZATION



Cells were then permeabilized with detergents and stained for **total Eph (or ephrin)** using a different primary antibody. Staining that appears exclusively after permeabilization represents the **intracellular pool of Eph (or ephrin)**

control 3T3 EphB2 +EphrinB1



Endocytosis of EphrinB1–Fc

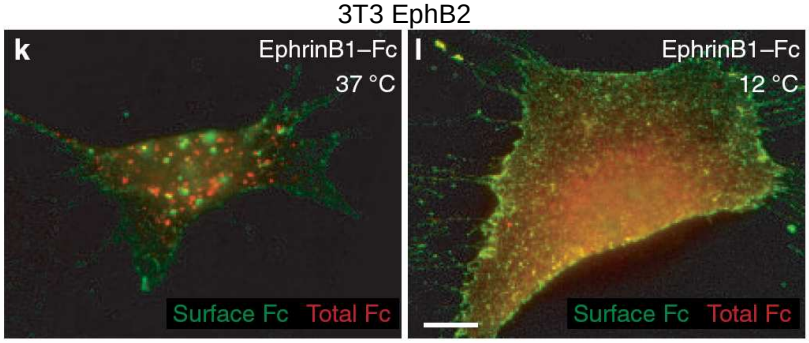
(Stimulation at 12 ℃, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization)

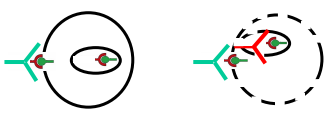
NIH3T3 cells stably expressing EphB2 were stimulated with unfused **Fc** (**control**) or **EphrinB1–Fc**, fixed in the absence of detergents, and immunolabelled for **EphB2 on the cell surface**.

Cells were then permeabilized and stained for **total EphB2** using a different primary antibody. Staining that appears exclusively after permeabilization represents the **intracellular pool of EphB2**.

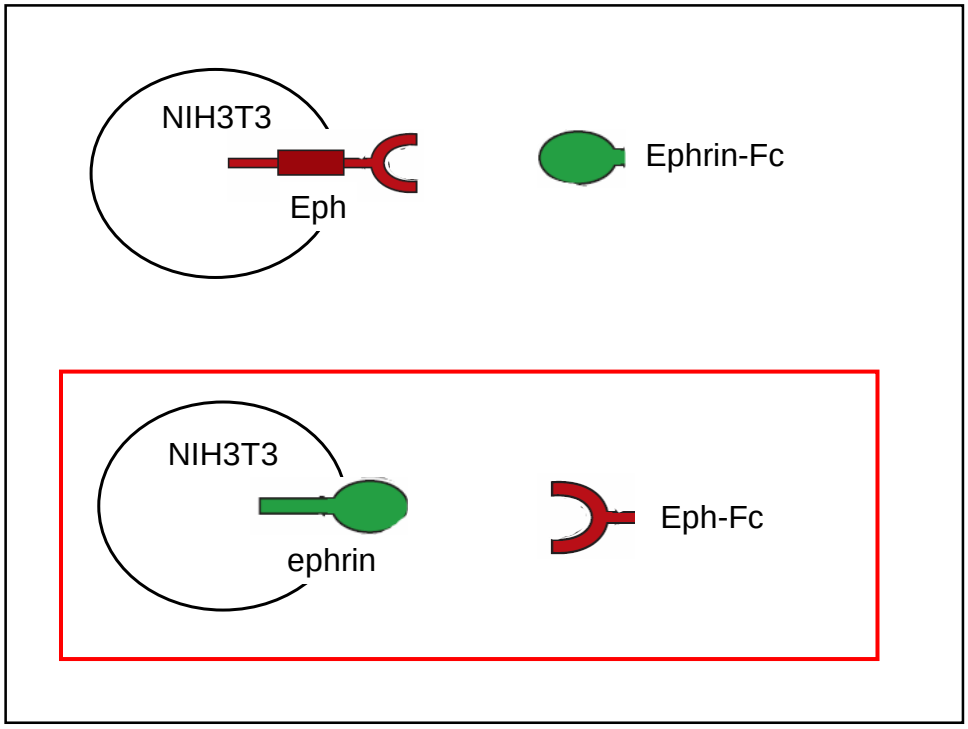
EphrinB1–Fc, bound to surface EphB2, was visualized with a FITC-conjugated antibody directed against human-Fc

3T3 EphB2



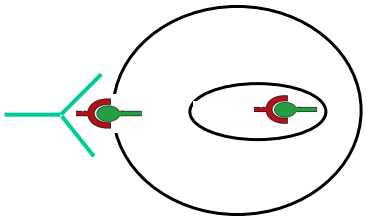


Stimulation at 12 ℃, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization.

