nature cell biology

ARTICLES

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. Here we show that 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. Here, we show a mechanism for the termination of adhesion and the promotion of cell repulsion after intercellular (trans) interaction between two transmembrane proteins.

Many axon guidance molecules, including ephrins, netrins, semaphorins and slits, elicit repulsive responses when bound to their receptors. When expressed by intermediate targets in the trajectory of a pathfinding axon, these guidance cues steer the growth cone by retraction of individual filopodia[1]. 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[2,3]. 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 bind A-type (ephrinA) and B-type (ephrinB) ephrins, which are anchored to the membrane through a glycosylphosphatidylinositol (GPI)-anchor or a transmembrane domain, respectively. Axon repulsion by GPI-anchored ephrinAs requires proteolytic cleavage of the ephrinA ectodomain by the A-Disintegrin-And-Metalloprotease (ADAM)-10 (ref. 2). 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. Endocytosis of plasma membrane at the growth cone seems to be activated during repulsive guidance as demonstrated for semaphorin3A and ephrinA2 (refs 4,5). Here we show that both EphB2 and ephrinB1 are endocytosed after to binding their respective partners when presented in soluble form and when EphB2-expressing cells encounter ephrinB1-expressing cells in culture. Clusters of EphB2 and ephrinB1 initially form locally at sites of cell–cell contact and are subsequently endocytosed into both cells. Both Ephs and ephrins are able to signal into the cells that express them, resulting in the notion of bi-directional signalling[6]. We demonstrate that endocytosis by EphB2 and ephrinB1 requires signalling through their respective cytoplasmic domains. Our data indicates that endocytosis is both sufficient and necessary for cell detachment, which we define as the termination of adhesion between two cells. Intercellular (trans) interaction of truncated forms of ephrinB and EphB promote strong adhesion. This response is terminated (converted into detachment) when one of the interaction partners is full-length and competent for endocytosis. Growth cone collapse, mediated by ephrinB reverse signalling in primary neurons, occurs after contact with cells expressing EphB2. When EphB2 endocytosis is blocked in these cells, axon detachment is delayed. Our results suggest that bi-directional endocytosis of ephrinB–EphB complexes represents a mechanism to terminate adhesion, thereby allowing contact-mediated repulsion.

RESULTS

Regulated endocytosis of ephrinB and EphB proteins

NIH3T3 cells stably expressing ephrinB1 (3T3 ephrinB1 cells) 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 (see Methods). Staining that

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Published online: 14 September 2003; DOI: 10.1038/ncb1045.

