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]

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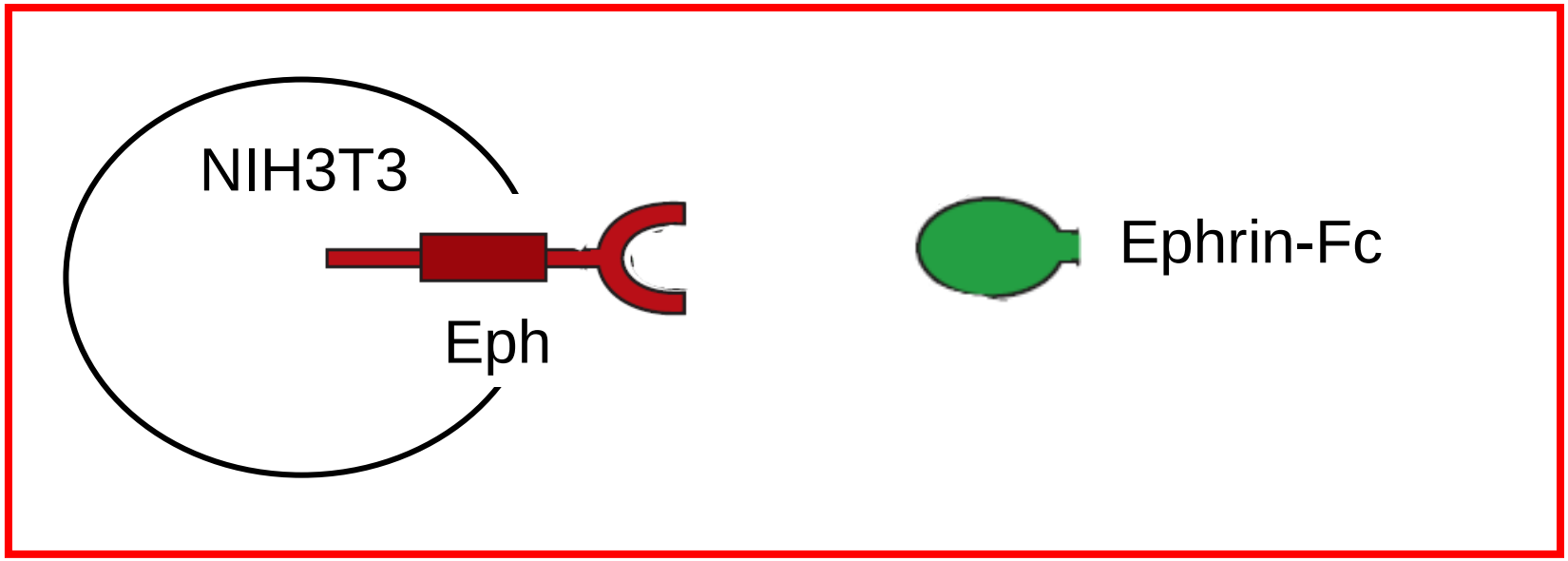
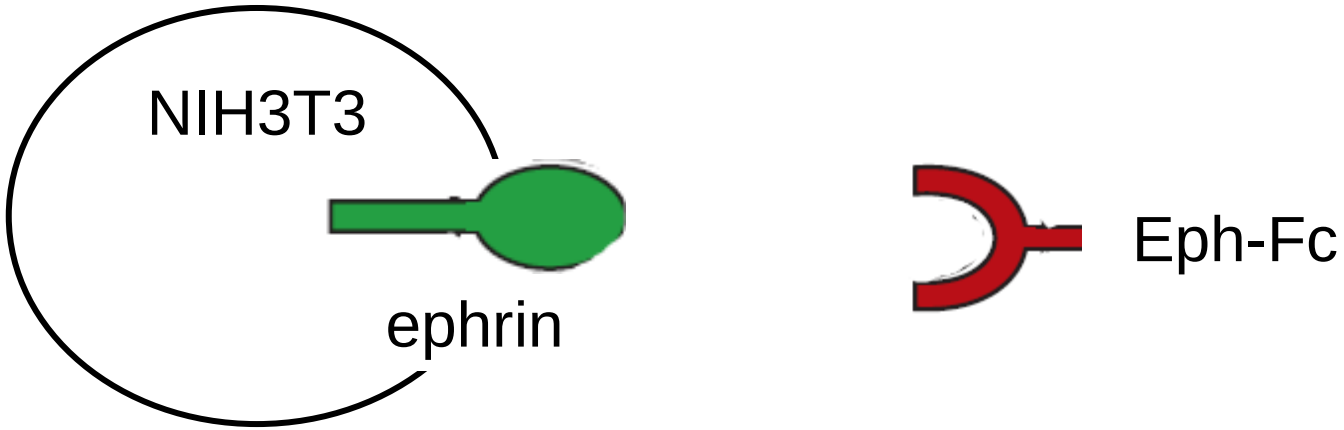
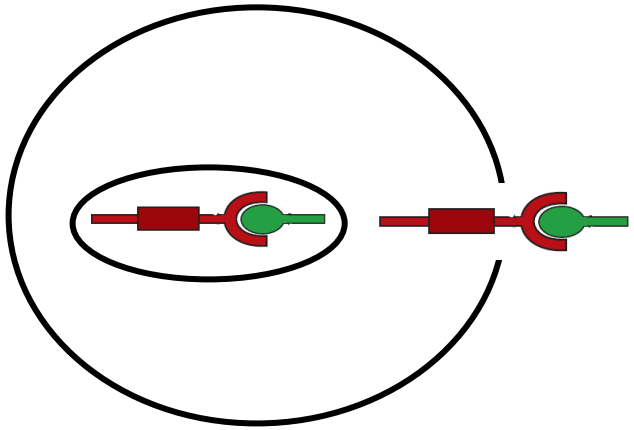
- Eph receptors and their membrane-associated ephrin ligands mediate cell–cell repulsion to guide migrating cells and axons
- repulsion requires that the ligand–receptor complex be removed from the cell surface, for example by PROTEOLYTIC PROCESSING of the ephrin ectodomain
- cell contact-induced EphB–ephrinB 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





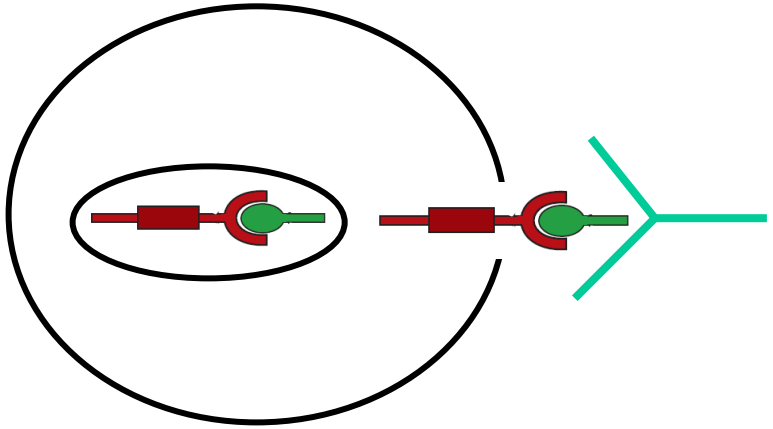
?

Ipotizzo che il complesso recettore-ligando possa essere internalizzato



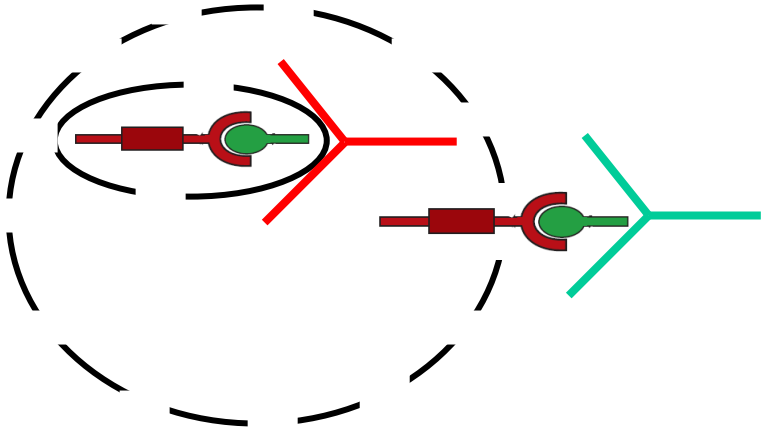
-ho a disposizione anticorpi primari in grado di riconoscere il recettore Eph

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



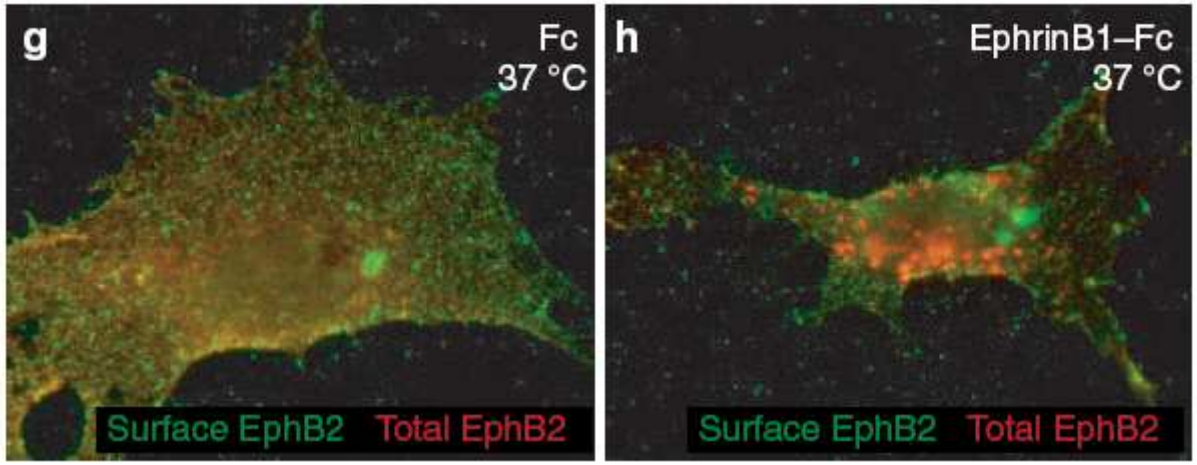
Cells were fixed <u>in the absence of detergents</u> and immunolabelled for **Eph (or ephrin) on the cell surface**.

PERMEABILIZATION



Cells were then <u>permeabilized</u> 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

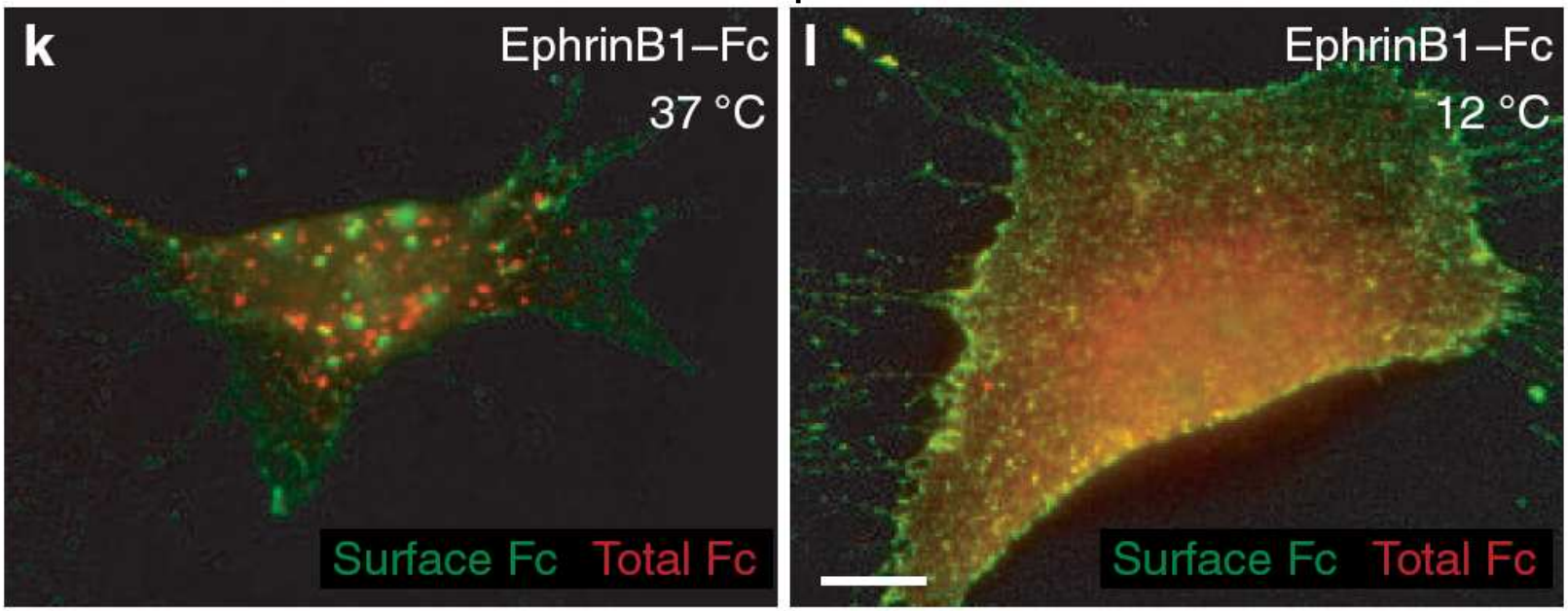
(Stimulation at 12 °C, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization)

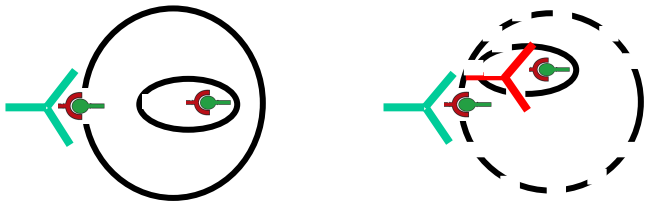
NIH3T3 cells stably expressing EphB2 were stimulated with unfused **Fc** (**control**) or **EphrinB1–Fc**, fixed in the absence of detergents, and immunolabelled for **EphB2 on the cell surface.**