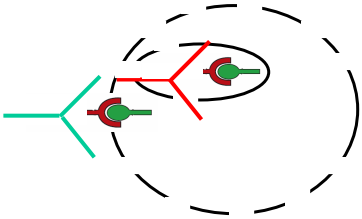


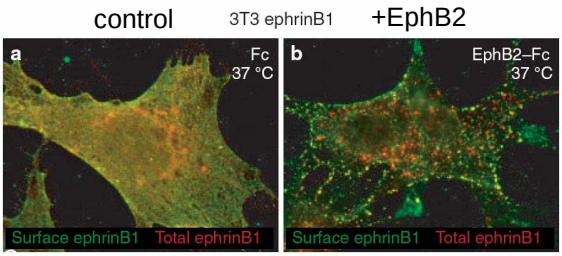
Cells were fixed <u>in the absence of detergents</u> and immunolabelled for **ephrin on the cell surface**.

PERMEABILIZATION

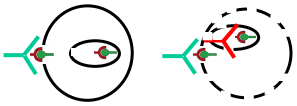


Cells were then <u>permeabilized</u> with detergents and stained for **total ephrin** using a different primary antibody. Staining that appears exclusively after permeabilization represents the **intracellular pool of ephrin**



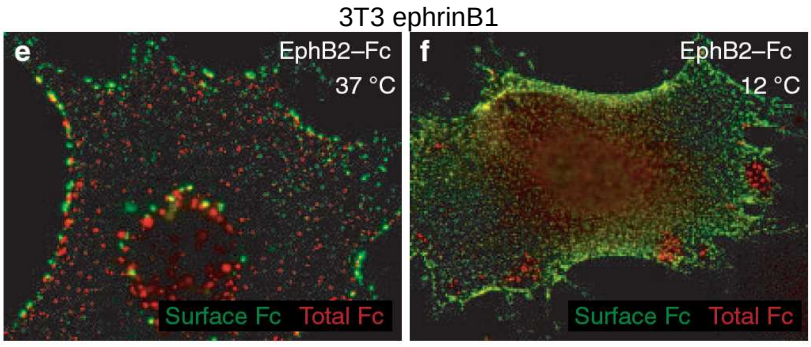


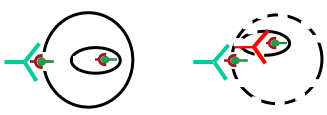
Endocytosis of EphB2–Fc



NIH3T3 cells stably expressing ephrinB1 were stimulated with unfused **Fc** (**control**) or **EphB2–Fc**, fixed in the absence of detergents, and immunolabelled for **ephrinB1 on the cell surface**.
Cells were then permeabilized and stained for **total ephrinB1** using a different primary antibody. Staining that appears exclusively after permeabilization represents the **intracellular pool of ephrinB1.**

EphB2–Fc, bound to surface ephrinB1, was visualized with a FITC-conjugated antibody directed against human-Fc

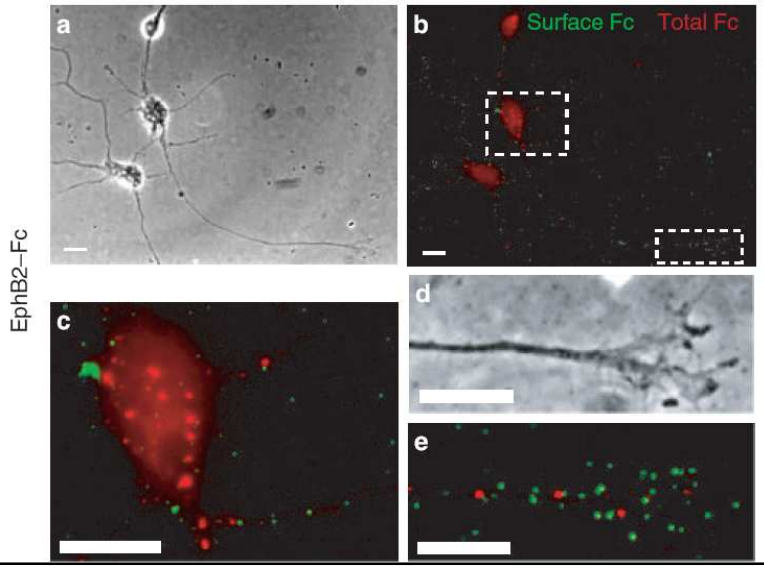




Stimulation at 12 ℃, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization.