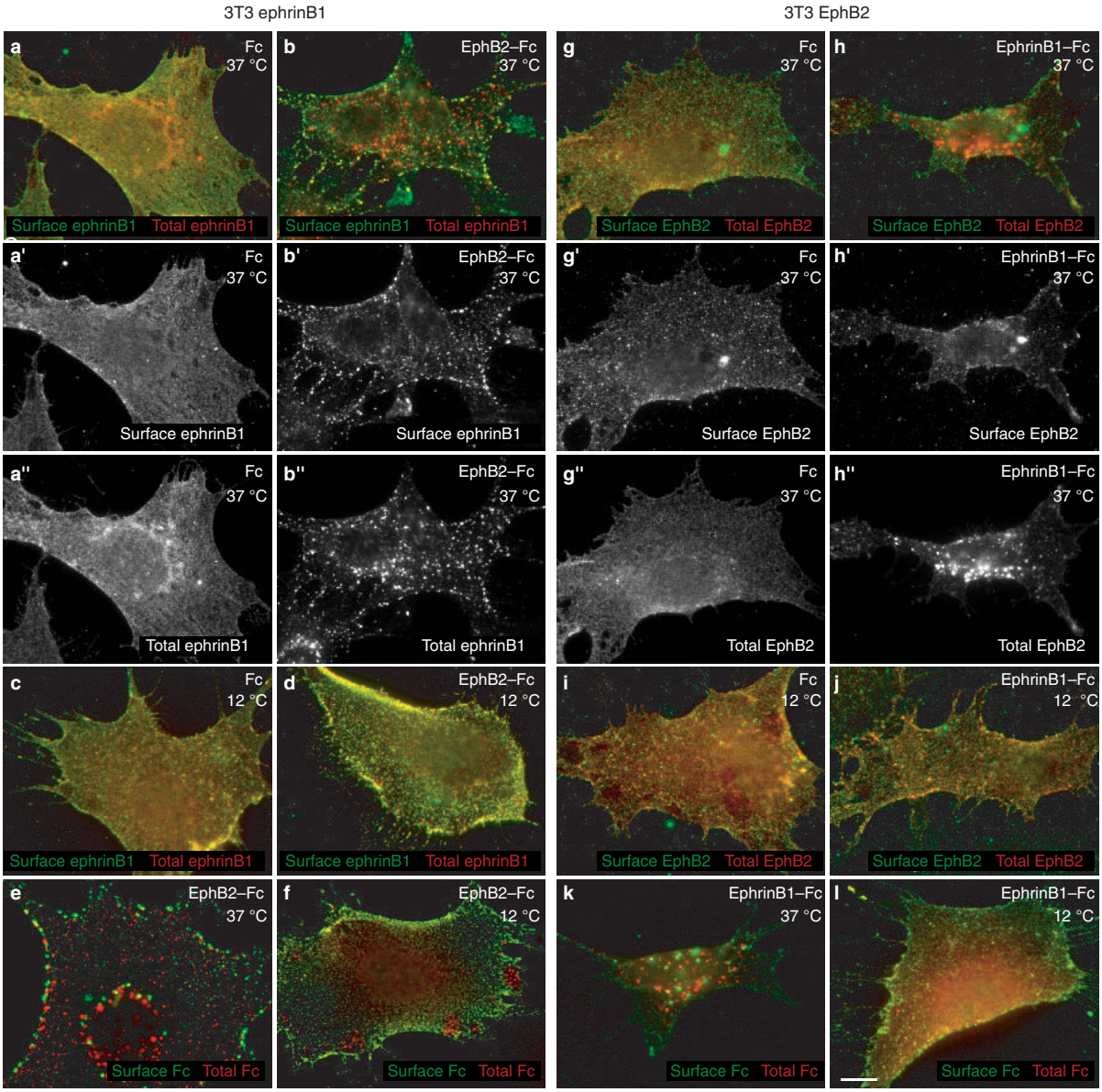

Figure 1 Endocytosis of EphB2–Fc and ephrinB1–Fc. (**a**–**f**) Reverse endocytosis. NIH3T3 cells stably expressing full-length ephrinB1 (3T3 ephrinB1) were stimulated with either control Fc protein (**a**, **a′**, **a″**, **c**), or with EphB2–Fc (**b**, **b′**,**b″**,**d**, **e**, **f**) at the indicated temperatures. After 15 min, cells were fixed and ephrinB1 was immunolabelled on the surface (**a**, **b**, **c**, **d**, **a′** and **b′**, green label). The total pool of ephrinB1 was visualized after cell permeabilization (**a**, **b**, **c**, **d**, **a″** and **b″**, red label). EphB2–Fc, bound to surface ephrinB1, was visualized with a FITC-conjugated antibody directed against human-Fc (**e**, **f**, green label). After permeabilization, EphB2–Fc was stained with a Texas-Red conjugated antibody directed against human Fc (**e**, **f**, red labels). Stimulation results in internalization of clustered receptor ligand complexes. (**g**–**l**) Forward endocytosis. NIH3T3 cells stably expressing full-length EphB2 (3T3 EphB2) were stimulated with either control Fc protein (**g**, **i**, **g′** and **g″**), or with ephrinB1–Fc (**h**, **j**, **k**, **l**, **h′**, **h″**) at the indicated temperatures. After 15 min, cells were fixed and surface EphB2 was immunolabelled (**g**, **h**, **i**, **j**, **g′** and **h′**, green label). The total pool of EphB2 was visualized after cell permeabilization (**g**, **h**, **i**, **j**, **g″** and **h″**, red label). EphrinB1–Fc was stained against human Fc as above (**k**, **l**). Scale bars: 10 μm.

appears exclusively after permeabilization represents the intracellular pool of ephrinB1. Stimulation of 3T3 ephrinB1 cells with EphB2–Fc, but not with Fc, at 37 °C for 10 min resulted in abundant intracellular ephrinB1 clusters (Fig. 1a, b, red staining). Stimulation at 12 °C, a temperature that prevents vesicular trafficking[7], did not block ephrinB1 clustering at the cell surface (although clusters appeared smaller), but blocked internalization (Fig. 1c, d). To determine whether the entire complex of EphB2–Fc bound to ephrinB1 was internalized, we used antibodies against the Fc portion for staining. At 37 °C, numerous Fc-positive intracellular vesicles appeared exclusively after permeabilization, whereas at 12 °C, only few focal internalized clusters were seen close to the cell periphery (Fig. 1e, f). EphB2 receptors also undergo internalization after stimulation with soluble ephrinB1–Fc. Stimulation of NIH3T3 cells stably expressing EphB2 (3T3 EphB2) with soluble ephrinB1–Fc at 37 °C, but not at 12 °C, caused clustering and accumulation of intracellular EphB2 in complex with ephrinB1–Fc (Fig. 1g–l).

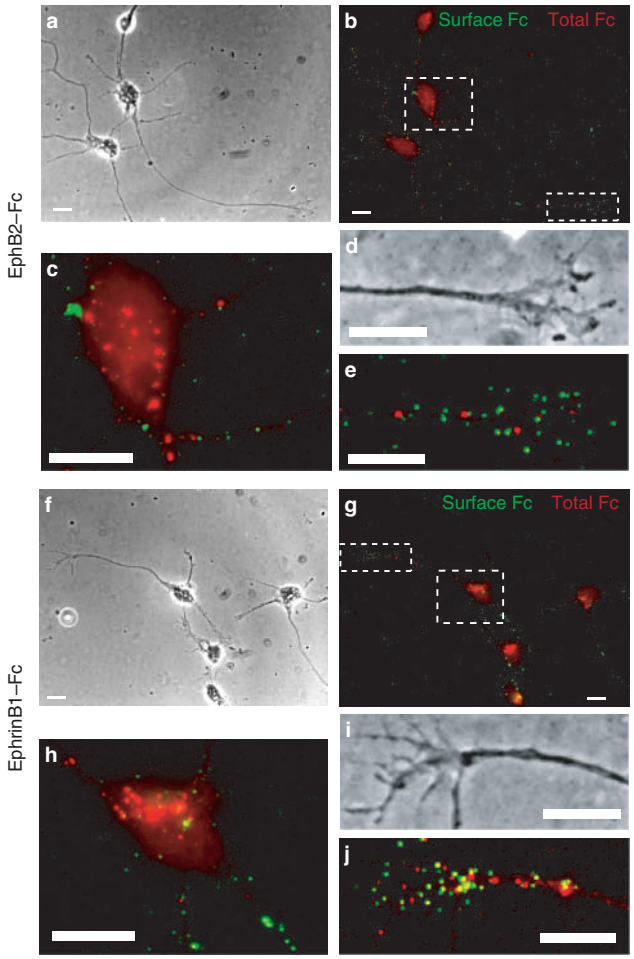

Figure 2 Endocytosis of EphB2–Fc and ephrinB1–Fc in primary telencephalic neurons. Neurons were dissected from E13.5 mouse embryonic forebrain, dissociated and cultured for 2 d *in vitro* to allow differentiation to occur. Cells were stimulated for 15 min with either EphB2–Fc (**a**–**e**) or ephrinB1–Fc (**f**–**j**). Staining for surface and total Fc fusion proteins was done as described (Fig. 1). (**a**, **d**, **f**, **I**) Phase contrast images of stained neurons. Stippled boxes in **b** and **g** indicate those areas, which are zoomed in **c**–**e** and **h**–**j**. Cell bodies are shown in **c** and **h**. Growth cones are shown in both, phase contrast in **d**, **i** and immunofluorescence in **e**, **j**. Scale bars: 10 μm.

Next, we asked whether internalization of ephrins and Ephs occurs in primary cultured neurons. Primary neurons from mice forebrains expressing endogenous EphB receptors and ephrinB ligands[8–13] were dissected from E13.5 embryos and cultured for 2 d *in vitro*. Cells were stimulated with either EphB2–Fc or ephrinB1–Fc at 37 °C for 10 min and stained as above. Stimulation caused the rapid accumulation of internalized ligand–receptor complexes all over the cells (Fig. 2, red staining). Growth cones contain few small, internalized clusters that increase in number and size with proximity to the cell bodies, suggesting retrograde transport (compare Fig. 2e with 2c and Fig. 2j with 2h).

EphrinB and EphB proteins co-cluster at sites of cell-to-cell contact and are endocytosed bi-directionally

We developed a 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 as above. If we use 3T3 EphB2 stimulator cells with 3T3 ephrinB1 recipient cells, we observe rapid and localized co-clustering of ephrinB1 and EphB2 at the site of cell–cell contact. (Fig. 3a, b, g and h, purple staining). Comparison of the surface localization of ephrinB1 (Fig. 3f) with its total distribution (Fig. 3g) indicated that these clusters were partially endocytosed (Fig. 3c, d, red staining). The direction of internalization was in a reverse manner, that is, into the recipient 3T3 ephrinB1 cells. No internalization or clustering is observed when untransfected 3T3 cells are used as stimulator cells (data not shown). The pool of internalized ephrinB1 co-clusters with EphB2 (Fig. 3a, b; compare Fig. 3g with 3h). EphB2 was detected with an antibody against its intracellular domain, indicating that the full-length protein was transcytosed into 3T3 ephrinB1 cells. Next, we 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 (Fig. 3i, j, red staining; compare Fig. 3k with 3l). Immunostaining of total ephrinB1 was carried out with an antibody against its carboxy terminus, indicating that full-length ephrinB1 was transcytosed into the EphB2 cell. Seeding of 3T3 ephrinB1 cells onto wild-type control 3T3 cells did not cause clustering and internalization (Fig. 3m, n; compare Fig. 3o with 3p). These findings using transfected cells indicate localized and bi-directional endocytosis of complexes that comprise of full-length EphB2 and ephrinB1.