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.**

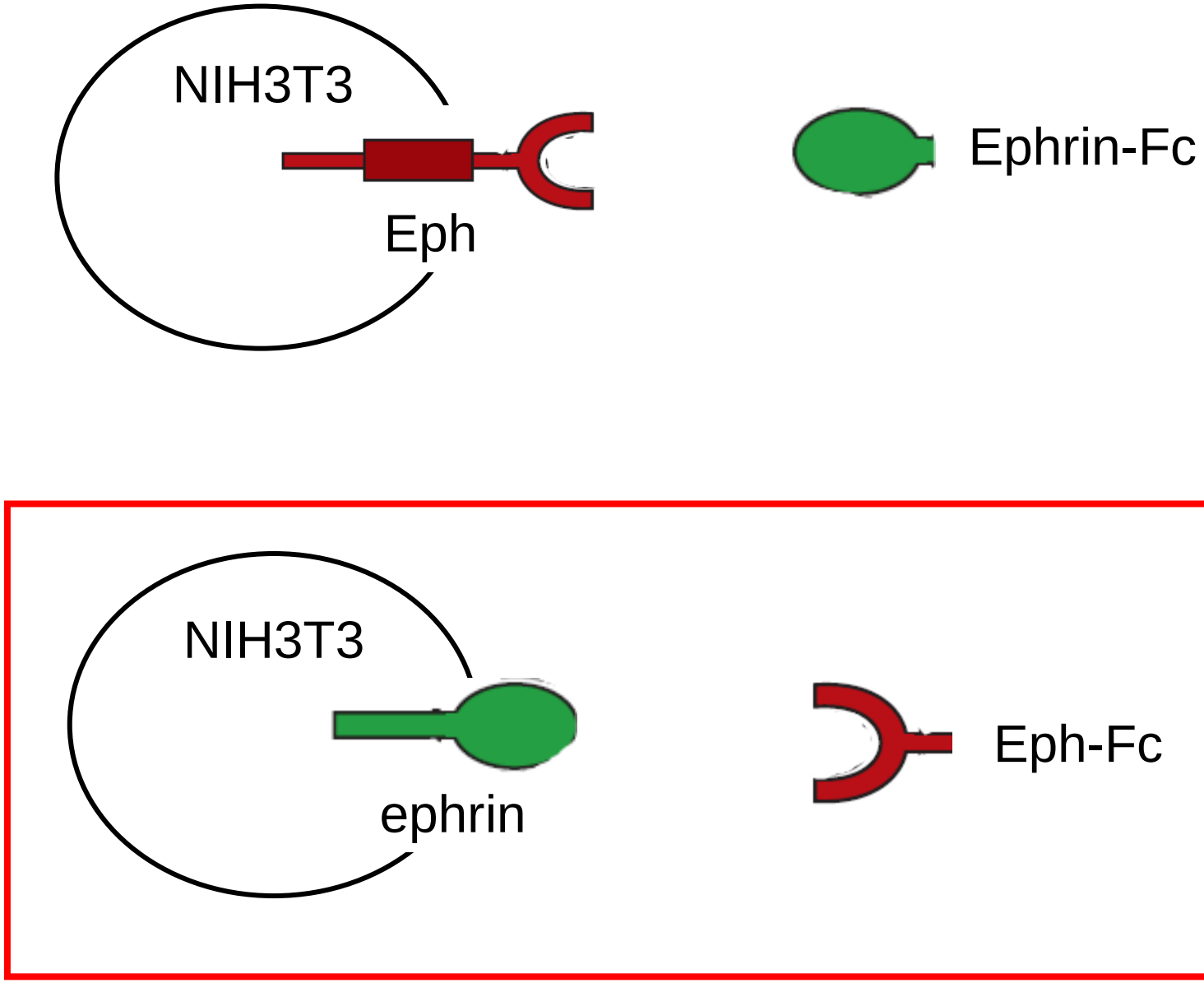
EphrinB1–Fc, bound to surface EphB2, was visualized with a FITC-conjugated antibody directed against human-Fc

3T3 EphB2

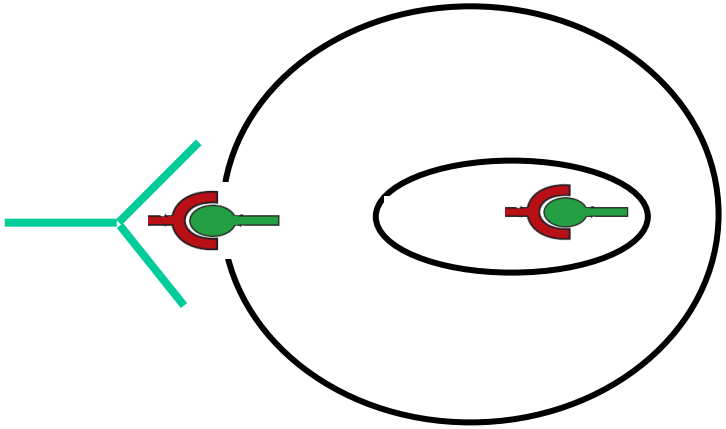




Stimulation at 12 °C, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization.

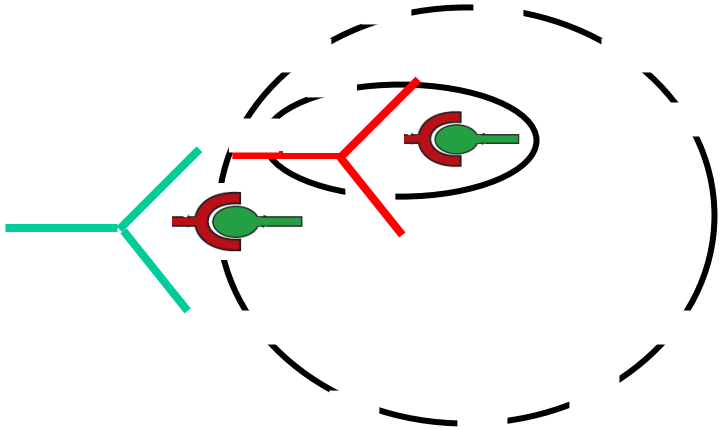


Cells were fixed in the absence of detergents and immunolabelled for **ephrin on the cell surface**.

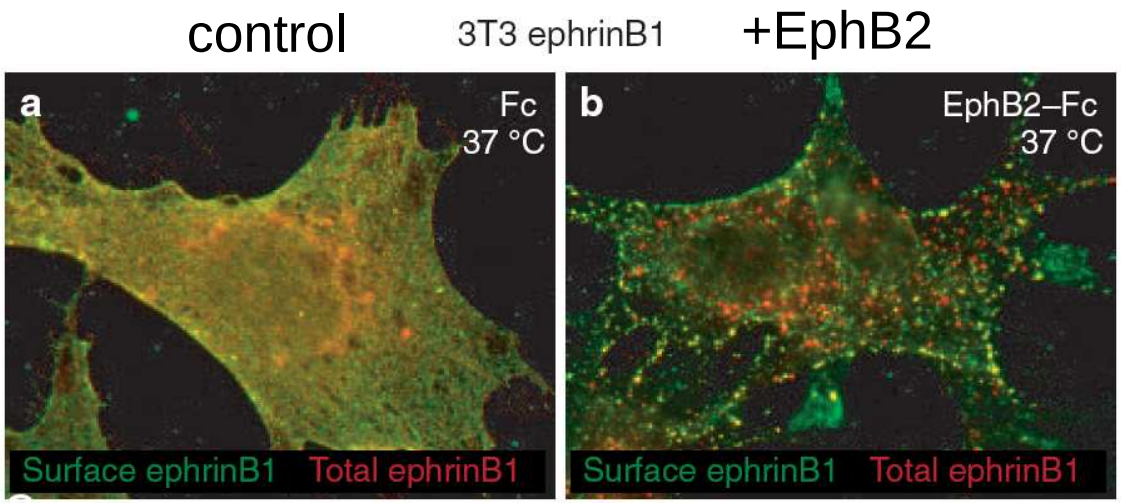


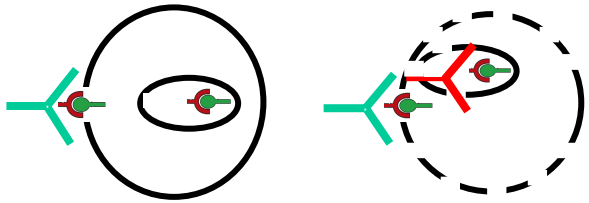
PERMEABILIZATION

Cells were then permeabilized 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

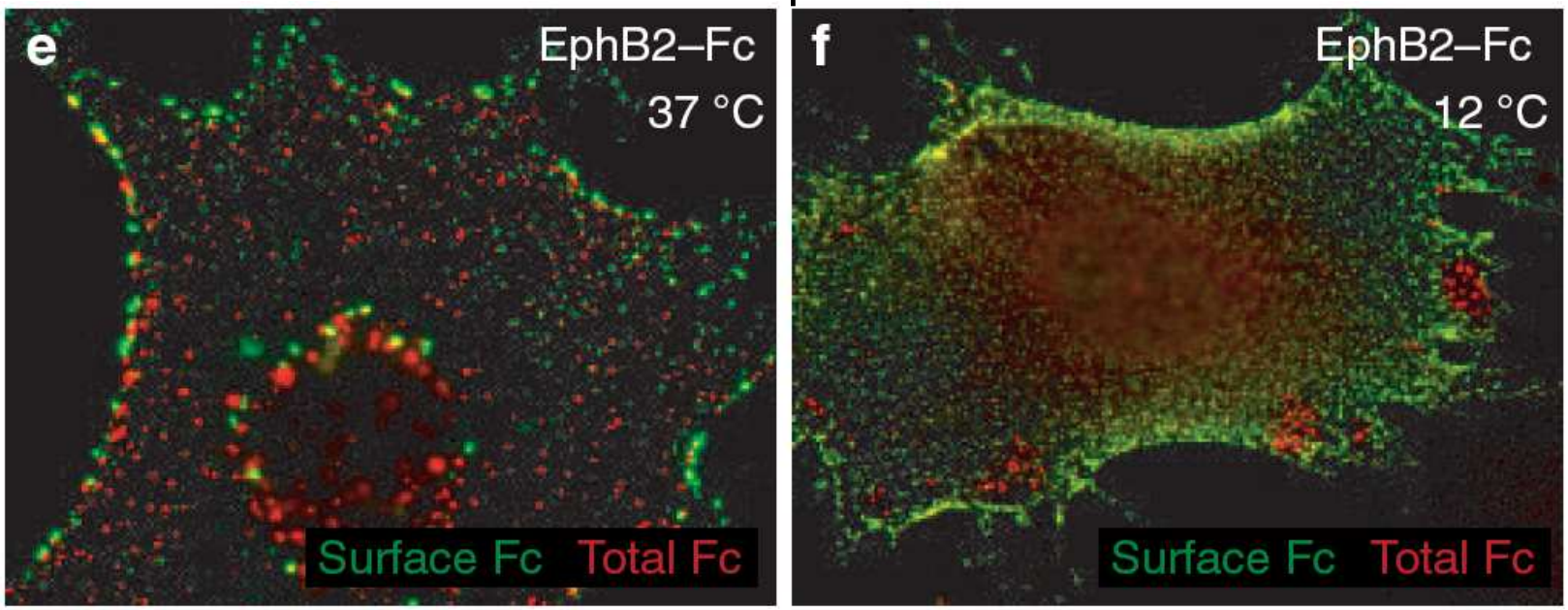


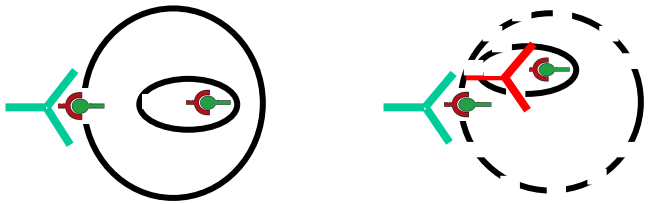


NIH3T3 cells stably expressing ephrinB1 were stimulated with unfused **Fc** (**control**) or **EphB2–Fc**, fixed in the absence of detergents, and immunolabelled for **ephrinB1 on the cell surface**.
Cells were then permeabilized and stained for **total ephrinB1** using a different primary antibody.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

3T3 ephrinB1



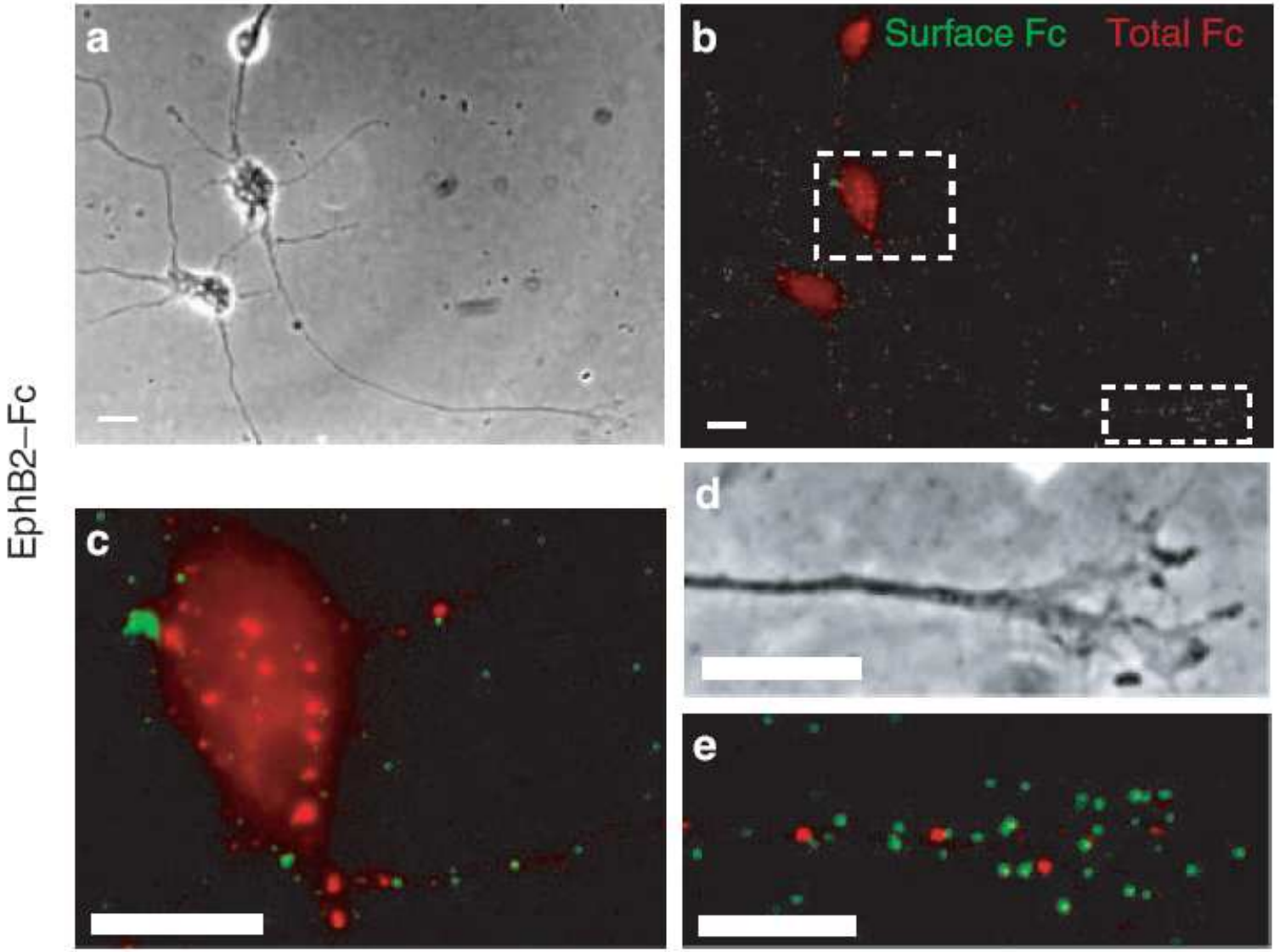


Stimulation at 12 °C, a temperature that prevents vesicular trafficking, did not block clustering at the cell surface but blocked internalization.