Endocytosis of EphB2–Fc and ephrinB1–Fc in primary telencephalic neurons.

Neurons were stimulated for 15 min with EphB2–Fc.
EphB2–Fc, was visualized with a FITC-conjugated antibody directed against human-Fc



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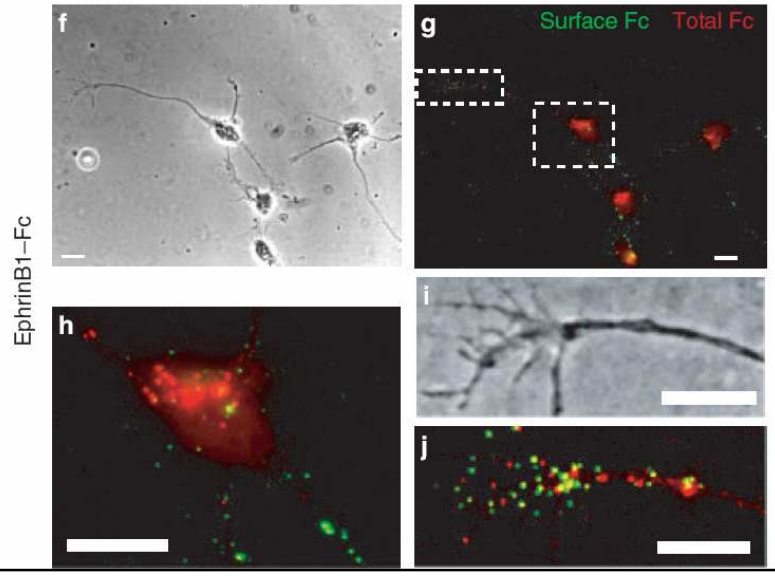
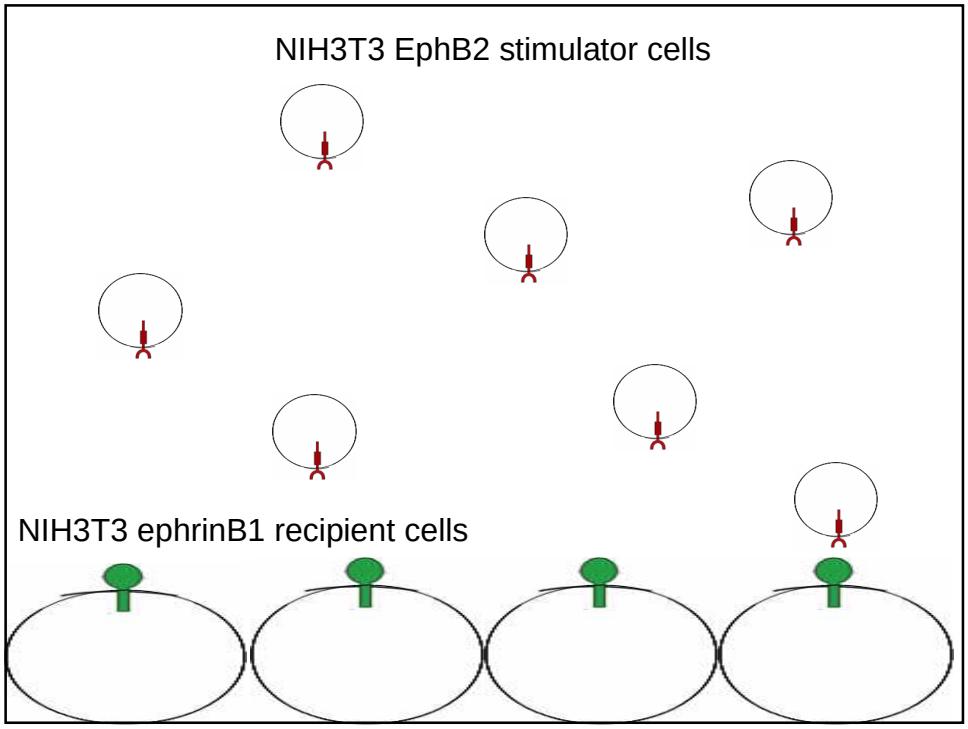
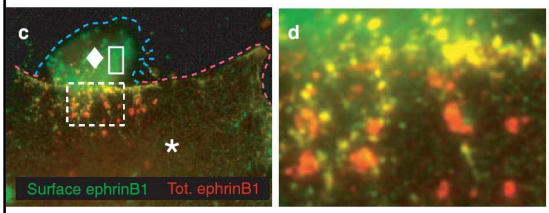