EphrinB–EphB uptake and transport by primary neurons

To determine if membrane attached B-ephrins and EphBs are taken up in a regulated fashion by primary neurons, we co-cultured cortical neurons (E14.5 after 1 d *in vitro*) with HeLa cells expressing an EphB2– yellow fluorescent protein (YFP) fusion protein. This modification does not interfere with the normal regulation and kinase signalling of EphB2 (J.K., R.K., unpublished observations). Neuronal growth cones touching HeLa–EphB2–YFP cells were imaged using time-lapse microscopy, with a sequence of 7 images taken for the indicated times (Fig. 4a, b). During the first 10 min, the growth cone collapses (Fig. 4a; see Supplementary Information, Movie 1). During collapse, a small fluorescent cluster of EphB2 can be seen at the tip of a HeLa cell protrusion that becomes extended (Fig. 4b, arrow). In subsequent images, two clusters of EphB2 are taken up by the neuron and are retrogradely transported along the neurite. HeLa cells expressing a membrane-targeted version of YFP (memYFP) did not cause comparable effects (data not shown). 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 look at the reverse situation, we co-cultured a stable 3T3 cell line expressing an amino-terminally HA-tagged version of ephrinB1 with hippocampal neurons incubated for 7 d *in vitro*. Near the contact site of a 3T3 HA-ephrinB1 cell, clusters of ephrinB protein were visualized along a dendrite by immunostaining with an antibody against ephrinB (Fig. 4e). Most of these clusters also stained with an antibody against the HA epitope, suggesting that the neuron has transcytosed ephrinB1 from the 3T3 cell (Fig. 4d–f). In no case did we observe HA-positive clusters in neurons co-cultured with untransfected 3T3 cells (Fig. 4g, h).

EphrinB- and EphB-mediated endocytosis requires cytoplasmic determinants

To gain insights into the molecular mechanisms that regulate endocytosis of the ephrin–Eph complex, we investigated whether the cytoplasmic tails of ephrinB1 and EphB2 are required for bidirectional endocytosis. When both partners are full-length, stimulation of a 3T3 ephrinB1 recipient cell with a 3T3 EphB2 stimulator cell causes the rapid (10 min) internalization of ephrinB1 clusters near the contact site of the two cells into predominantly the 3T3 ephrinB1 recipient cell