Endocytosis of EphB2–Fc in primary telencephalic neurons.

Neurons were stimulated for 15 min with EphB2–Fc.

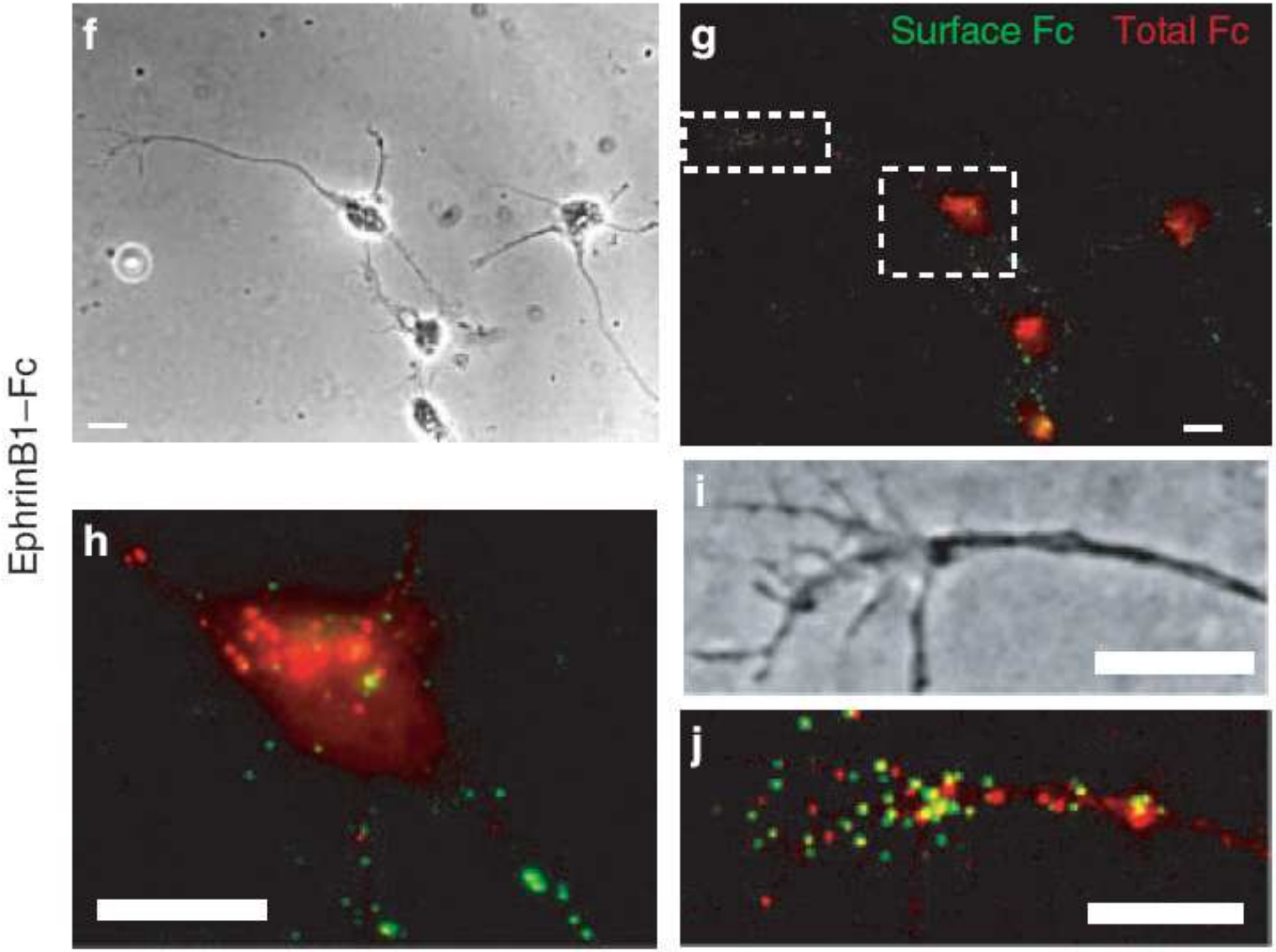
EphB2–Fc, was visualized with a FITC-conjugated antibody directed against human-Fc



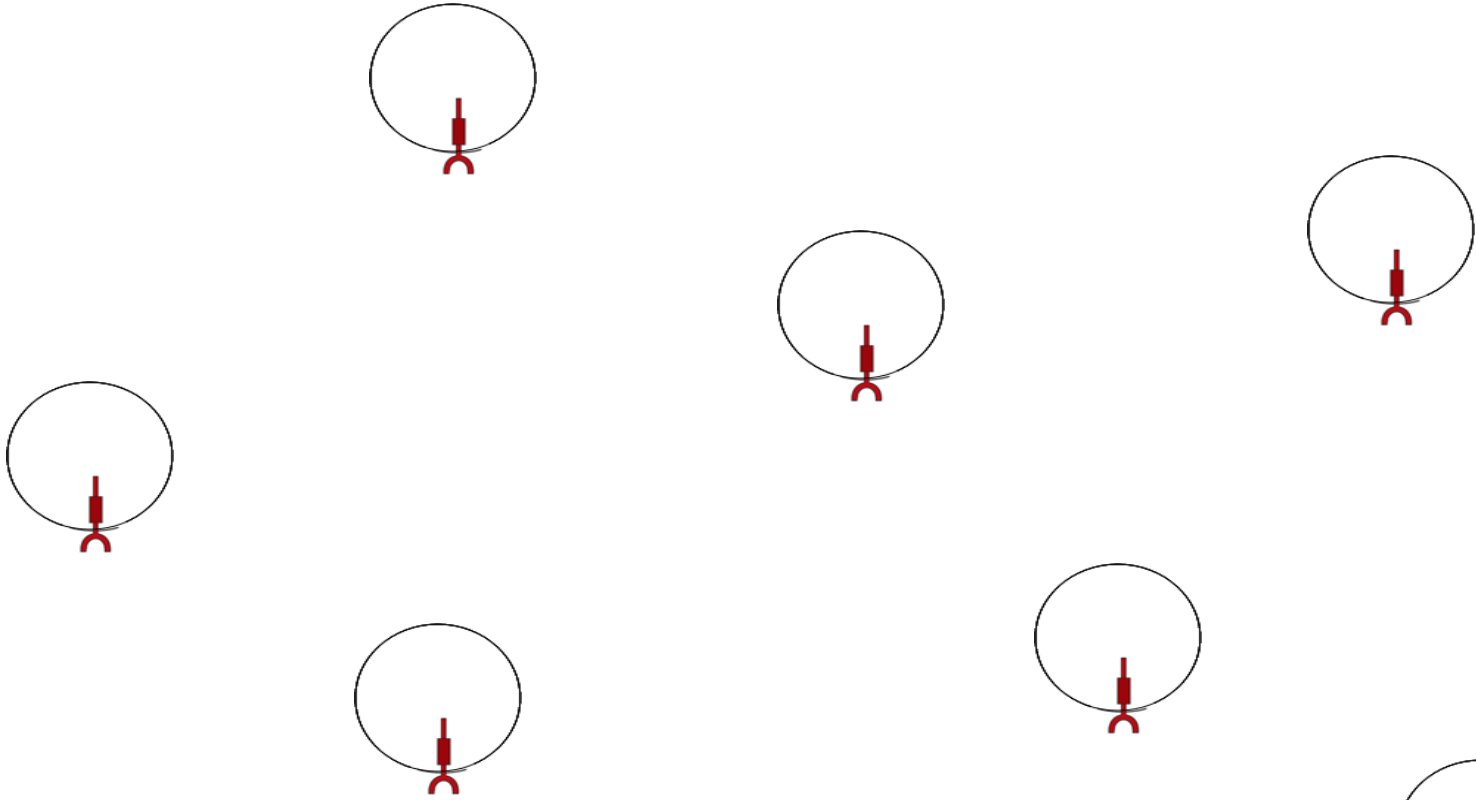
Endocytosis of ephrinB1–Fc in primary telencephalic neurons

Neurons were stimulated for 15 min with ephrinB1–Fc.

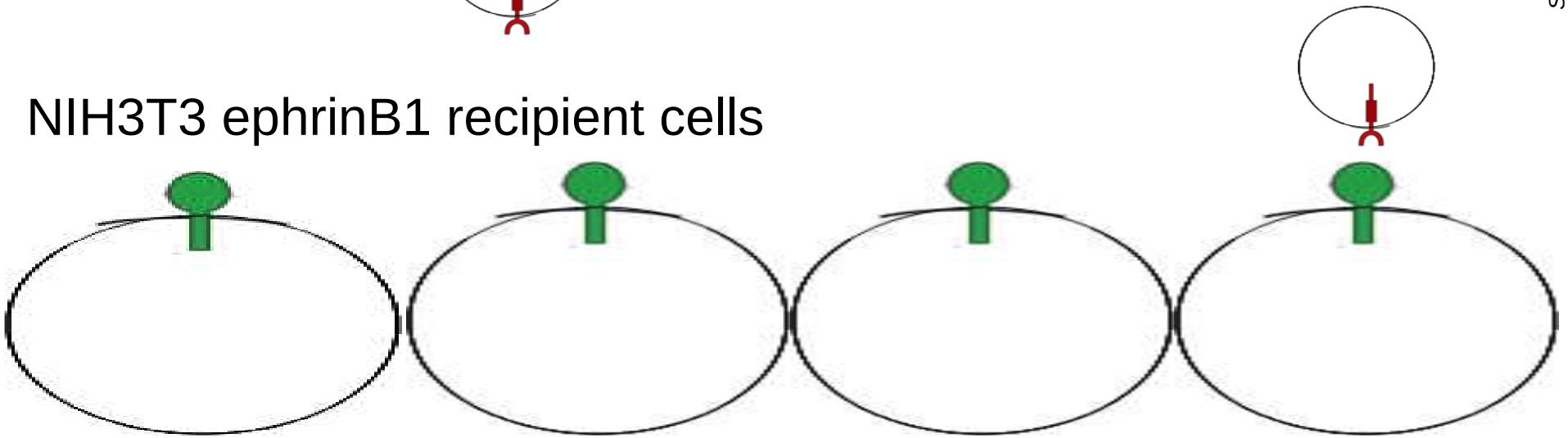
EphrinB1–Fc, was visualized with a FITC-conjugated antibody directed against human-Fc



NIH3T3 EphB2 stimulator cells

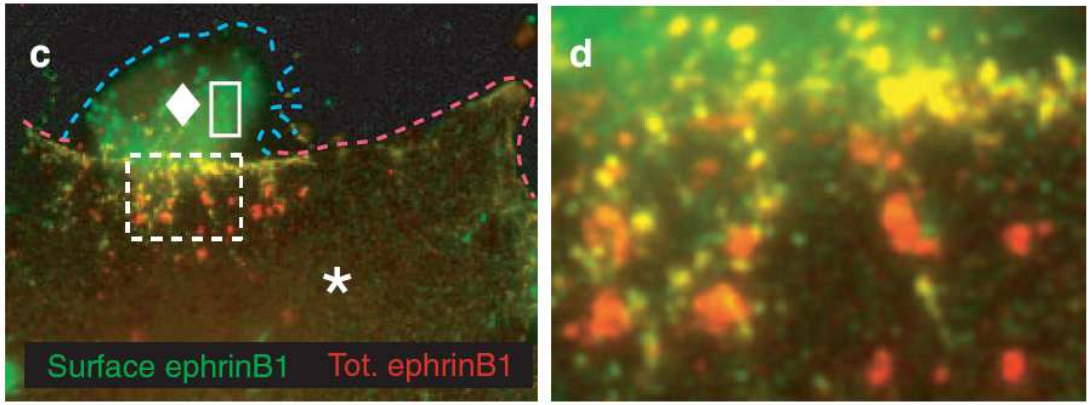


NIH3T3 ephrinB1 recipient cells

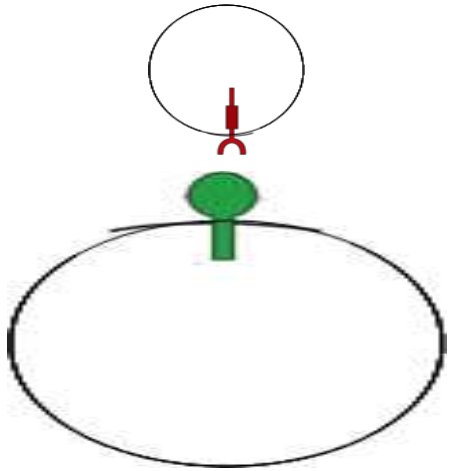


cell–cell stimulation assay to investigate whether membrane-bound ephrinB–EphB complexes co-cluster and subsequently internalize

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.