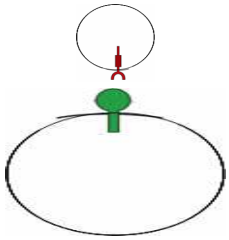


cell–cell stimulation assay to investigate whether membrane-bound ephrinB–EphB complexes co-cluster and subsequently internalize

A sparse monolayer of 'recipient cells', is first cultured on glass cover slips. Next, 'stimulator cells' are taken in suspension by a mild treatment and added onto the recipient cells. After 10 min, all cells are fixed and stained.



If they use 3T3 EphB2 (♦)stimulator cells with 3T3 ephrinB1(✶) recipient cells, they observe rapid and localized co-clustering of ephrinB1 and EphB2 at the site of cell–cell contact. These clusters were partially endocytosed and the direction of internalization was in a *reverse* manner, that is, into the recipient 3T3 ephrinB1 cells



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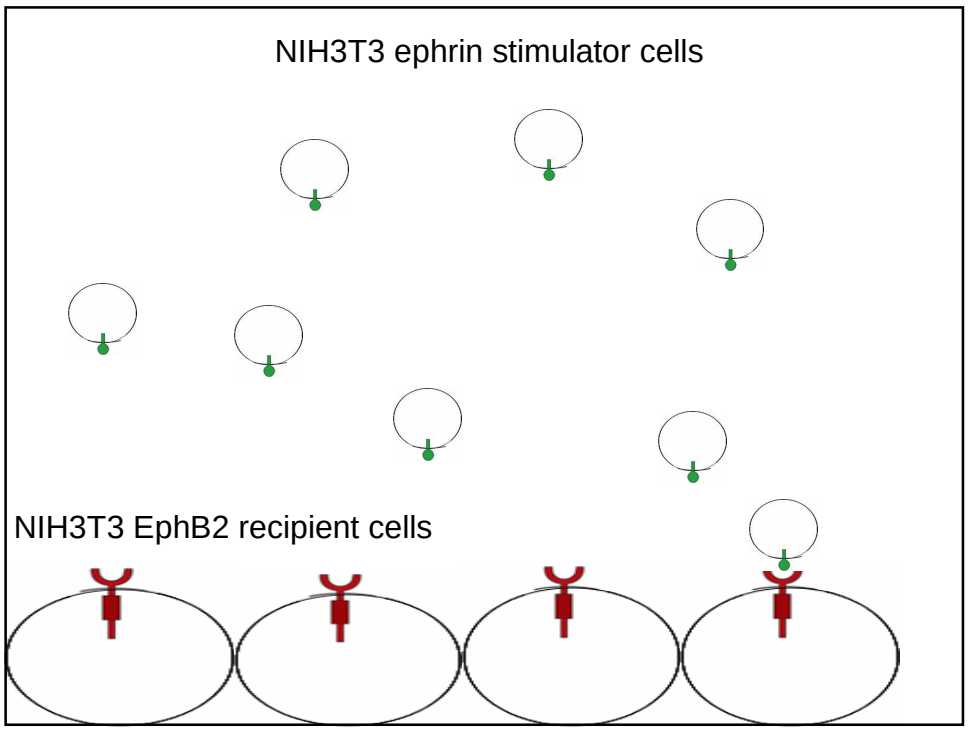

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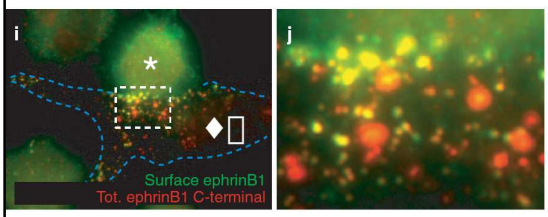
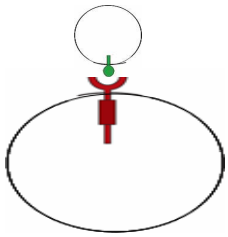


Next, they did the reverse experiment and used 3T3 ephrinB1(✱) as stimulator cells and 3T3 EphB2 (♦) as recipient cells. EphrinB1 was internalized in a *forward* manner by 3T3 EphB2 cells



These findings using transfected cells indicate localized and bi-directional endocytosis of complexes that comprise of full-length EphB2 and ephrinB1.