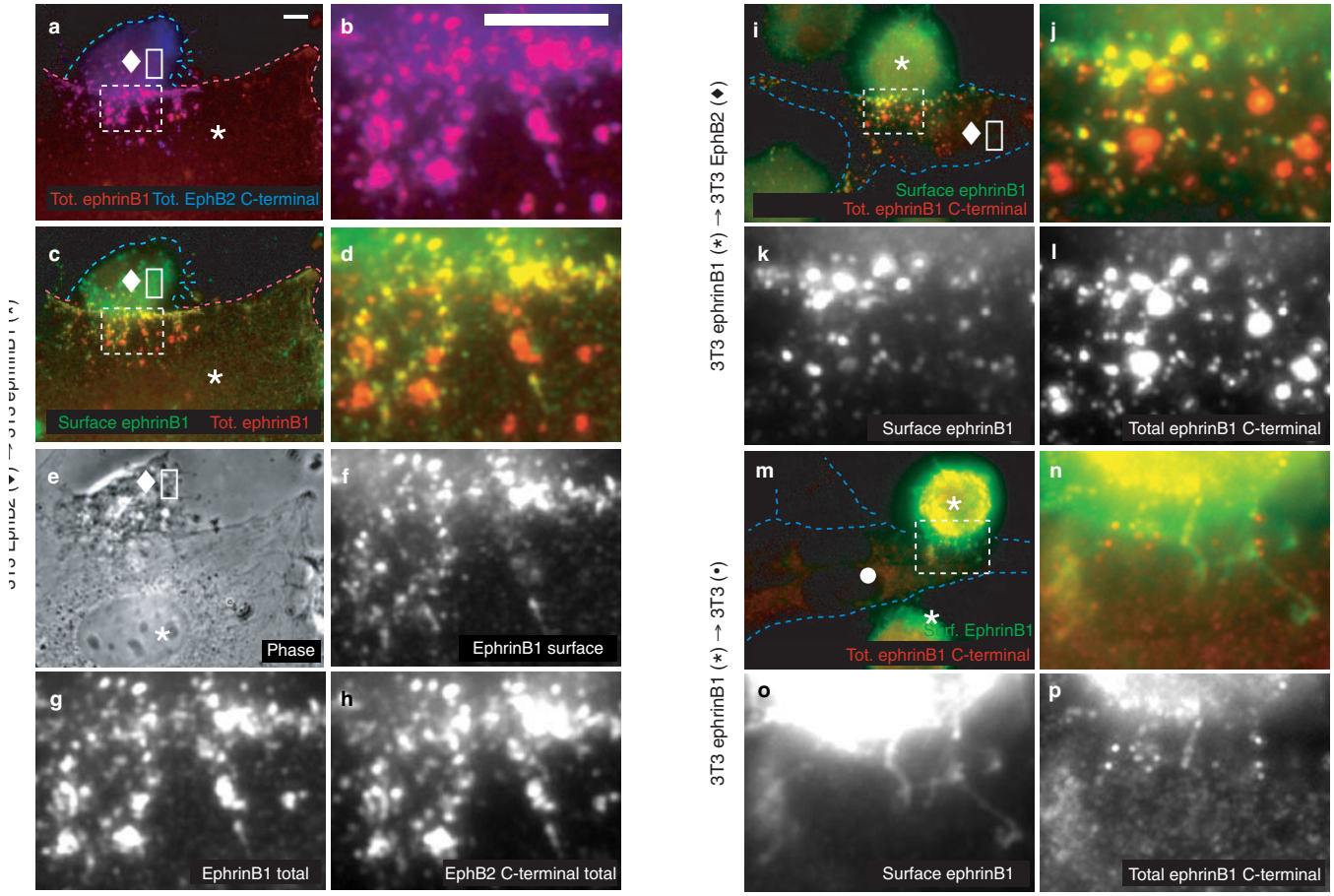

Figure 3 EphB2 and ephrinB1 cluster and endocytose at sites of cell–cell contact. (**a**–**h**) 3T3 ephrinB1 recipient cells (asterisks) were cultured on glass cover slips and then stimulated with 3T3 EphB2 cells (diamonds). After 10 min, cells were fixed and immunostained for ephrinB1 on the surface (**c**–**d**, green label; **f**) and for its total distribution (**a**–**d** red label; **g**). The total distribution of EphB2 was visualized with an antibody directed against the EphB2 cytoplasmic domain (**a**, **b**, blue label; **h**). EphrinB1–EphB2 co-clusters are detected at the interface of the two cells and over the 3T3 ephrinB1 cell (**a**, **b**, purple colour). Surface clusters are seen only at the direct contact sites of the two cells (**c**, **d**, yellow staining). Endocytosed ephrinB1 is seen only in the ephrinB1-expressing cell (**c**, **d**, red staining). **i**–**p**, 3T3 EphB2 (diamond) or untransfected recipient cells (filled circle) were stimulated with 3T3 ephrinB1 cells (asterisk). After 10 min, cells were fixed and immunostained for ephrinB1 on the surface (**i**, **j**, **m** and **n**, green label; **k**, **o**) or for its total distribution using an antibody directed against the C-terminus (**i**, **j**, **m**, **n**, red label; **l**, **p**). Surface clusters are seen exclusively at the interface of the two cells (**i**, **j**, yellow staining). Endocytosed full-length ephrinB1 is seen in the EphB2 recipient cells (**i**, **j**, red staining). In control cultures, almost no clustering is observed with untransfected 3T3 cells (**m**–**p**). Scale bars: 5 μm.

(Fig. 5a, b, red staining). After 60 min, internalized ephrinB1 is seen mostly in the 3T3 EphB2 cell (Fig. 5c, d).However, if the recipient cell expresses truncated ephrinB1–ΔC, strong ephrinB1–ΔC clustering is induced by the 3T3 EphB2 stimulator cell, but no internalization into the recipient cell occurs (Fig. 5e, f, yellow staining). Instead, at 60 min the adhered 3T3 EphB2 cell has internalized ephrinB1–ΔC (Fig. 5g, h). Endocytosis was not inhibited by the blocking of either Tyr phosphorylation of ephrinB1 or its interaction with PDZ-domain proteins (data not shown).

In the opposite situation, where the recipient cell expresses a truncated (EphB2–ΔC) or kinase deficient (EphB2-KD) EphB2 receptor, transcytosis of ephrinB1 clusters into the 3T3 EphB2–ΔC or EphB2–KD cells is not observed at 10 min (Fig. 5i, j, m and n) and very rare at 60 min (Fig. 5k, l,o and p). Instead, at 60 min, internalization of ephrinB1 shifted towards the 3T3 ephrinB1 stimulator cell (Fig. 5k, l, o and p, large red clusters). These results indicate that the cytoplasmic tails of ephrinB1 and EphB2 are required for endocytosis. C-terminal truncation of one protein does not impair transcytosis into the neighbouring cell. EphB2 receptor-mediated forward endocytosis requires the kinase activity of the receptor.

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

To determine if bi-directional endocytosis affects repulsive cell migration, we developed an *in vitro* assay in which cells expressing fluorescently tagged EphB2 receptor (EphB2–YFP) were co-cultured with cells expressing fluorescently tagged ephrinB1 (cyan fluorescent protein (CFP)–ephrinB1). HeLa cells were chosen because they express low levels of endogenous ephrinB and EphB proteins (similar to NIH3T3 cells; see Supplementary Information Figs S1 and S2) 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 (11 out of 13) when a ruffling lamellipodium of an EphB2 cell collides with an ephrinB1 cell, strong co-clustering of receptor with ligand occurs within 1 min. The initial clusters always appear in filopodia-like protrusions. During the retraction of EphB2 positive lamellipodia,