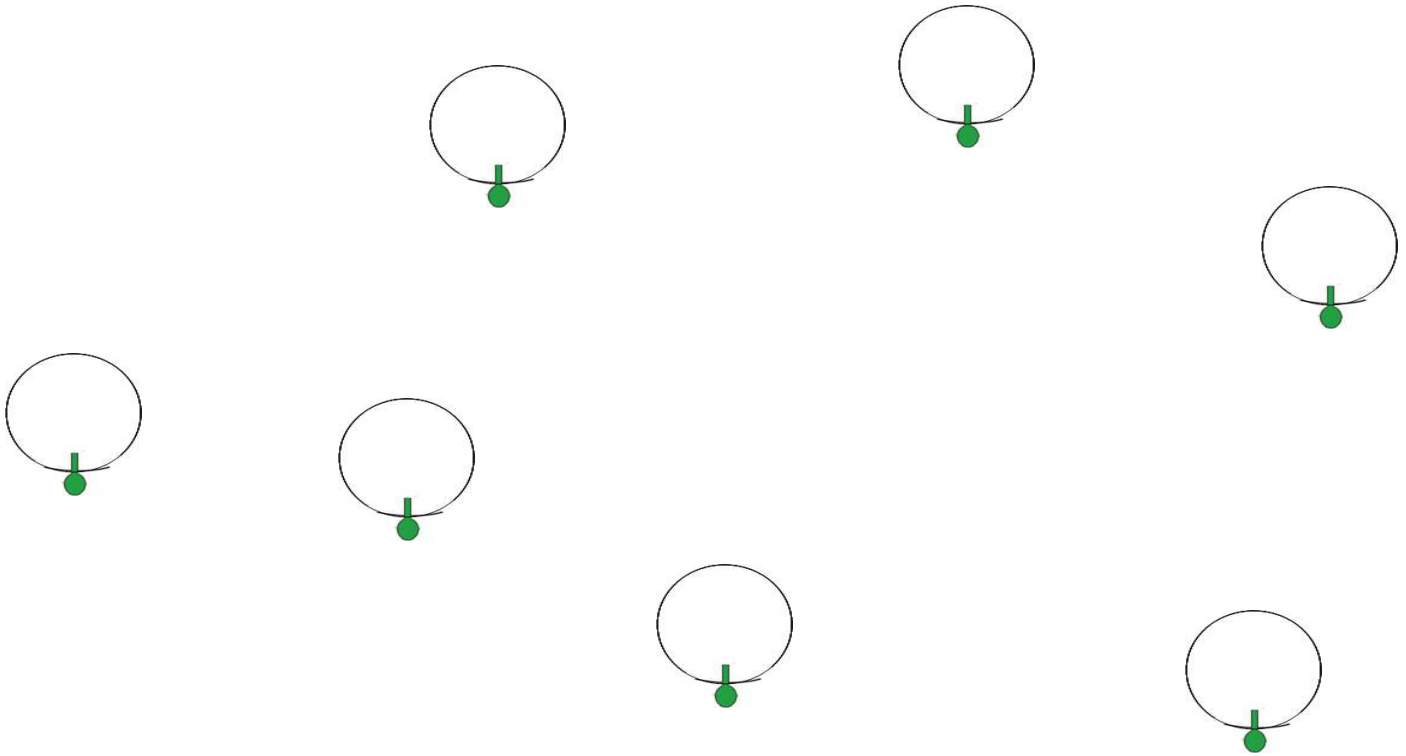


If they use 3T3 EphB2 (♦)stimulator cells with 3T3 ephrinB1(*) recipient cells, they observe rapid and localized co-clustering of ephrinB1 and EphB2 at the site of cell–cell contact. These clusters were partially endocytosed and the direction of internalization was in a *reverse* manner, that is, into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

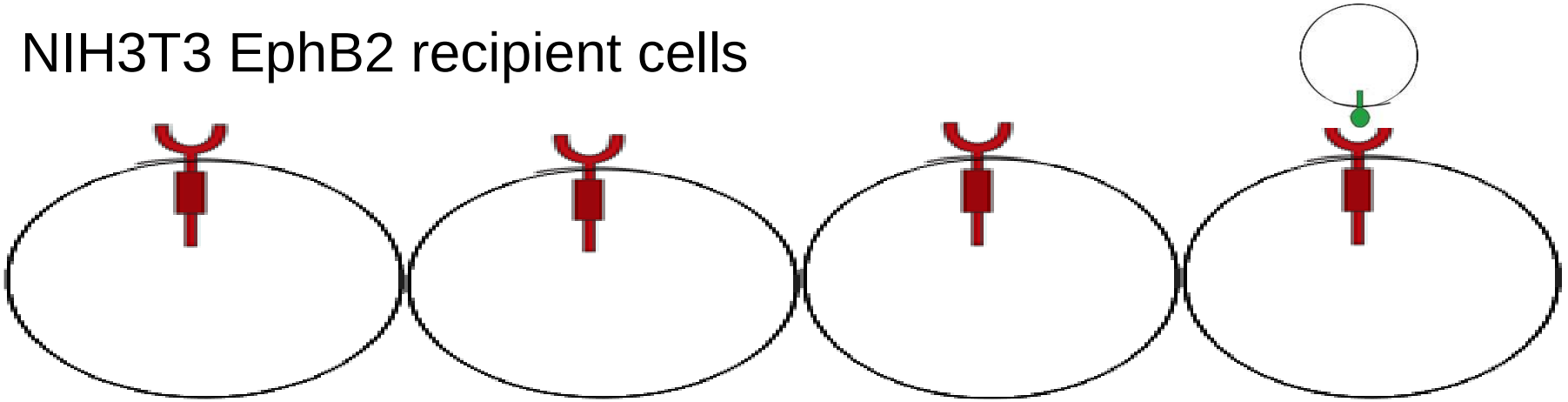


*

NIH3T3 ephrin stimulator cells



NIH3T3 EphB2 recipient cells



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

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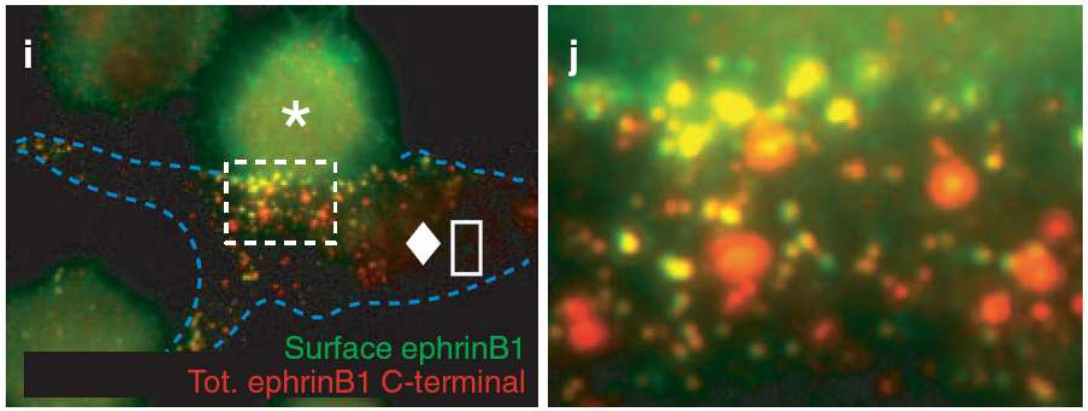
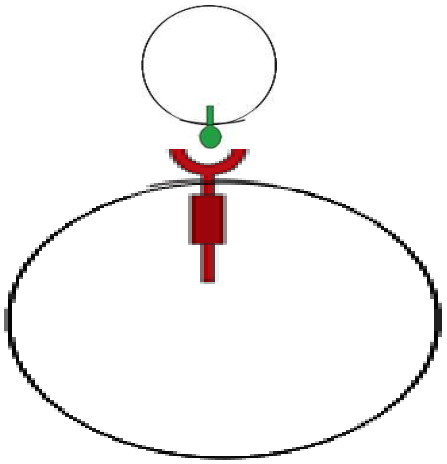


Next, they did the reverse experiment and used 3T3 ephrinB1(*) as stimulator cells and 3T3 EphB2 (♦) as recipient cells. EphrinB1 was internalized in a *forward* manner by 3T3 EphB2 cells



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

why ?

● 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

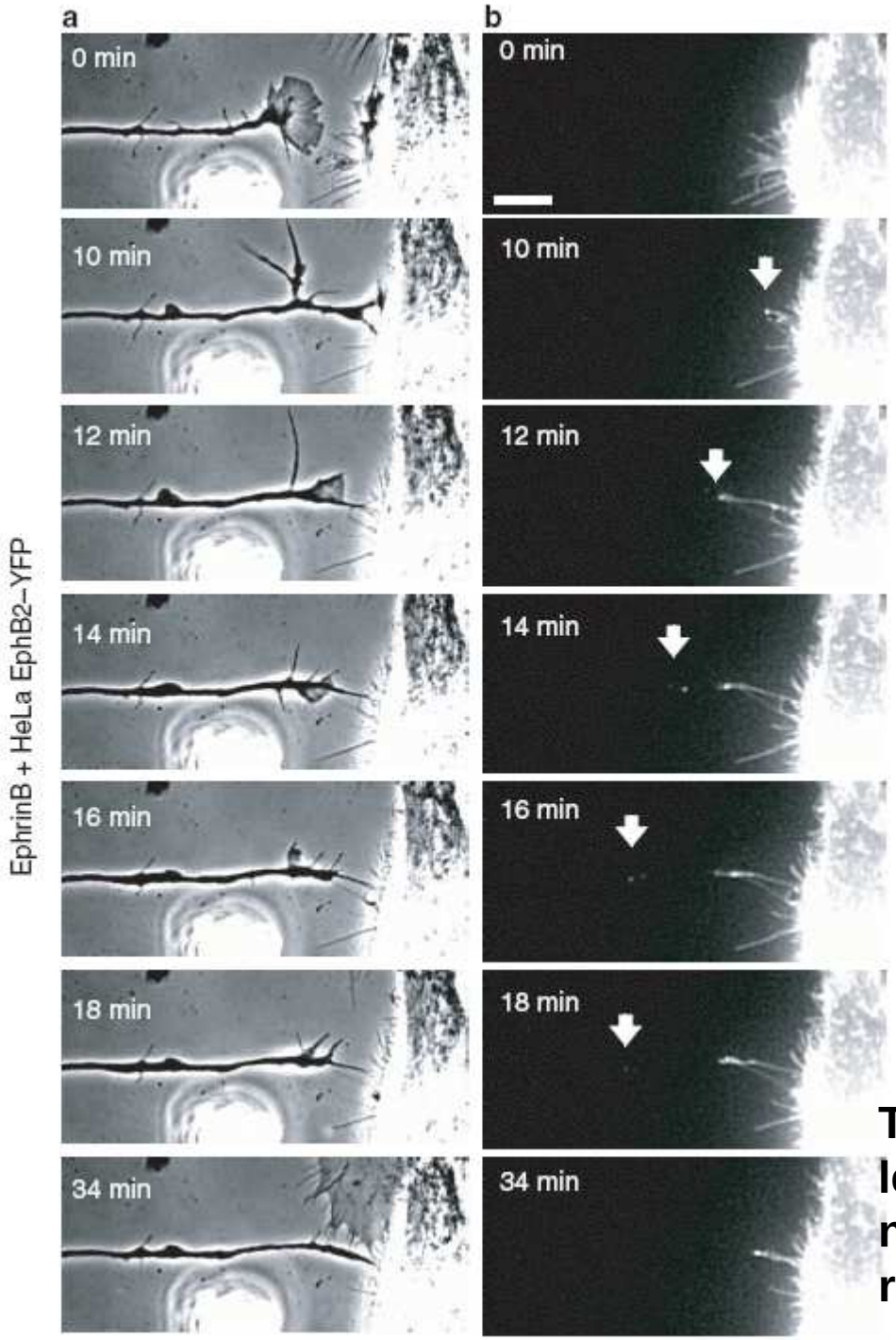
● weakening the receptor ability to signal shifted endocytosis towards ephrinB reverse signalling

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).

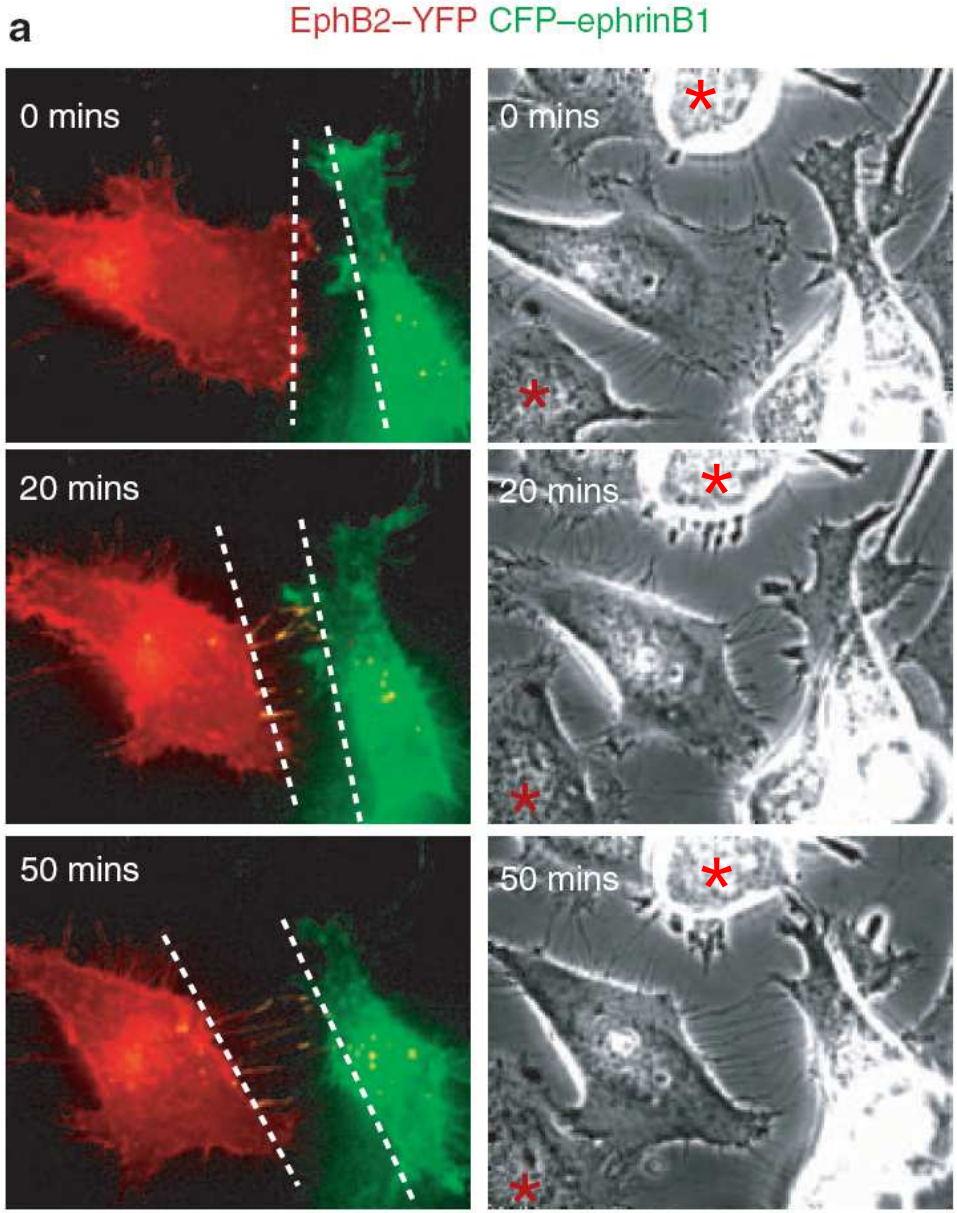
See **Movie 1**.

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 cell repulsion response and cell detachment



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

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

Right, phase contrast images.