• this experiment involved the stimulation with cells in suspension

• endocytosis was predominant in the preplated recipient cells

perché ?

● it is possible that the recipient cells have an advantage in their organization of the endocytic and membrane trafficking machinery over the freshly seeded stimulator cells as the endocytic machinery might be linked to the actin cytoskeleton

● after the stimulator cells had spread out, endocytosis was favoured in the EphB2 forward direction

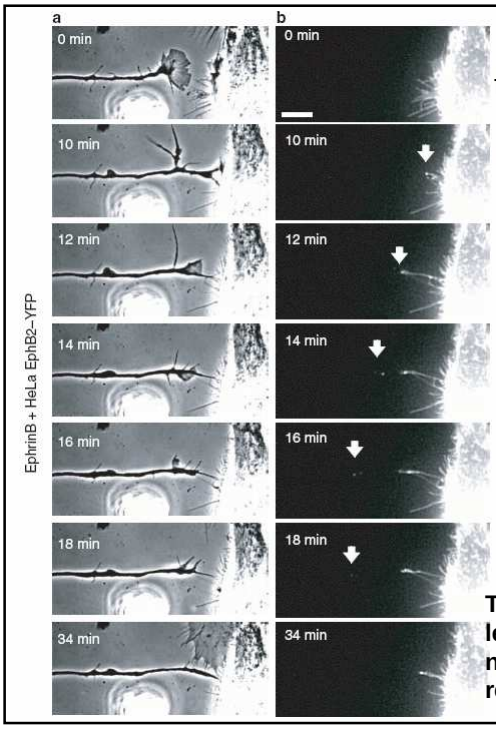
● weakening the receptor's ability to signal shifted endocytosis towards ephrinB reverse signalling

Nell'esperimento descritto nella diapositiva seguente, si utilizza il costrutto per esprimere EphB2–YFP

Come posso ottenere questo costrutto?

In esperimenti successivi si parla anche di EphB2–YFP-ΔC

Quali strategie posso seguire per ottenere questo costrutto che codifica per un recettore privo della regione C terminale?



EphrinB1 and EphB2 uptake and transport by primary neurons.

(**a**, **b**) Forebrain neurons from E14.5 mouse embryos (cultured for 1 d *in vitro*) were co-cultured with HeLa cells transiently expressing **EphB2–YFP**. Growth cones were imaged by time-lapse microscopy at 1 frame per min. The presented selection of images shows a neuronal growth cone before contact with a HeLa cell and collapse of the growth cone within 10 min after contact. At the time of collapse, a fluorescent cluster of EphB2 forms at the tip of a single protrusion of the HeLa cell (arrow at 10 min). The growth cone partially retracts and pulls a protrusion out. Two EphB2 clusters are retrogradely transported into the neurite (arrows).

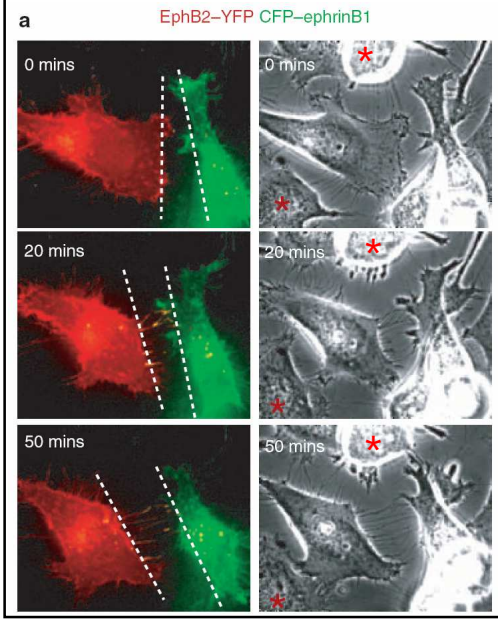
See **Movie 1**.

These results demonstrate that the full-length EphB2 receptor is taken up by the neuron, probably owing to ephrinB reverse endocytosis in the growth cones.