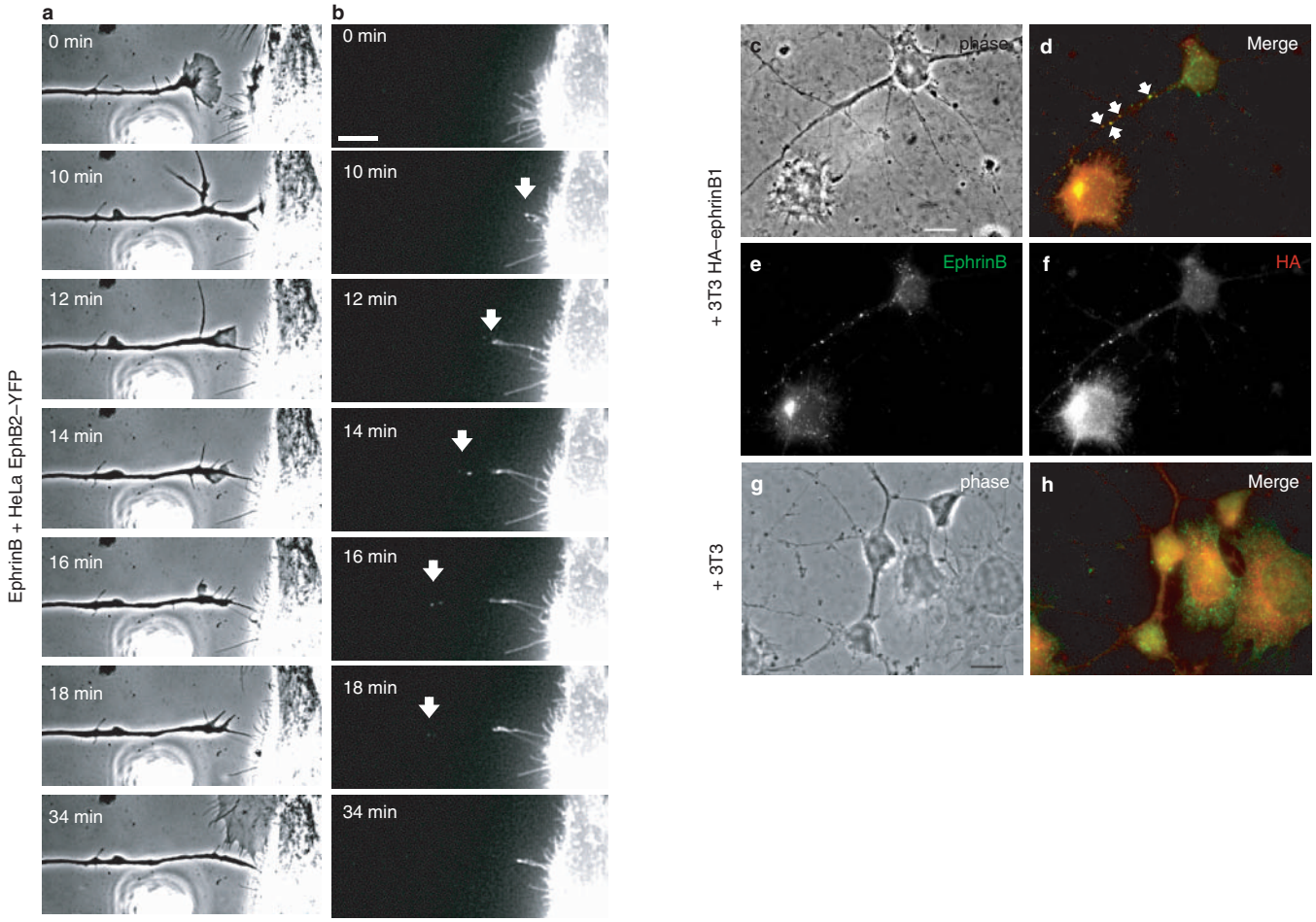

Figure 4 EphrinB1 and EphB2 uptake and transport by primary neurons. (**a**, **b**) Forebrain neurons from E14.5 mouse embryos (cultured for 1 d *in vitro*) were co-cultured with HeLa cells transiently expressing EphB2–YFP. Growth cones were imaged by time-lapse microscopy at 1 frame per min. The presented 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 also Supplementary Information, Movie 1. (**c**–**h**), Hippocampal neurons (E17.5, cultured for 7 d *in vitro*) were cultured on glass cover slips and allowed to mature. Neurons were either stimulated with 3T3 HA–ephrinB1 cells (**c**–**f**) or with 3T3 cells (**g**, **h**). After 2 h, cells were fixed and stained for ephrinB (**e**; **d**, **h**, green label) and for the HA epitope tag (**f**; **d**, **h**, red label). Clusters of ephrinB protein co-stain for HA indicating uptake of HA–ephrinB1 into the neuron. No clusters are seen when control 3T3 cells were co-cultured with untransfected 3T3 cells. Scale bars: 10 μm.

receptor–ligand complexes endocytose bi-directionally (Fig. 6a, see Supplementary Information, Movie 2). Contacts of EphB2- or ephrinB1-transfected cells with untransfected cells in the same culture do not result in clustering nor cell retraction (Fig. 6a, asterisk; see Supplementary Information Movie 2).

When ephrinB1 endocytosis was blocked by a C-terminal truncation, markedly different cell behaviour was observed. Rapid co-clustering with EphB2 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 (Fig. 6b, left, large yellow clusters; see Supplementary Information, Movie 3; compare Fig. 6f with 6g). The EphB2 cell engulfs the clusters vigorously, retracts strongly, and in most cases (8 out of 14) even rounds up, a behaviour rarely (3 out of 11) observed with wild-type ephrinB1 (Fig. 6b, e; see Supplementary Information, Movie 3). Therefore, a mutation that blocks ephrinB1 endocytosis results in a stronger EphB2 cell retraction response. When both ephrinB1 and EphB2 are truncated at the C-terminal, the cells strongly adhere to each other and large receptor-and ligand-bearing fascicles are formed at the contact zone (Fig. 6c). These findings indicate that ephrinB and EphB proteins can function as adhesion molecules if endocytosis and other signalling events are blocked.

Next, we determined how cells reacted to uni-directional ephrinB reverse signalling. We investigated cell behaviour in the presence of truncated EphB2 and wild type ephrinB1. As expected, ephrinB1 cells strongly endocytose receptor–ligand clusters, whereas EphB2–ΔC cells fail to endocytose these complexes (Fig. 6d; see Supplementary Information, Movie 4). However, the cells neither retract nor adhere to each other. Cell behaviour is indistinguishable from non-transfected cells (Fig. 6d, phase images; see Supplementary Information, Movie 4). EphrinB1 reverse endocytosis is therefore sufficient to terminate adhesion and to cause cell detachment.

EphB2 forward endocytosis is required for efficient axon detachment

We next asked if endocytosis may also be required for ephrinB-mediated growth cone collapse and retraction. Both cells expressing full-