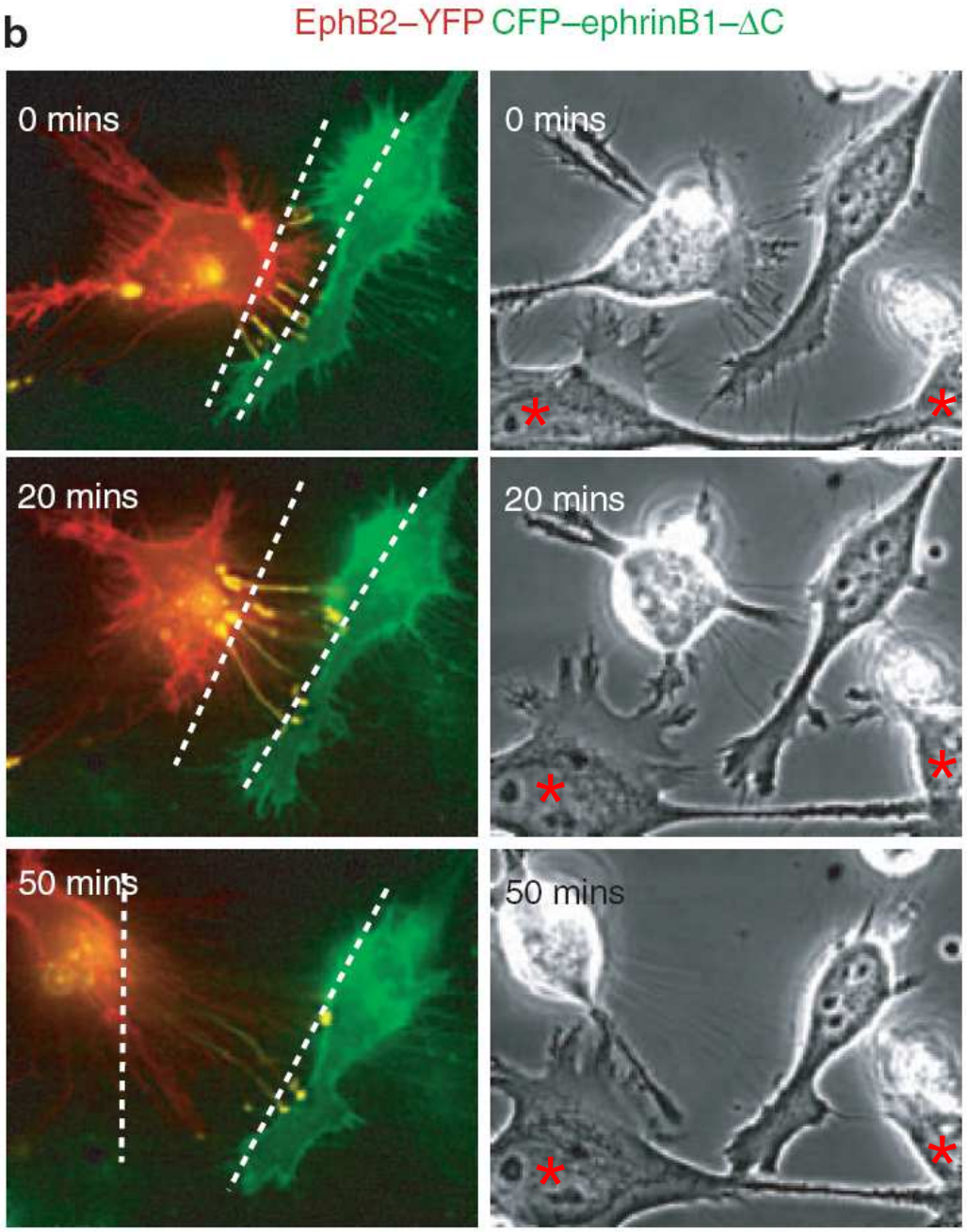
Intense clustering of EphB2 and ephrinB1 is seen at the contact site between the two cells at 20 min, the **EphB2–YFP** cell retracts a lamellipodium from the **ephrinB1 cell** (indicated by the distance between the two stippled lines).

(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.

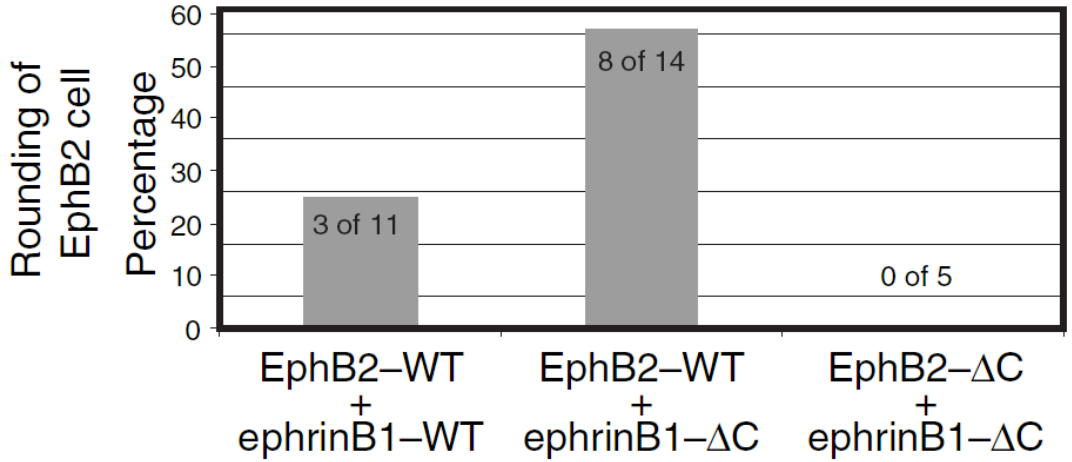
Strong repulsion and rounding of EphB2–YFP expressing cell is observed.

(see **Movie 3**).

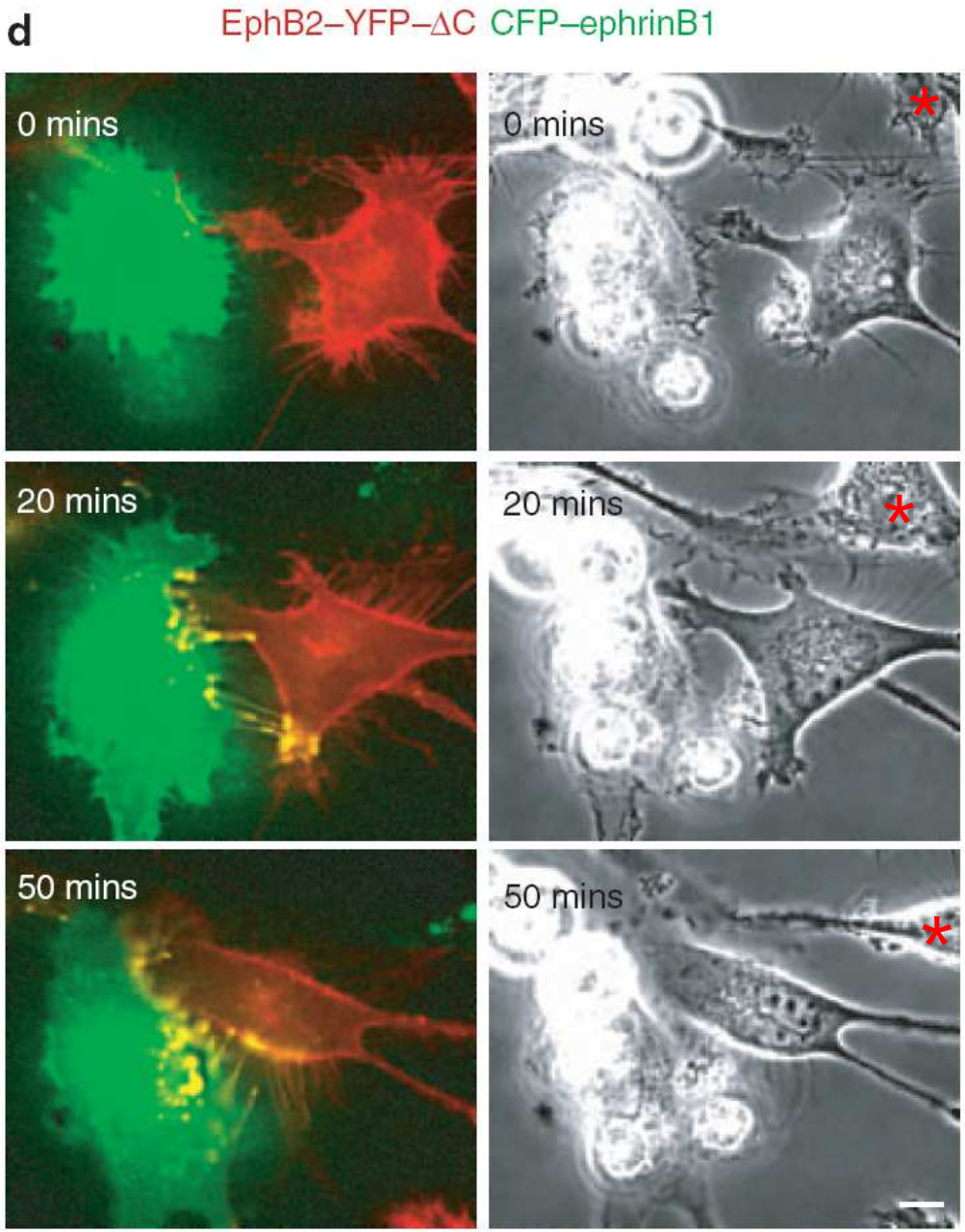
• when ephrinB1 endocytosis was blocked by a C-terminal truncation (**CFP–ephrinB1-ΔC**), markedly different cell behaviour was observed

• 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**

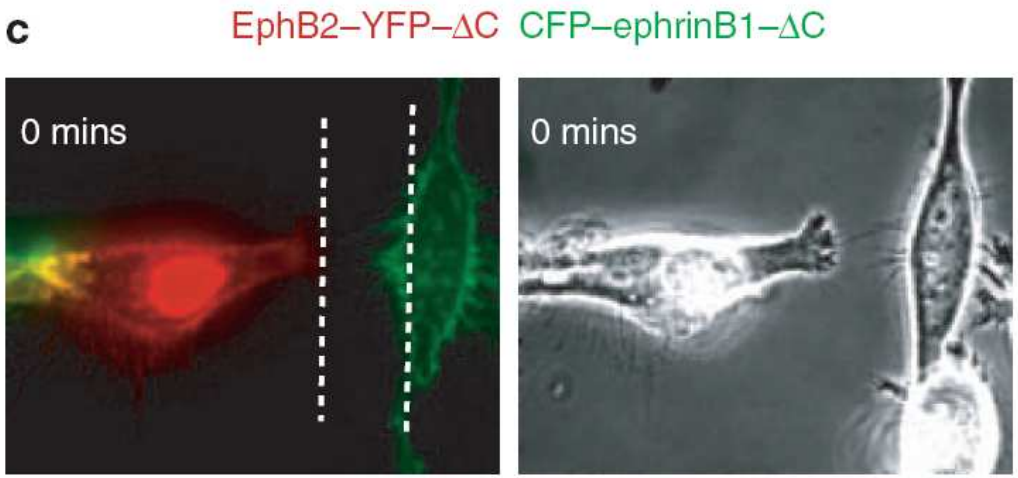
How do cells react to unidirectional ephrinB reverse signalling?

• 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

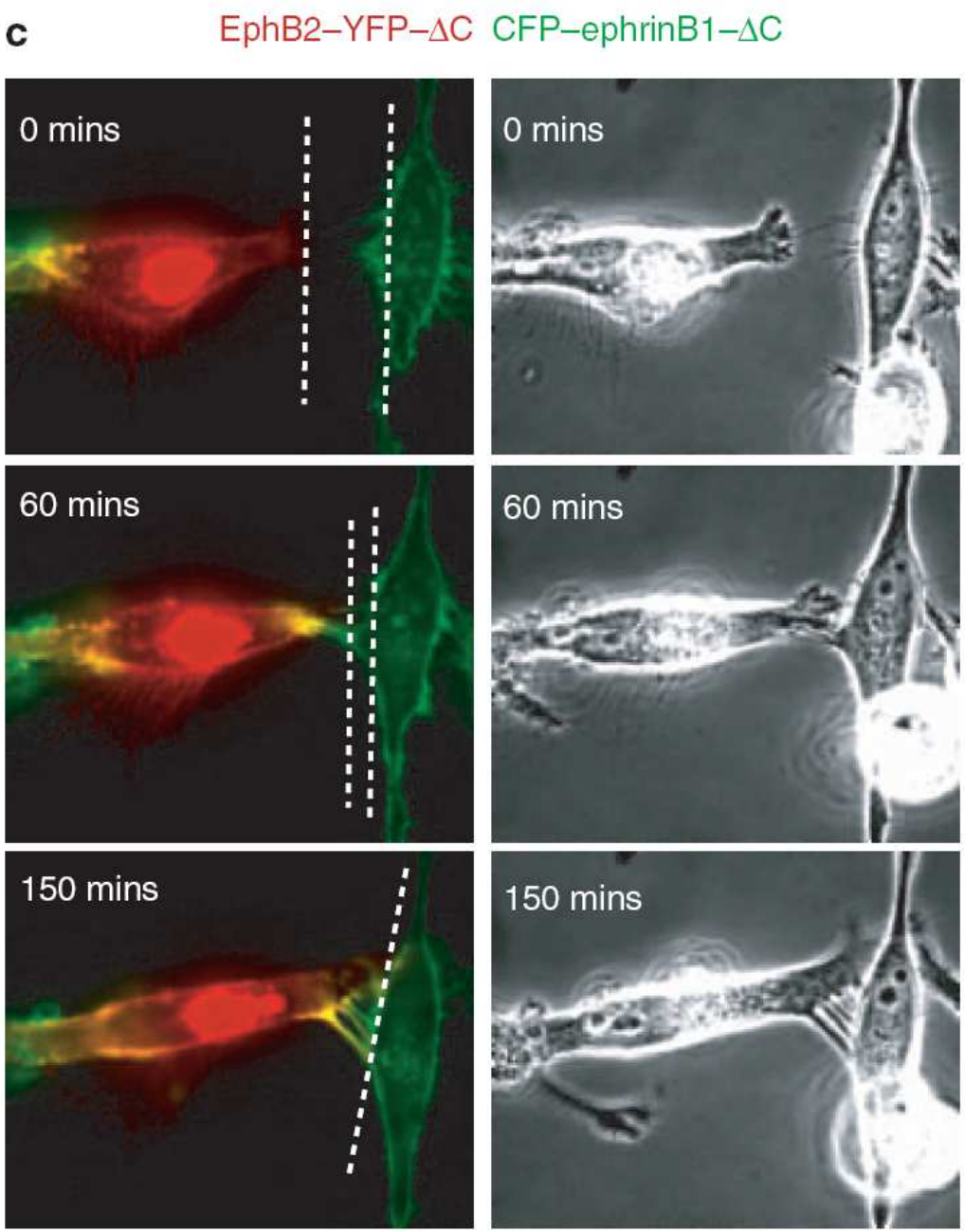
→ **ephrinB1 reverse endocytosis is sufficient to terminate adhesion and to cause cell detachment, but not retraction**



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.**

- Secondo voi, cosa succede se facciamo esprimere **CFP–ephrinB1-ΔC** e **EphB2–YFP- ΔC**?