• to determine whether bi-directional endocytosis affects repulsive cell migration, an *in vitro* assay was developed in which cells expressing fluorescently tagged EphB2 receptor (**EphB2–YFP**) were co-cultured with cells expressing fluorescently tagged ephrinB1 (**CFP–ephrinB1**)

• HeLa cells were chosen because they express low levels of endogenous ephrinB and EphB proteins and high levels of transfected proteins; they are also very motile, which makes them ideal for fluorescence time-lapse imaging.

Bi-directional endocytosis regulates the cell repulsion response and cell detachment



a. HeLa cells were transiently transfected with full-length **EphB2–YFP** and full-length **CFP–ephrinB1** and then cocultured before time-lapse imaging.

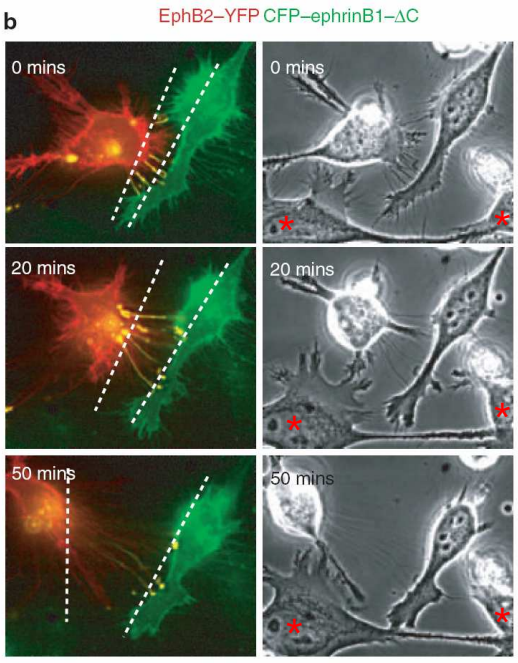
Left, selected fluorescence images with **EphB2–YFP** in **red** and **CFP–ephrinB1** in **green**.

Right, phase contrast images.

Intense clustering of EphB2 and ephrinB1 is seen at the contact site between the two cells at 20 min, the **EphB2–YFP** cell retracts a lamellipodium from the **ephrinB1 cell** (indicated by the distance between the two stippled lines).

(see **Movie 2**).

- in almost all observed cases, when a ruffling lamellipodium of an **EphB2–YFP** cell collides with an **CFP–ephrinB1** cell, strong co-clustering of receptor with ligand occurs within 1 min and the initial clusters always appear in filopodia-like protrusions.
- during the retraction of **EphB2–YFP** positive lamellipodia, receptor–ligand complexes endocytose bi-directionally
- contacts of **EphB2–YFP-** or **CFP–ephrinB1-** transfected cells with untransfected cells in the same culture do not result in clustering nor cell retraction (asterisks in the figure)



b. HeLa cells were transiently transfected with full length **EphB2–YFP** and C-terminally truncated **CFP–ephrinB1-ΔC** then cocultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP** in **red** and **CFP–ephrinB1- ΔC** in **green.**

Right, phase contrast images.

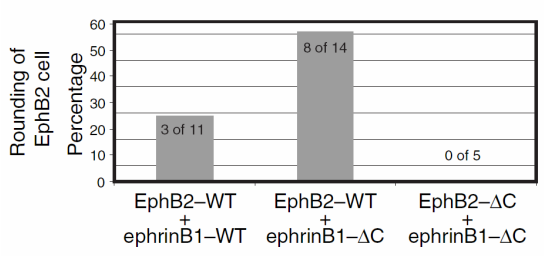
EphB2–YFP clusters (in **yellow**) are uni-directionally endocytosed into the **EphB2–YFP** expressing cell. Strong repulsion and rounding of **EphB2–YFP** expressing cell is observed.

(see **Movie 3**).