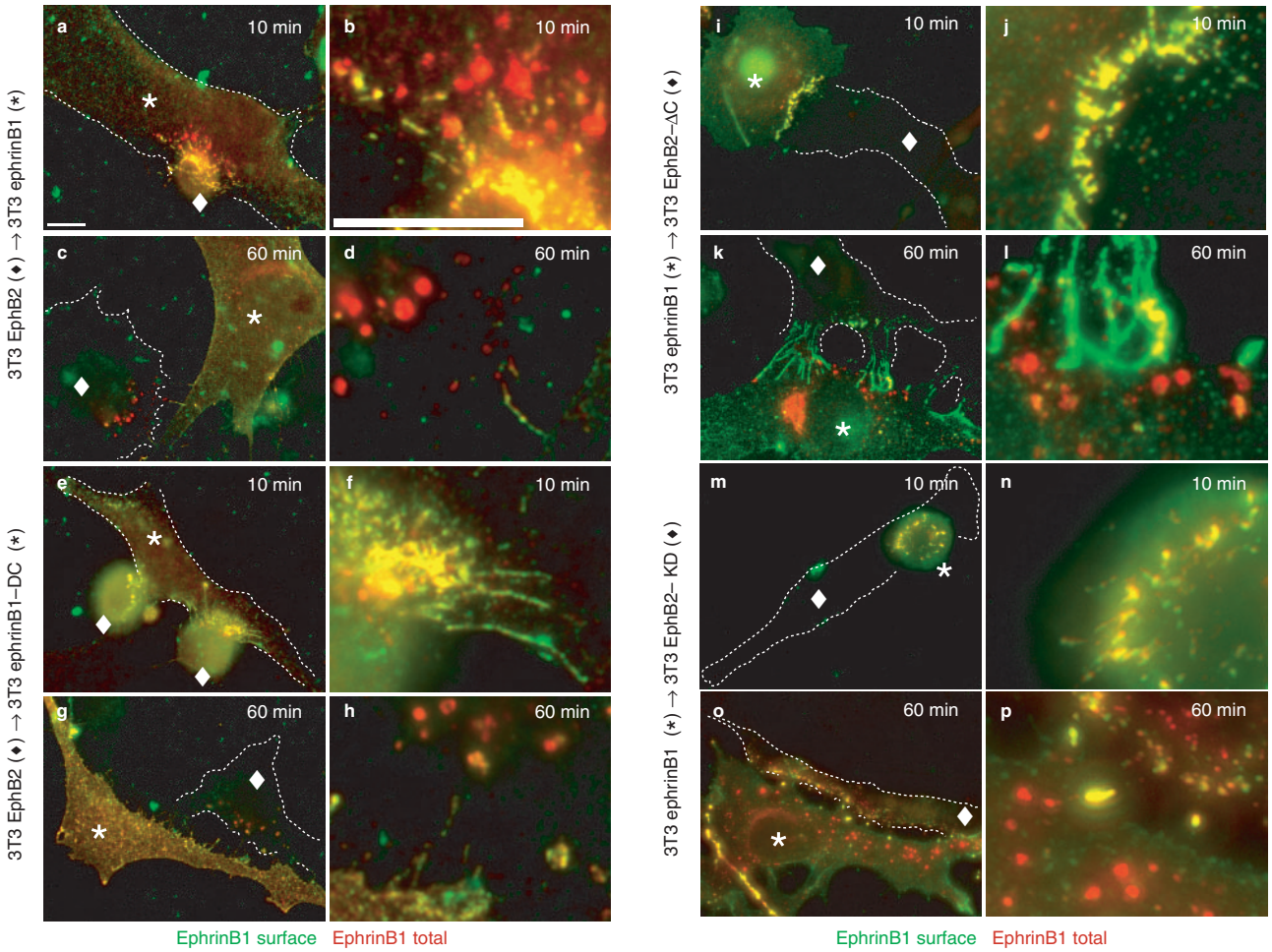

Figure 5 Endocytosis requires cytoplasmic determinants. (**a**–**d**) 3T3 ephrinB1 recipient cells (asterisks) were stimulated with 3T3 EphB2 cells (diamonds). Cells were fixed 10 min (**a**–**b**) or 60 min (**c**–**d**) after seeding and stained for surface (green) and total ephrinB1 (red). After 10 min of stimulation, ephrinB1 clusters are predominantly detected in the ephrinB1 cells. After 60 min, ephrinB1 clusters are seen in 3T3 EphB2 stimulator cells. (**e**–**h**) 3T3 ephrinB1-ΔC recipient cells (asterisk) were stimulated with 3T3 EphB2 cells (diamonds), for 10 and 60 min. At 10 min, ephrinB1 clusters appear on the surface of the contact site between the cells. No endocytosis into the 3T3 ephrinB1-ΔC recipient cell is observed. At 60 min, internal ephrinB1-ΔC clusters are seen in 3T3 EphB2 stimulator cells (**g**, **h**, red labels). (**i**–**l**) 3T3 EphB2-ΔC recipient cells (diamonds) were stimulated with 3T3 ephrinB1 cells (asterisks) for 10 min or 60 min . Intense staining of surface ephrinB1 is seen at the contact sites of both cells. Endocytosis into the 3T3 EphB2-ΔC recipient cell is largely reduced after 10 min as well as after 60 min co-culture. After 60 min ephrinB1 clusters, unlike in **c**–**d**, are now seen in the 3T3 ephrinB1 stimulator cell (**k**, **l**, red label). (**m**–**p**) Recipient cells (diamonds) expressing a kinase inactive mutant EphB2 receptor (3T3 EphB2–KD) were stimulated with 3T3 ephrinB1 cells (asterisks) for 10 min (**m**, **n**) or 60 min (**o**, **p**). Endocytosis into 3T3 EphB2-KD cells is largely reduced. After 60 min co-culture, large endocytosed clusters can be seen in the 3T3 ephrinB1 cells. Scale bars: 10 μm.

length EphB2–YFP and truncated EphB2–YFP–ΔC cause collapse of neuronal growth cones within 5–10 min after contact (Fig. 7a, b). Therefore, uni-directional ephrinB reverse endocytosis is sufficient to allow collapse of the growth cone. In most cases, the contacting protrusion (carrying small EphB2 clusters) is pulled out by the retracting growth cone (Fig. 7a, b). Using time-lapse imaging, we 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 (Fig. 7c; 15.5 versus 8 μm, respectively; $p < 0.002$; two-tailed t-test). The time it took for protrusions to rupture was significantly longer in EphB2–YFP–ΔC expressing cells ($p < 0.03$) (Fig. 7d). The speed of extension of protrusions was not different between the groups (Fig. 7e). We conclude that axon detachment from EphB2–YFP–ΔC-expressing cells is delayed compared with EphB2–YFP-expressing cells. Our results suggest that forward endocytosis by EphB2 is required for efficient ephrinB-mediated axon detachment of forebrain neurons.

DISCUSSION

Endocytosis of protein complexes involving the intercellular (trans) interaction of two transmembrane proteins is unusual[14] 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[15]. 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[16]. Notch receptor binding to its membrane-anchored ligand, Delta, triggers proteolytic shedding of the Notch ectodomain and endocytosis of