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.**

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 and cell detachment

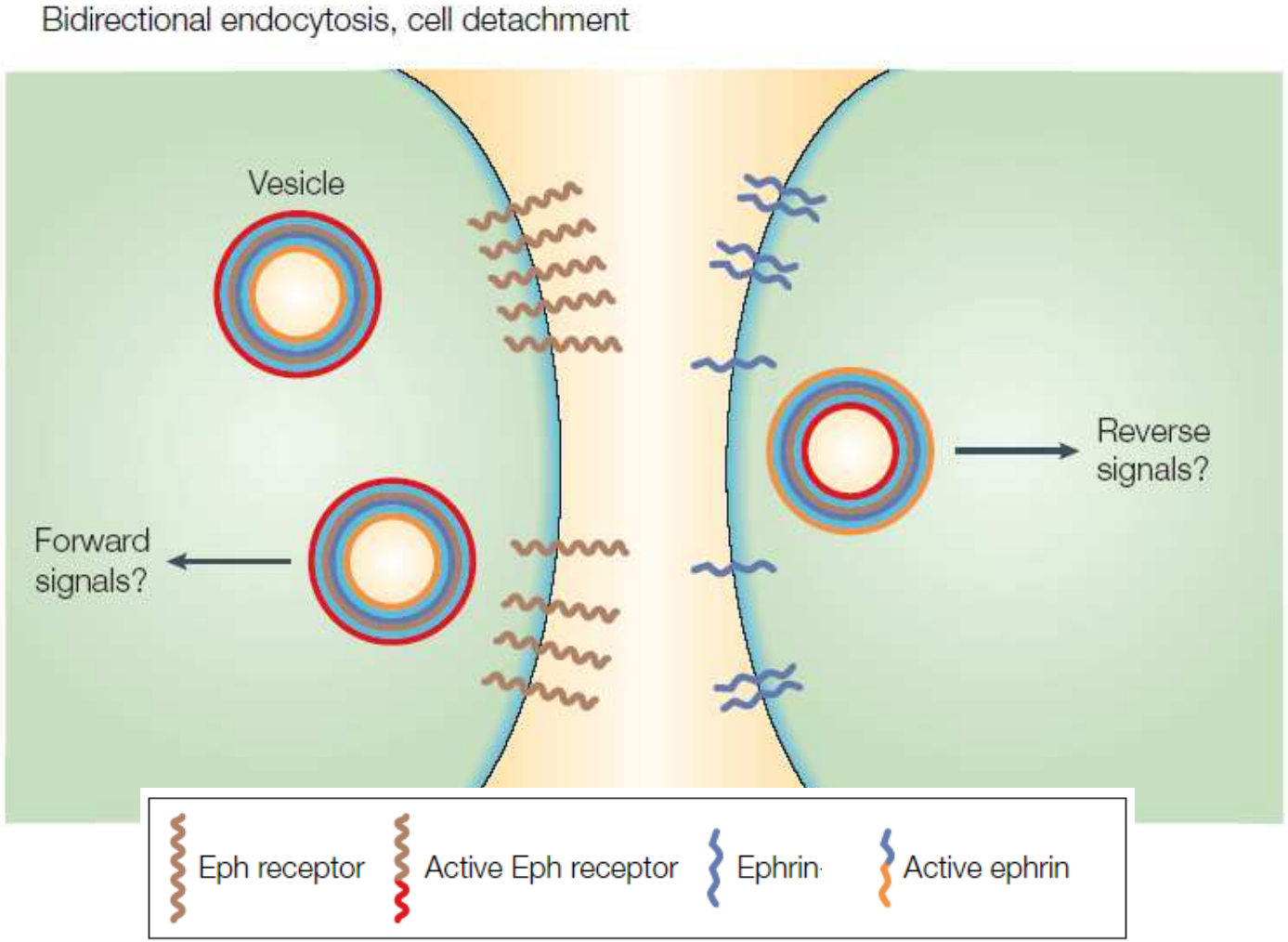
- in the absence of reverse endocytosis a gain-of-function phenotype is observed: enhancement of repulsion by EphB receptor forward signalling

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

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

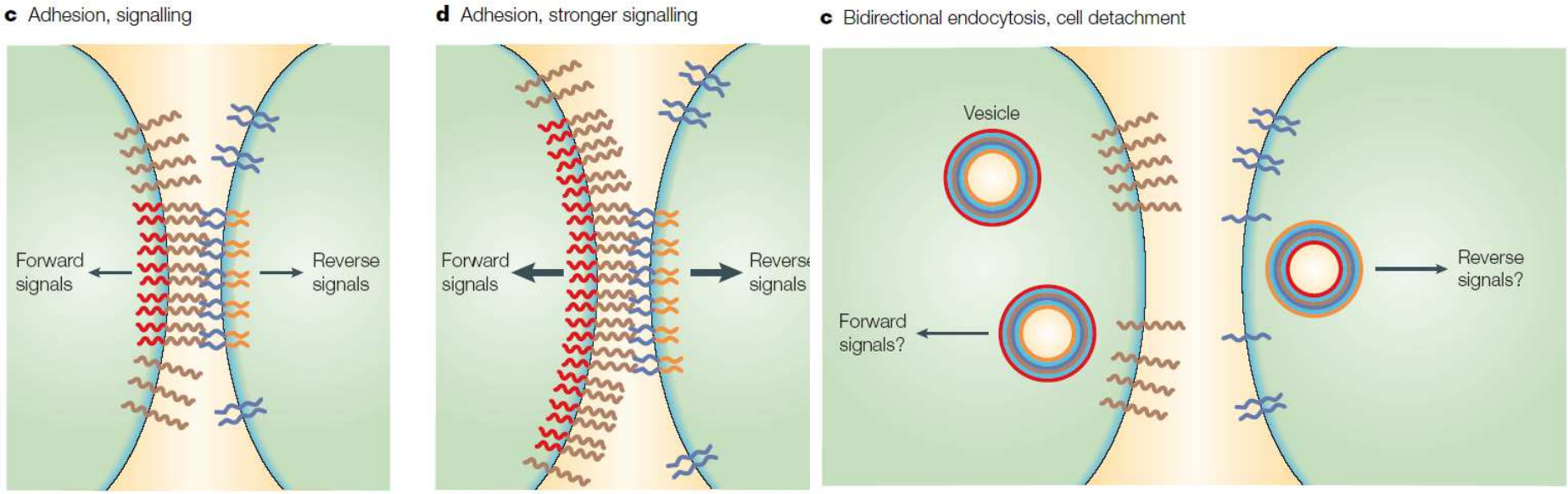
- the underlying mechanism of EphB2 endocytosis may resemble phagocytosis or macropinocytosis

Mechanisms of Eph signal attenuation and termination.

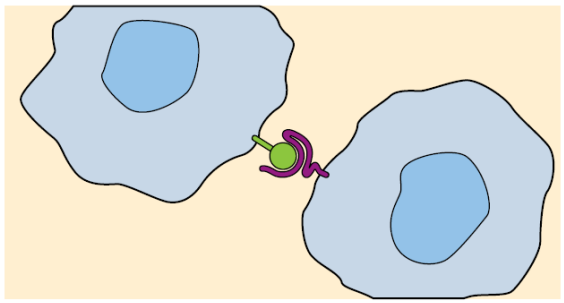


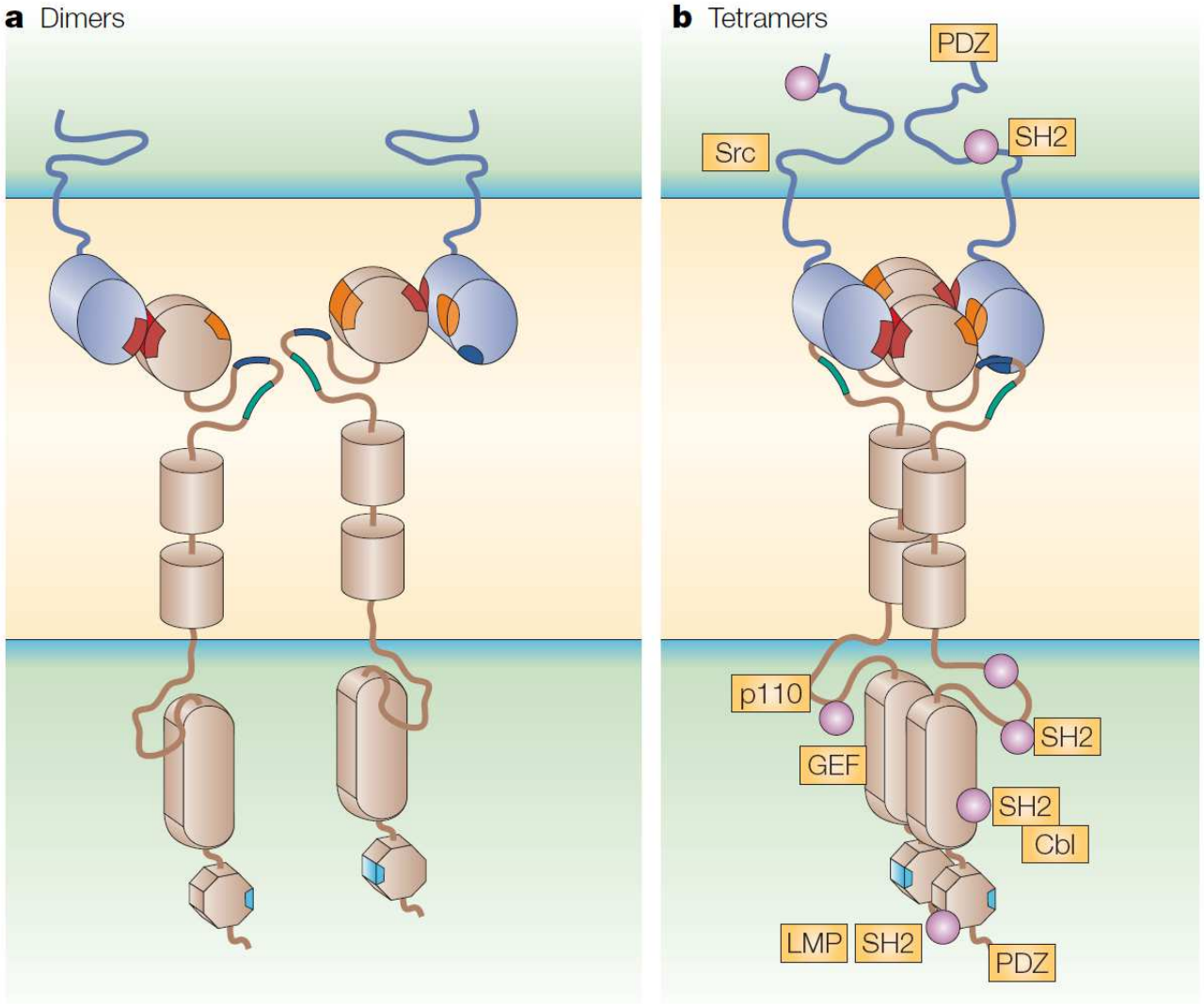
Internalization of Eph-receptor–ephrin complexes together with their surrounding plasma membranes, which can occur into the receptor- and the ligand-expressing cell, allows disengagement of the two cells and gives rise to internalized double-membrane vesicles.

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



Comunicazione bidirezionale: come studiare in maniera quantitativa le proteine coinvolte nella segnalazione nelle due cellule?



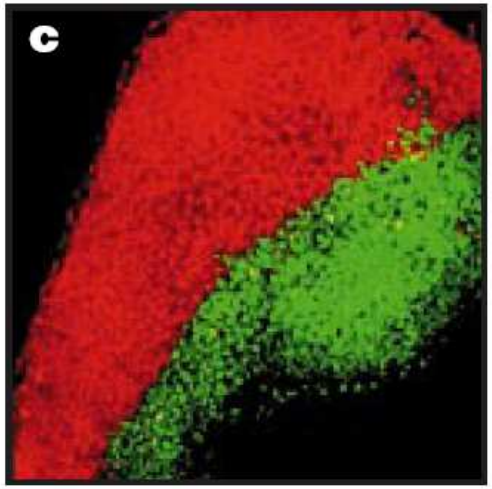


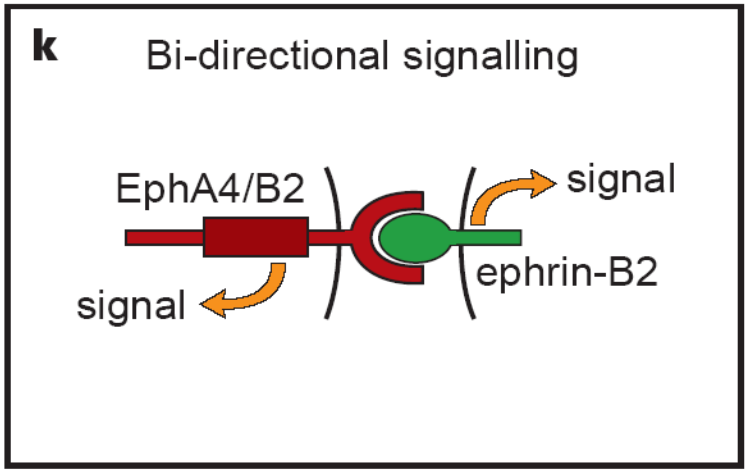
- la trasduzione del segnale di solito viene studiata stimolando le cellule bersaglio con un ligando solubile extracellulare, come ad esempio un fattore di crescita

- non è detto che questo approccio ripercorra il processo fisiologico della comunicazione intercellulare, in parte perché le proteine che fungono da ligandi per alcuni recettori sono esse stesse ancorate alla membrana della cellula