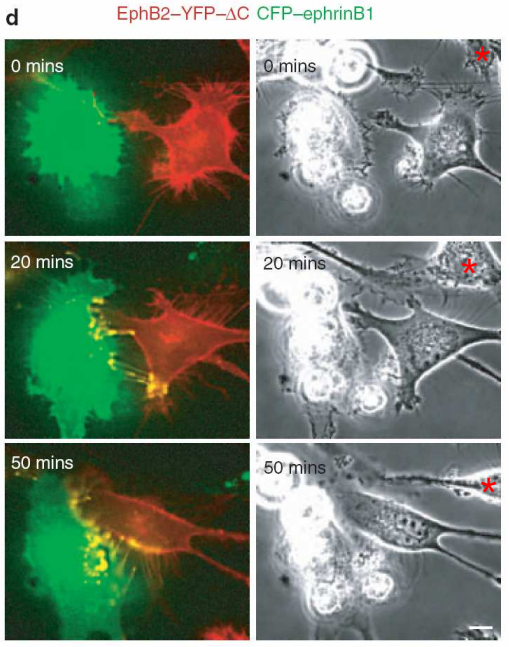
• when ephrinB1 endocytosis was blocked by a C-terminal truncation (**CFP–ephrinB1-ΔC**), markedly different cell behaviour was observed

• rapid co-clustering with **EphB2–YFP** occurs after contact, but these clusters remain in part localized to the surface of the ligand expressing cell, where they grow to much larger complexes

• the **EphB2–YFP** cell engulfs the clusters vigorously, retracts strongly, and in most cases even rounds up, a behaviour rarely observed with wild-type ephrinB1



→ therefore, a mutation that blocks ephrinB1 endocytosis results in a stronger EphB2 cell retraction response



c. HeLa cells were transiently transfected with C-terminally truncated **EphB2–YFP- ΔC** and full length **CFP–ephrinB1** then cocultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP- ΔC** in **red** and **CFP–ephrinB1** in **green.**

Right, phase contrast images.

EphB2–YFP clusters (in **yellow**) are strongly uni-directionally endocytosed into the **CFP–ephrinB1** expressing cell.

Otherwise normal cell behaviour similar to un-transfected cells is observed.

See **Movie 4**

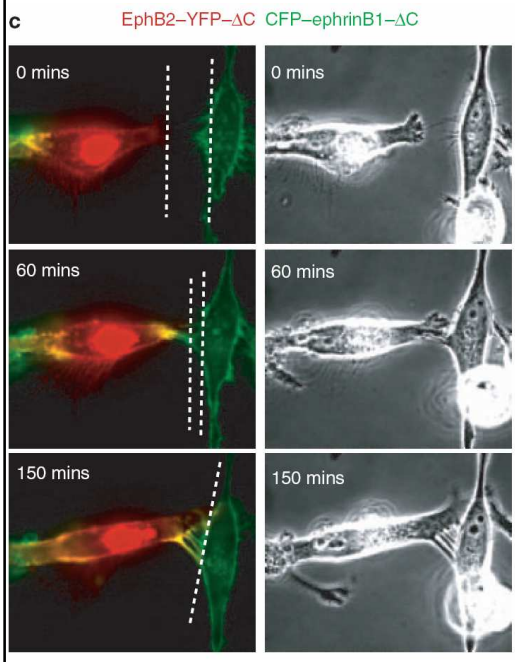
How do cells react to unidirectional ephrinB reverse signalling?

• as expected, **CFP–ephrinB1** cells strongly endocytose receptor–ligand clusters, whereas **EphB2–YFP- ΔC** cells fail to endocytose these complexes

• however, the cells neither retract nor adhere to each other

• cell behaviour is indistinguishable from non-transfected cells

→ ephrinB1 reverse endocytosis is sufficient to terminate adhesion and to cause cell detachment



c. HeLa cells were transiently transfected with C-terminally truncated **EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC** then co-cultured before time-lapse imaging.

Left, selected fluorescence images with **EphB2–YFP- ΔC** in **red** and **CFP–ephrinB1- ΔC** in **green**.

Right, phase contrast images.