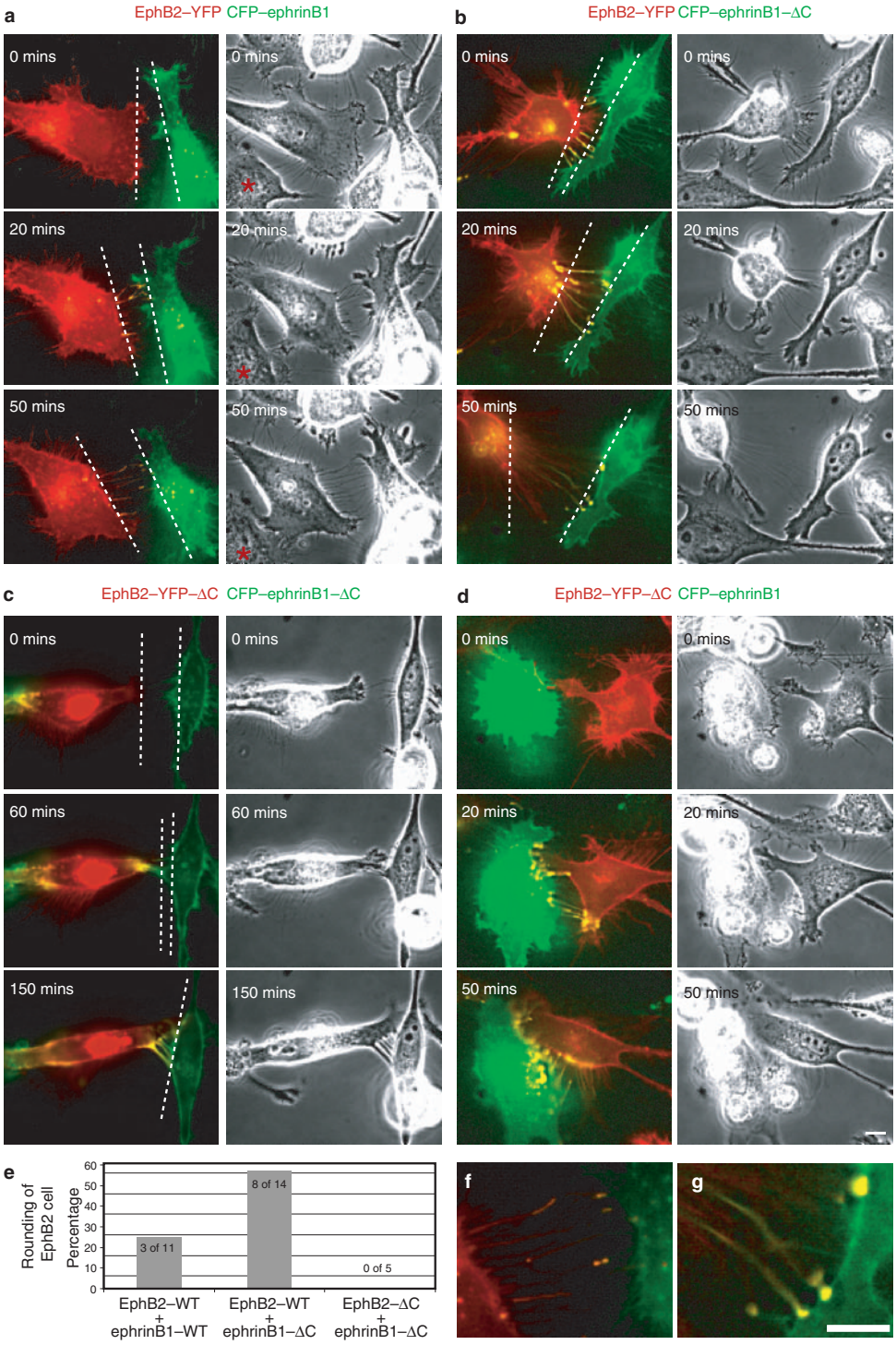

Figure 6 Bi-directional endocytosis regulates the cell repulsion response and cell detachment. (**a**) HeLa cells were transiently transfected with full-length EphB2–YFP and full-length CFP–ephrinB1 and then co-cultured before time-lapse imaging (see also Supplementary Information, Movie 2). Left, selected fluorescence images with EphB2–YFP in red and CFP–ephrinB1 in green (corresponding times are indicated). 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). (**b**) As in **a**, except that C-terminally truncated CFP–ephrinB1ΔC was used (See also Supplementary Information, Movie 3). 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. (**c**) As in **a**, except that C-terminally truncated CFP–ephrinB1ΔC and EphB2–YFP-ΔC were used. Cells strongly adhere to each other forming large fascicles filled with EphB2–YFP complexes. (**d**) As in **a**, except that wild-type CFP–ephrinB1 and EphB2–YFP-ΔC were used (see also Supplementary Information, Movie 4). Strong reverse endocytosis of ephrinB–EphB complexes, but otherwise normal cell behaviour similar to un-transfected cells is observed. (**e**) Quantification of cell rounding. (**f**, **g**) Higher power images of 50 min from **a** and **b**. Note the accumulation of brightly fluorescing complexes on the surface of CFP–ephrinB1ΔC-expressing cells (**g**).

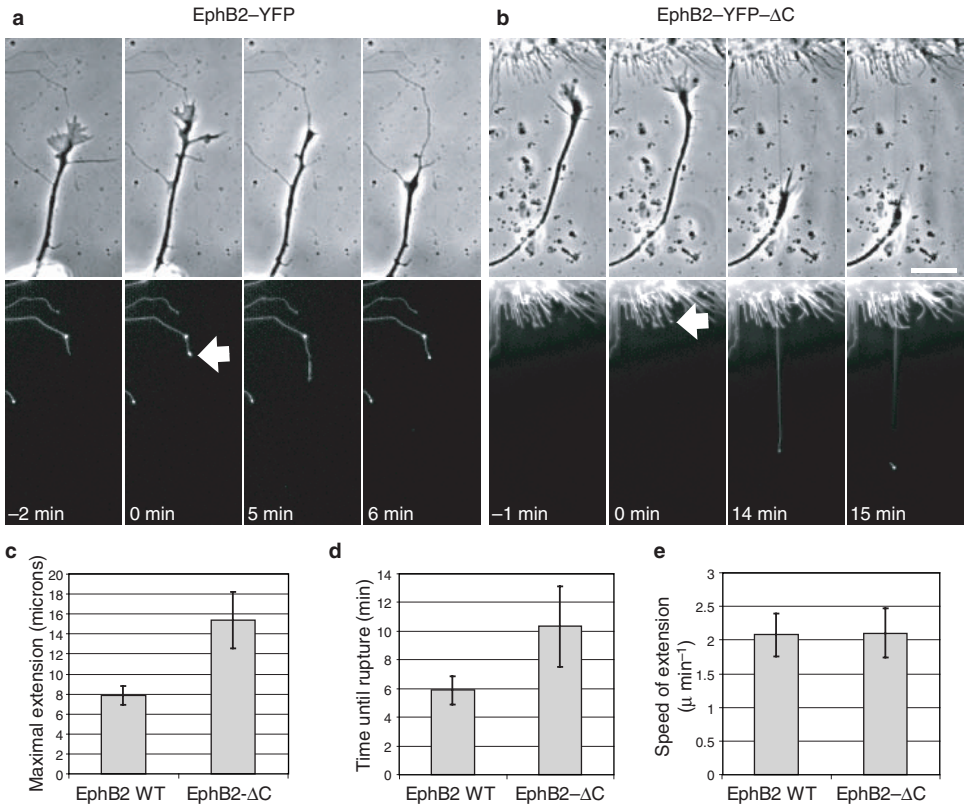

Figure 7 EphB2 C-terminal truncation impairs growth cone detachment. Primary forebrain neurons were co-cultured with HeLa cells expressing either EphB2–YFP (**a**) or EphB2–YFP-ΔC (**b**). After 1 d *in vitro*, growth cones were imaged by time-lapse microscopy at 1 frame per min. (**a**) Growth cone (left, 2 min before contact) collapses after contact with a protrusion of a HeLa cell expressing EphB2–YFP (fluorescent cluster indicated by arrow). Five minutes after contact the collapsing growth cone pulls the HeLa protrusion to its maximal length. After 1 min the protrusion detaches (6 min). (**b**) Growth cone (left, one minute before contact) collapses after contact with a protrusion of a HeLa cell expressing EphB2–YFP-ΔC (fluorescent cluster indicated by arrow). One protrusion is pulled out to its maximal length by the retracting growth cone after 14 min, before it ruptures after 15 min. (**c**) Quantification of average maximal extension of protrusion. (**d**) Average time until rupture. (**e**) Average speed of filopodia extension in the two groups. Scale bar: 10 μm.

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[17]. Therefore, in this case endocytosis is bi-directional, but involves proteolytic cleavage of one of the proteins. 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. NIH3T3 and HeLa cells ectopically expressing EphB and ephrinB are capable of mediating ephrinB reverse and EphB forward endocytosis, which was further confirmed by time-lapse imaging of ephrinB and EphB expressing HeLa cells (Fig. 6; see Supplementary Information, Movie 2). Swiss 3T3 and primary human endothelial (HUVECs) cells primarily undergo forward endocytosis (see accompanying paper by Marston *et al.*[27]). However, in that case the experimental setup was different. Endocytosis was studied several hours after microinjection of cells with expression constructs. Prolonged cell–cell contact may favour forward endocytosis by receptor-expressing cells, as was also observed in our study (Fig. 5). Very immature primary neurons from mouse forebrain demonstrate little forward, but rather pronounced ephrinB reverse endocytosis (Fig. 4; data not shown). Similar results were obtained with primary neurons from *Xenopus laevis* retina when stimulated with soluble Fc fusion proteins[18].

One of our experimental setups involved the stimulation with cells in suspension. In this case, endocytosis was predominant in the pre-plated 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[19]. 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.