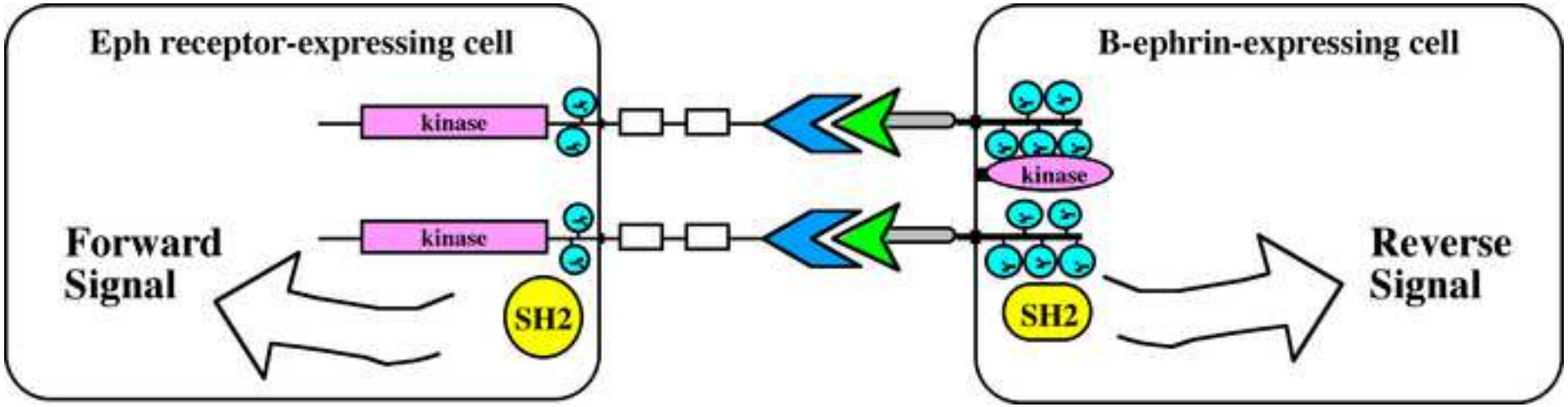
- la segnalazione iniziata dal contatto cellula-cellula di solito è un processo reciproco, nel quale due tipi cellulari si scambiano segnali distinti, che portano ad alterazioni mutualmente dipendenti nei loro rispettivi comportamenti

EphB2 /ephrin-B2

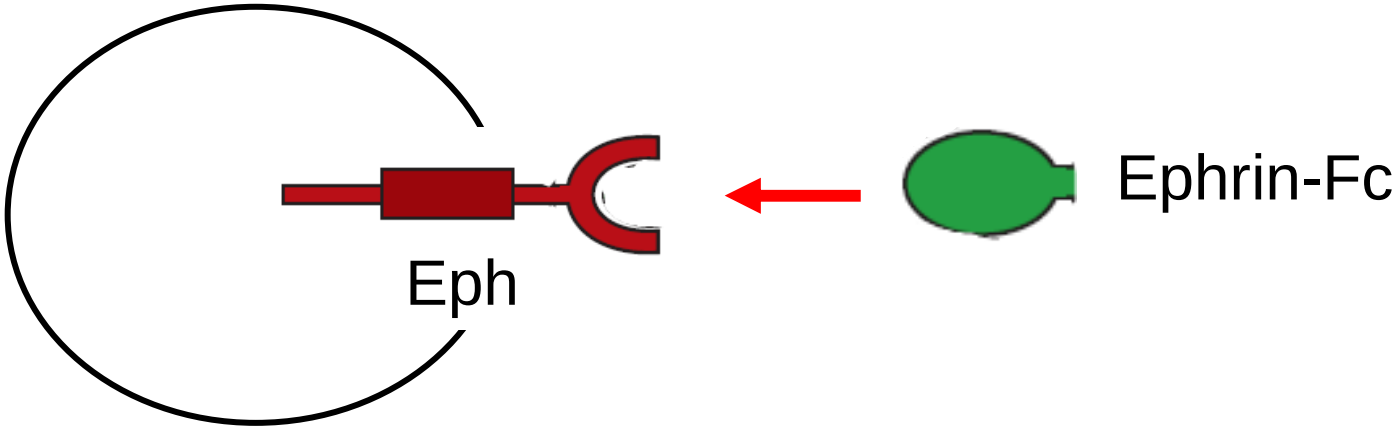




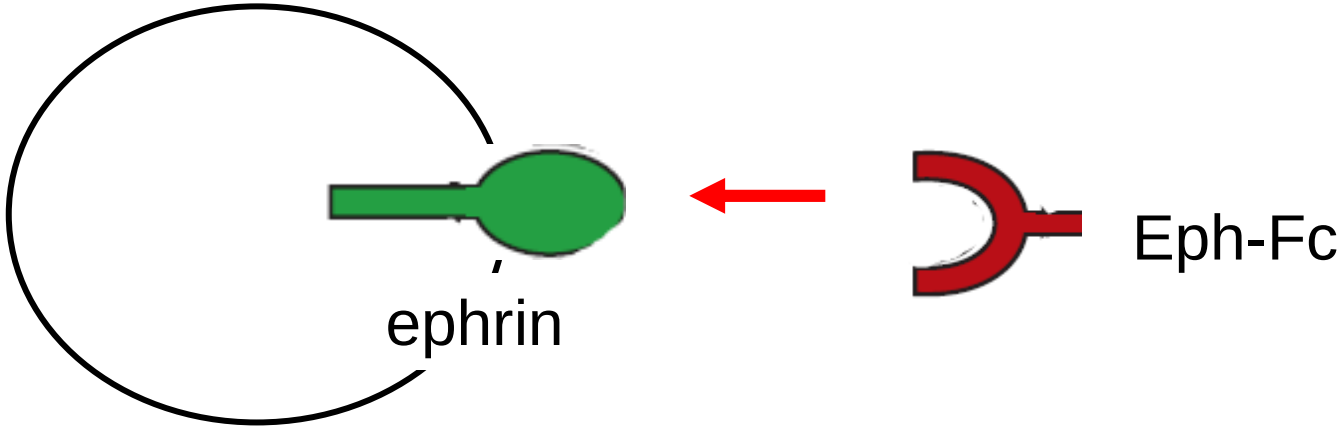
The Phenomenon of Bidirectional Signaling



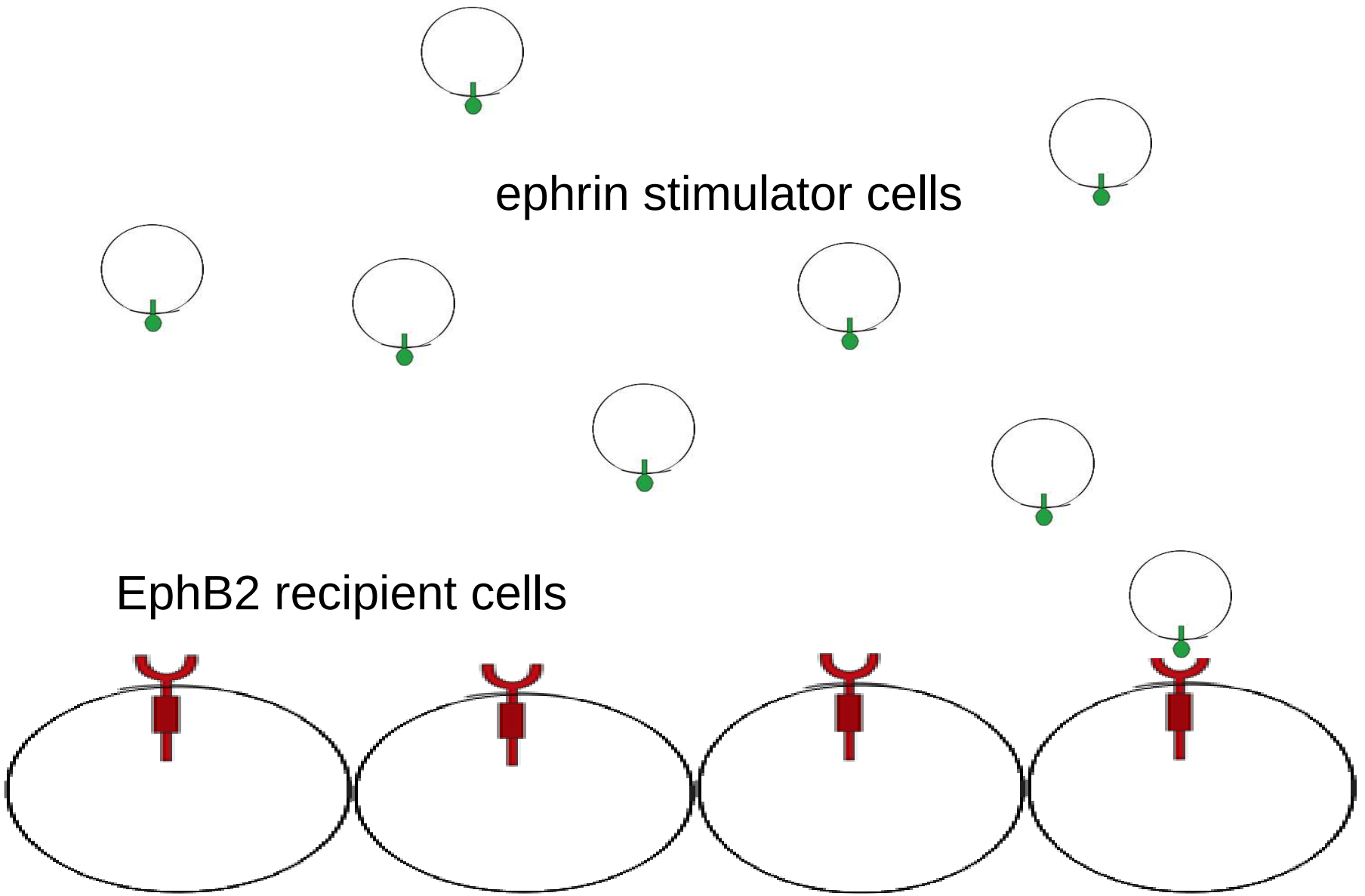
Stimolazione (con un ligando solubile) di cellule adese (che esprimono il recettore transmembrana)



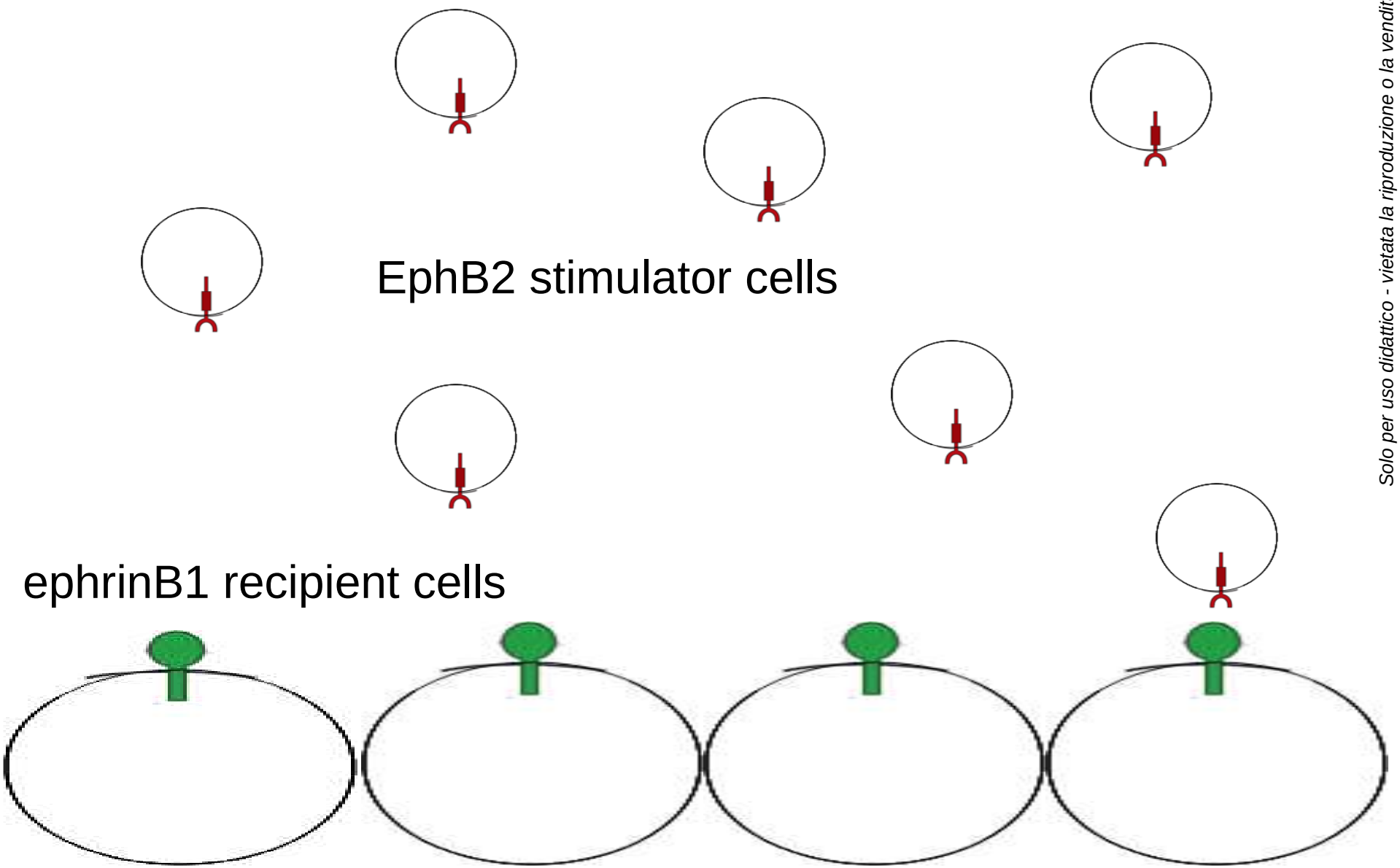
Stimolazione (con un recettore solubile) di cellule adese (che esprimono il ligando transmembrana)



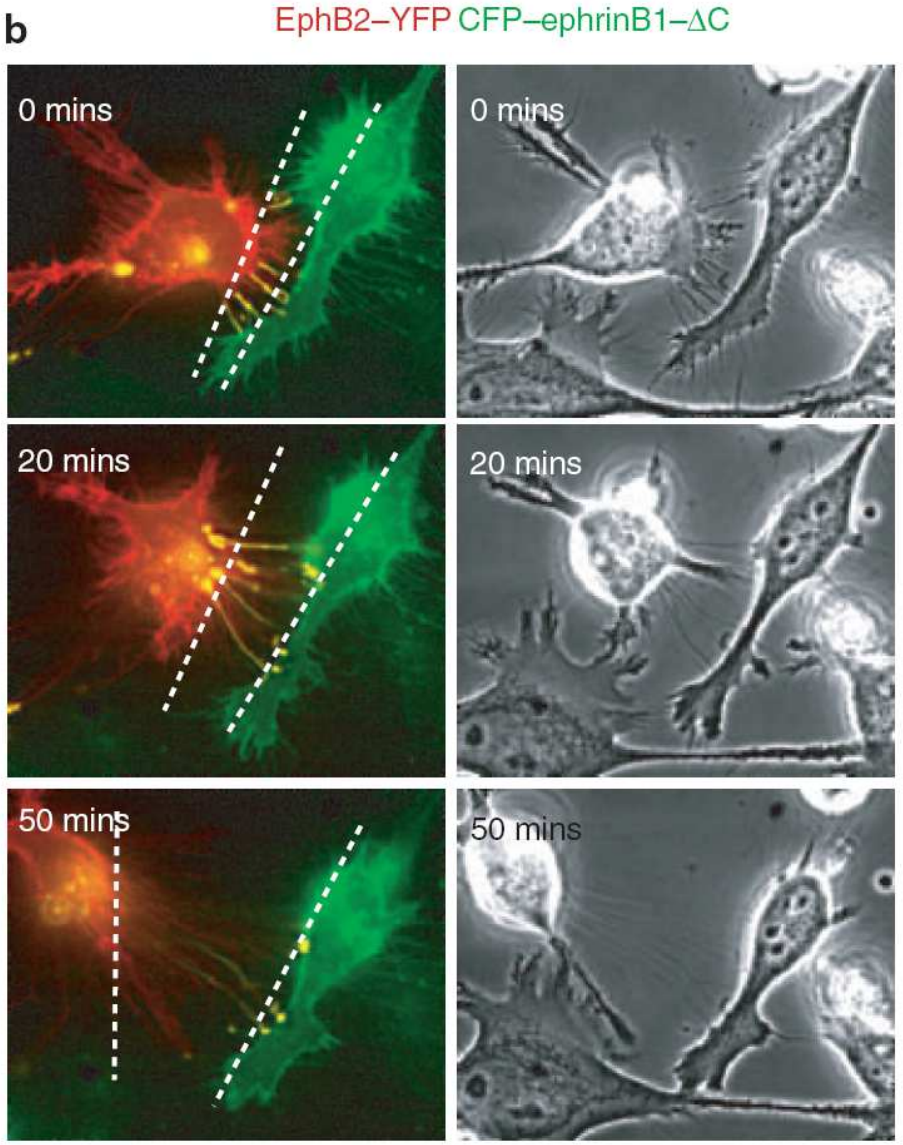
Stimolazione di cellule adese (che esprimono il recettore transmembrana) con cellule in sospensione (che esprimono il ligando transmembrana)



