• 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

• therefore, there must be a mechanism in place that overcomes adhesion immediately after cell–cell contact.

→ one mechanism that may remove ligand–receptor complexes from the cell surface is PROTEOLYTIC CLEAVAGE
Eph receptors and ephrin ligands

- EphA receptors bind ephrinA (glycosylphosphatidylinositol membrane anchored)
- EphB receptors bind ephrinB (transmembrane domain)
- Axon repulsion by GPI-anchored ephrinAs requires proteolytic cleavage of the ephrinA ectodomain by the A-Disintegrin-And-Metalloprotease (ADAM)-10
- The growth cones of neurons that encountered cells expressing the ligand ephrinA2 collapsed and withdrew
- When cleavage of ephrinA2 was prevented by mutations in the ephrinA2 ectodomain growth cones still collapsed, but withdrawal was greatly delayed
- It was suggested that this mechanism provides a means for efficient axon detachment and termination of signalling

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

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**Diagram:**

- **Eph**
- **ephrin**
- **INTERAZIONE**
- **PROTEOLISI**
- **ENDOCITOSI**
- **RISULTATO → REPULSIONE**

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• 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**

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- 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

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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**
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.

Stimulation at 12 °C, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface (although clusters appeared smaller), 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.

Stimulation at 12 °C, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface (although clusters appeared smaller), but blocked internalization.
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.

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
- in this case, endocytosis was predominant in the preplated recipient cells.
- 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
**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.

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**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.


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).
• 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.

• 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.


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 CFP–ephrinB1-ΔC then cocultured before time-lapse imaging.


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.

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


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

CONCLUSIONS

• in HeLa cells, EphB2 receptor forward signalling induces forward endocytosis of EphB2–ephrinB1 complexes and in addition a lamellipodial retraction response, whereas ephrinB1 reverse signalling only mediates reverse endocytosis

• in the absence of reverse endocytosis a gain-of-function phenotype is observed, that is, 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, that is, 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–YFP endocytosis may resemble phagocytosis or macropinocytosis

• it is possible that proteolysis and endocytosis have parallel functions in Eph–ephrin-mediated cell–cell communication