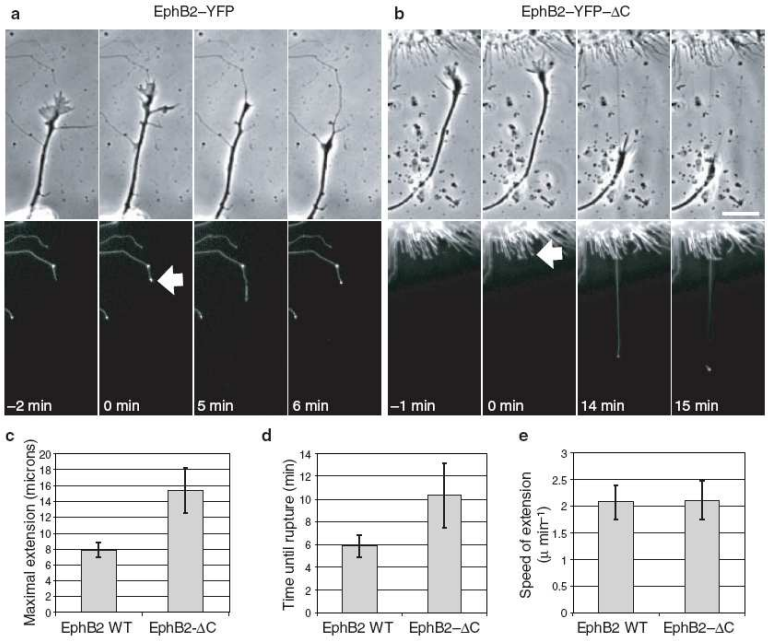
Cells strongly adhere to each other forming large fascicles filled with EphB2–YFP complexes.

• when both ephrinB1 and EphB2 are truncated at the C-terminal (**EphB2–YFP- ΔC** and **CFP–ephrinB1-ΔC**), the cells strongly adhere to each other and large receptor-and ligand-bearing fascicles are formed at the contact zone

→ **ephrinB and EphB proteins can function as adhesion molecules if endocytosis and other signalling events are blocked.**

Is endocytosis required for ephrinB-mediated growth cone collapse and retraction?

EphB2 C-terminal truncation impairs growth cone detachment.



- Both cells expressing full length EphB2–YFP and truncated EphB2–YFP–ΔC cause collapse of neuronal growth cones within 5–10 min after contact.
- Therefore, uni-directional ephrinB reverse endocytosis is sufficient to allow collapse of the growth cone.
- Using time-lapse imaging, they measured the maximal extension of the contacting protrusions just before detachment occurs.
- The average expansion of protrusions was approximately twice as long in EphB2–YFP–ΔC- than in EphB2–YFP-expressing cells

→ They conclude that axon detachment from EphB2–YFP–ΔC expressing cells is delayed compared with EphB2–YFP-expressing cells.

CONCLUSIONS

• in HeLa cells:
  •EphB2 forward signalling induces forward endocytosis of EphB2–ephrinB1 complexes **and** a lamellipodial retraction response
  •ephrinB1 reverse signalling only mediates reverse endocytosis

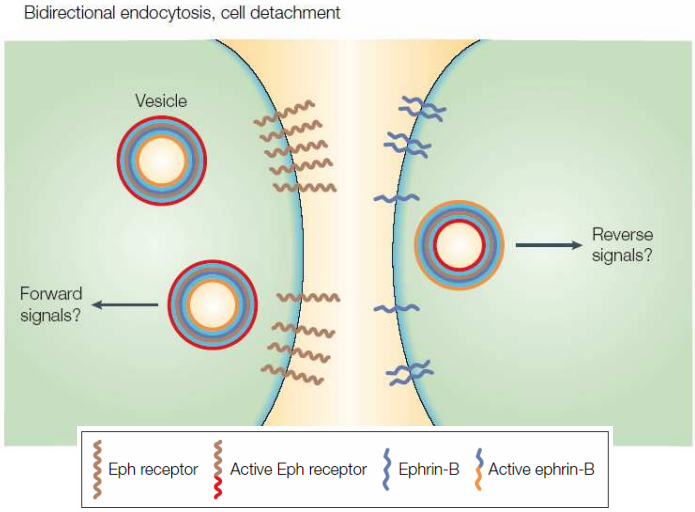
• in the absence of reverse endocytosis a gain-of-function phenotype is observed: enhancement of repulsion by EphB receptor forward signalling

• in the case of ephrinB–EphB complexes endocytosis occurs in a bi-directional fashion involving full-length proteins: one of the interaction partners is transcytosed from one cell to its neighbour

• **the relative contribution of reverse *versus* forward endocytosis may largely depend on cellular context**

• the underlying mechanism of EphB2 endocytosis may resemble phagocytosis or macropinocytosis

Mechanisms of Eph signal attenuation and termination.



Internalization of Eph-receptor–ephrin complexes together with their surrounding plasma membranes, which can occur into the receptor- and the ligand-expressing cell, allows disengagement of the two cells and gives rise to internalized double-membrane vesicles.

→ **TRANS-ENDOCYTOSIS** may provide an alternative mechanism for the removal of ligand–receptor complexes from the surface