Stimolazione di cellule adese (che esprimono il ligando transmembrana)
con cellule in sospensione (che esprimono il recettore transmembrana)



Interazione fra cellule adese (che esprimono il ligando transmembrana) e cellule adese (che esprimono il recettore transmembrana)



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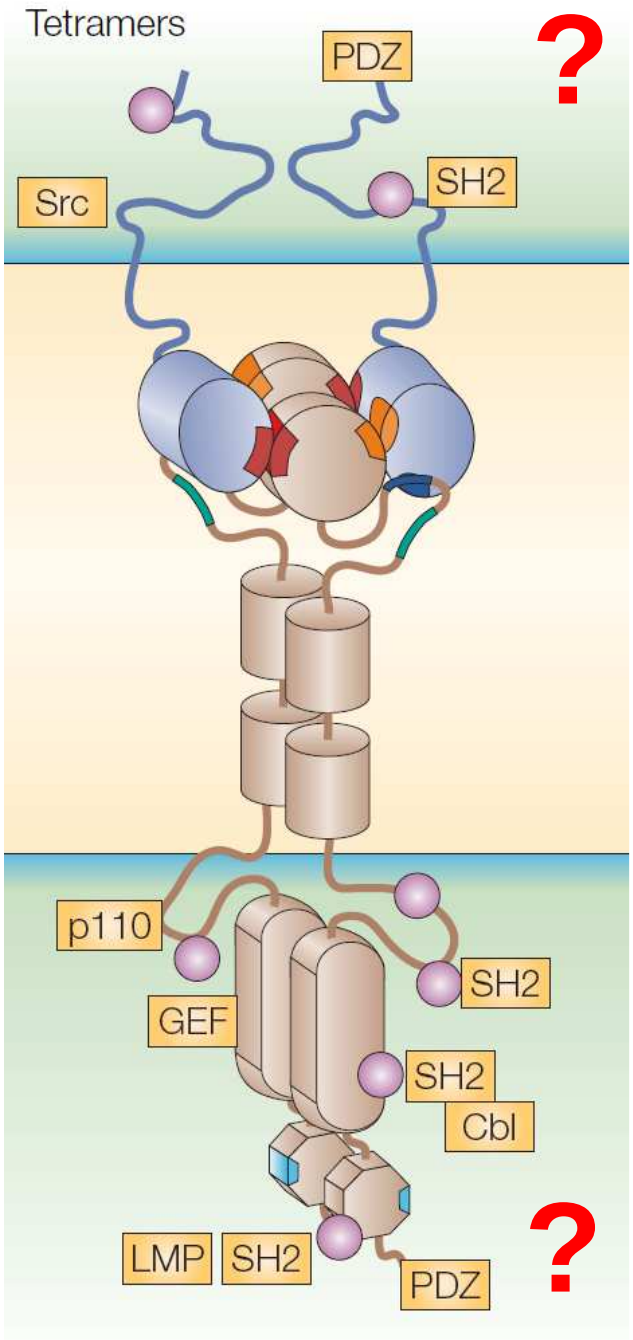
Cell-Specific Information Processing in Segregating Populations of Eph Receptor Ephrin–Expressing Cells

Claus Jørgensen,[1] Andrew Sherman,[1,2] Ginny I. Chen,[1,2] Adrian Pasculescu,[1] Alexei Poliakov,[3] Marilyn Hsiung,[1] Brett Larsen,[1] David G. Wilkinson,[3] Rune Linding,[4]* Tony Pawson[1,2]*



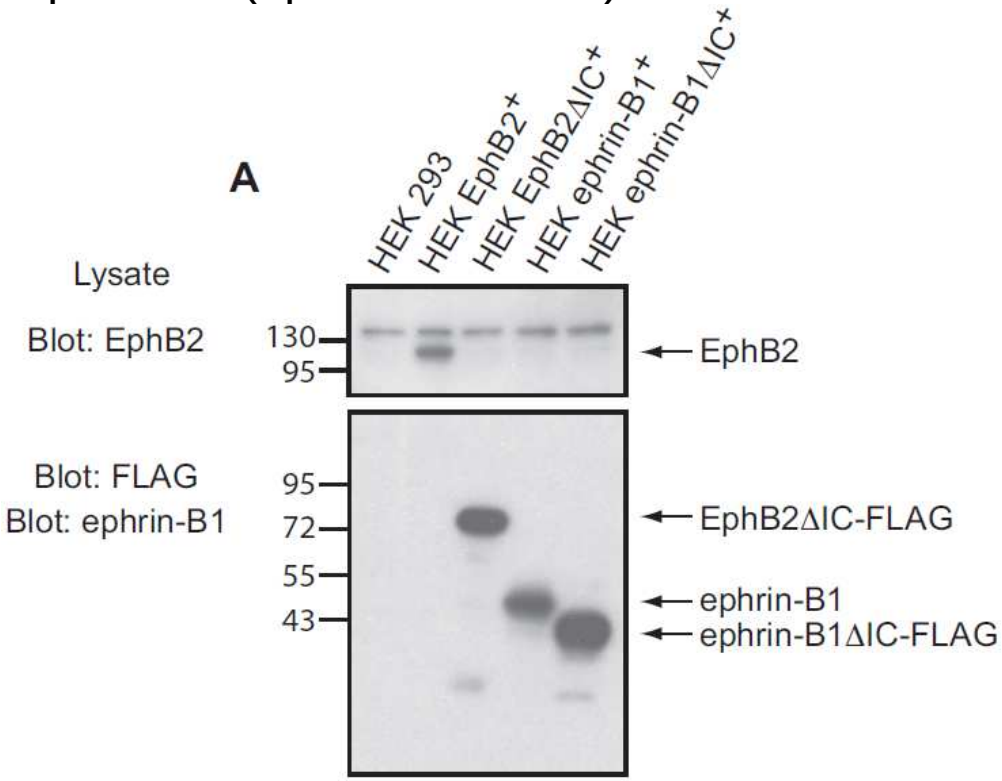
❁ direct interactions between transmembrane Eph receptor tyrosine kinases (EphRs) and their membrane bound ephrin ligands frequently lead to mutual **cell repulsion** and are important for **axon guidance** and **boundary formation** during tissue development

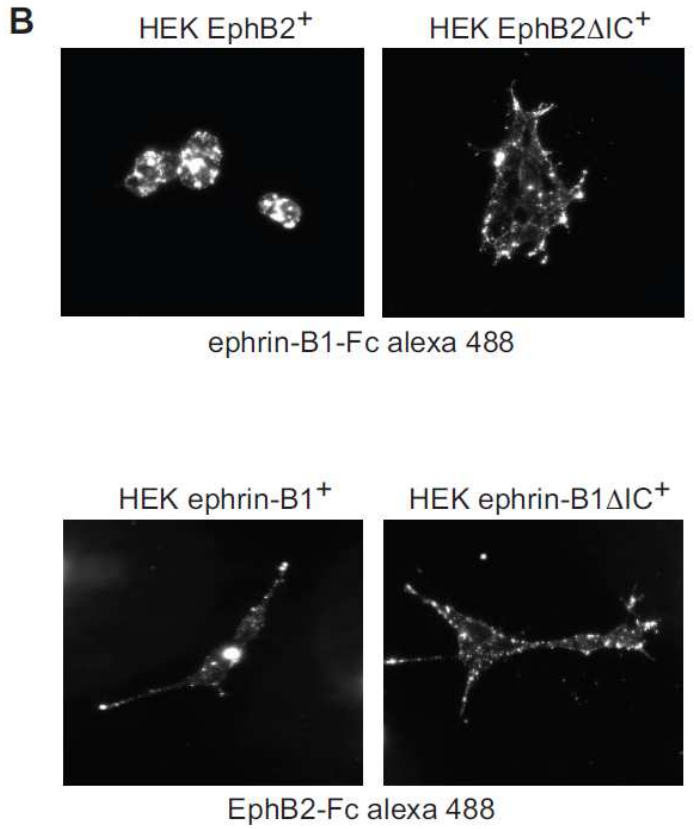
❁ clustering of B-type EphRs and ephrins at the surface of adjacent cells activates phosphotyrosine (pTyr) signaling in both the EphR- and ephrin-expressing cells, termed **forward** and **reverse** signaling, respectively

❁ systematic analysis of cell-specific networks in distinct populations of interacting cells is challenging primarily because the unique properties of each cell type are lost once co-cultured cells are processed for biochemical analysis, such as by immunoblotting

Quantitative analysis of Bidirectional Signaling (qBidS)

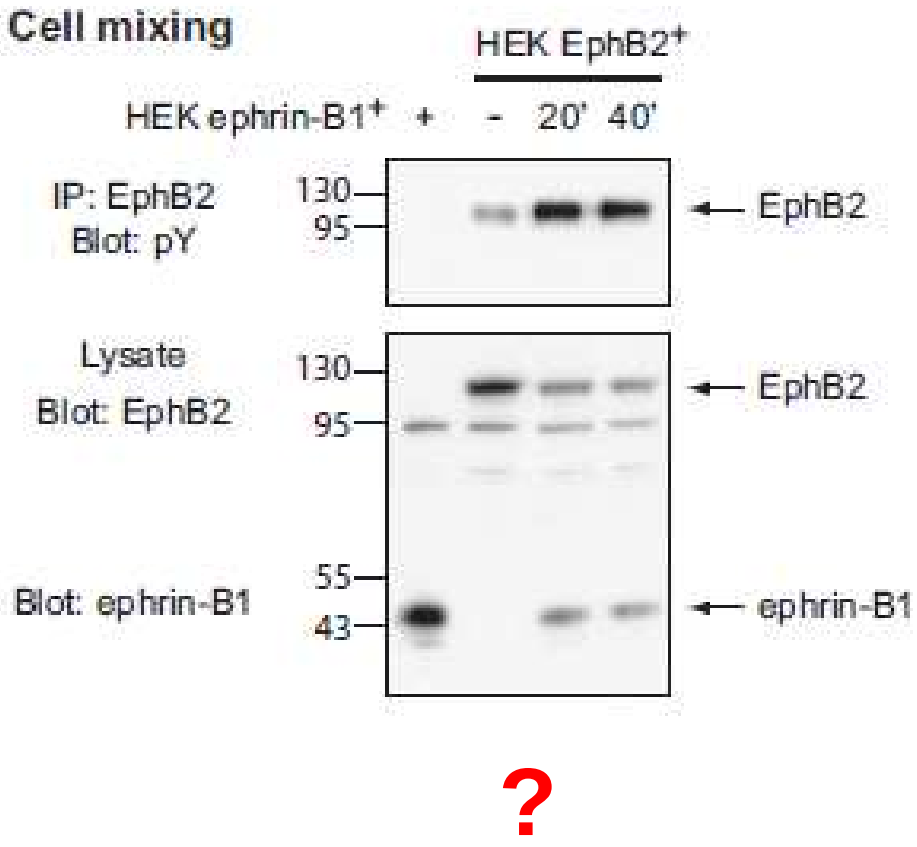
To study bidirectional EphR-ephrin signaling, they used the human embryonic kidney (HEK)293 cell line engineered to express either EphB2 (EphB2+ cells) or ephrin-B1 (ephrin-B1+ cells).





Relative surface levels of these proteins were tested using immunofluorescence (IF) and FACS. Ectodomain-Fc fusions of EphB2 or ephrin-B1 were labeled with Alexa-488 and used to mark surface exposed Eph receptor or ligand in live cells. Analysis by IF or FACS revealed similar surface levels of wild type or mutant forms of EphB2 and ephrin-B1 in all stable cell lines used.

To verify the ability of the EphB2+ and ephrin-B1+ cells to induce a pTyr response, signaling was initiated by cell mixing.
There is extensive tyrosine phosphorylation of both ephrin-B1 and EphB2, demonstrating a functional tyrosine kinase response.



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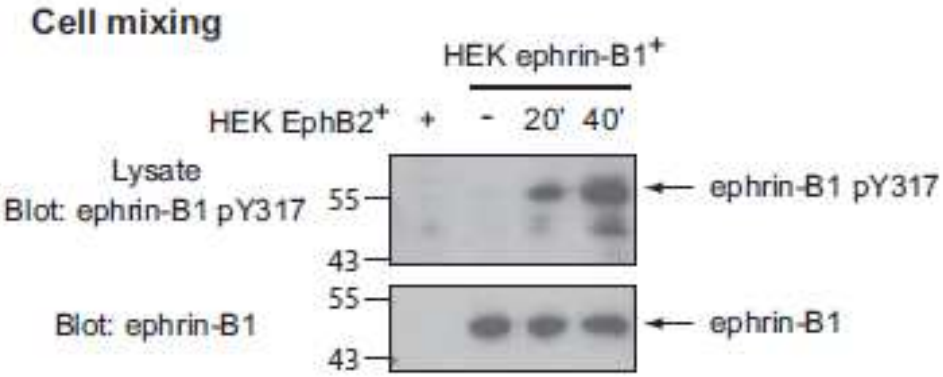

The ability of mixed populations of EphB2+ and ephrin-B1+ cells to sort and organize into distinct multi-cellular structures (colonies) was also confirmed, suggesting that all the relevant molecules required for this process are expressed within these cells.