Our results and those of Marston *et al.*[27] have demonstrated that the endocytosed complex contains full-length proteins indicating that one of the partners had to be transcytosed from one cell to its neighbour. In accordance with Marston *et al.*[27], we did not observe colocalization of ephrinB–EphB clusters with known markers of endocytic pathways including clathrin and caveolin (data not shown)[14]. We speculate that the underlying mechanism of EphB2–YFP endocytosis may resemble phagocytosis[20] or macropinocytosis[21].

EphrinA ligands are recognized and cleaved by ADAM-type metalloproteinases after binding of ephrinA by clustered EphA receptors[2]. We found that metalloproteinase cleavage of endogenous ephrinB ligands

is induced by phorbol ester treatment (G.A. Wilkinson, R.K., unpublished observations), a stimulus that promotes ectodomain shedding of a variety of transmembrane proteins. Instead, ephrinB cleavage induced by EphB receptor engagement was very inefficient and sometimes not detectable. The demonstration of full-length proteins in the internalized ephrinB–EphB complexes suggests that a major fraction of these proteins stays intact during endocytosis. Surprisingly, preliminary data on ephrinAs suggest that they also trigger endocytosis when engaged by EphA4 receptors on neighbouring cells, despite the fact they lack a cytoplasmic tail (see Supplementary Information, Fig. S3). It is possible that proteolysis and endocytosis have parallel functions in Eph–ephrin-mediated cell–cell communication.

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 we observe a gain-of-function phenotype, that is, enhancement of repulsion by EphB receptor forward signalling. It is likely that persistent ephrinB clusters on the surface of the ephrinB-positive cell result in enhanced activation of EphB2 receptors on the neighbouring cell. EphB2 receptors may continue to signal after endocytosis, as was demonstrated for internalized epidermal growth factor (EGF) and nerve growth factor (NGF) receptors[22,23]. Consistent with this idea, we observed that internal clusters containing EphB2 stain positive for phosphotyrosine (data not shown; see Marston *et al.*[27]). Interfering with repulsion and bi-directional endocytosis by truncation of the C-terminals on both sites results in a remarkable abnormal adhesion between the cells (Fig. 6c) that could be explained by the high affinity between receptor and ligand. Uni-directional reverse endocytosis alone efficiently switches adhesion to detachment by the removal of Eph–ephrin complexes from the surface (Fig. 6d; see Supplementary Information, Movie 4). However, in the case of reverse-signalling-mediated growth cone collapse in cortical neurons, detachment occurs in a delayed fashion when endocytosis is uni-directional and thereby less efficient.

What are the relative contributions to the retraction response of endocytosis and signalling to the cytoskeleton? Endocytosis alone cannot be sufficient to mediate cellular repulsion, as HeLa-ephrinB1 cells endocytose efficiently but do not retract after contact with EphB2 cells. Detailed knowledge about the molecular mechanisms of how Ephs and ephrins regulate their internalization will provide tools to specifically interfere with endocytosis while leaving other signalling mechanisms intact. In conclusion, we have demonstrated that cell–cell communication mediated by Eph–ephrins can result in bi-directional endocytosis of the Eph–ephrin ligand–receptor complex. In addition to previously described ligand ectodomain shedding[2] we propose endocytosis as a mechanism for cell and axon detachment during repulsive guidance. Both mechanisms may be relevant for other repulsive guidance systems. □

METHODS

Expression constructs. Expression constructs encoding full-length and truncated Flag–EphB2 and for EphA4 are described[16,13]. Kinase deficient mouse EphB2 was generated by site directed mutagenesis leading to exchange of Lys 660 to Arg. To generate ECFP–HA–ephrinB1 (pJK30) or ECFP–HA–ephrinB1ΔC (pJK32), the ECFP coding region of pECFP-N1 (Clontech, Palo Alto, CA) was cloned into pJP136 or pJP139 (ref. 24) using SalI and BsrGI restriction sites. To generate EphB2–YFP (pJK12), EYFP (Clontech) was cloned in frame into the juxtamembrane region of Flag–EphB2. The flanking amino acid sequence (single letter code) of the EphB2 juxtamembrane region is …GFERADSE–(EYFP)–YTDKLQHY…. To generate EphB2–YFP-ΔC (pJK18) a restriction site was inserted by mutagenesis into Flag–EphB2. The remaining amino acid sequence of the EphB2 cytoplasmic domain is ….GFERADSE followed by the EYFP sequence.

Tissue culture. NIH3T3 cells stably expressing full-length (MZ2-5), C-terminally truncated (MZ1-6), N-terminally HA-tagged ephrinB1 or full length EphB2 (K-2-6-9), were generated following standard procedures. NIH3T3 cells were transiently transfected with Flag–EphB2–ΔC and Flag–EphB2–KD using lipofectamine plus reagent (Invitrogen, Carlsbad, CA). HeLa cells were transfected after a calcium phosphate-DNA precipitation procedure[25]. Stimulations were done with unclustered soluble Fc-fusion proteins as described[8]. For cell–cell stimulation experiments, recipient cells were seeded at low density (12,500 cells per cm^2) on glass cover slips and left to attach overnight. Stimulator cells were grown to 60% confluency, washed once with D-PBS buffer without $Ca^{2+}Mg^{2+}$ (Dulbecco's PBS, Invitrogen) containing 2 mM EDTA and then left for 5 min in the same solution. Cells were gently rinsed from the dishes, washed twice in D-PBS containing $Ca^{2+}Mg^{2+}$ and seeded onto recipient cells (25,000 cells per cm^2). For co-cultures with primary neurons, hippocampi were dissected from E17.5 mouse embryos as described[26]. NIH3T3 cells were seeded at a density of 2,000 cells per cm^2 as above. For time-lapse experiments of neurons with HeLa cells, we used culture dishes with a glass bottom (Mattek Corporation, Ashland, MA; P35G-0-14-C) coated with 1 mg ml^{-1} poly-D-lysine and 10 μg ml^{-1} mouse laminin (Invitrogen). Transfected HeLa cells were seeded in growth medium at a density of 25,000–50,000 cells per dish and left to attach overnight. Cells were then washed three times with D-PBS containing $Ca^{2+}Mg^{2+}$ and left in neurobasal medium containing B27 supplement (Invitrogen). Neurons were obtained from telencephali of E14.5 mouse embryos, as described[26] and plated at a density of 500,000 cells per dish. After 4–36 h *in vitro*, time-lapse movies were taken. For co-cultures of transfected HeLa cells, EphB2–YFP cells were seeded on glass bottom dishes coated with laminin at a density of 25,000–50,000 cells per dish 1 d before imaging. Before imaging (30 min) CFP–ephrinB1 cells were seeded at a density of 50,000–100,000 per dish and cell migration was stimulated with 20 ng ml^{-1} hepatocyte growth factor.

Immunocytochemistry. Cell were fixed with 4% paraformaldehyde, 4% sucrose in D-PBS for 15 min at room temperature, rinsed once with D-PBS, then incubated with 50 mM ammonium chloride in D-PBS for 10 min at room temperature and rinsed again before blocking for 30 min at room temperature with 2% bovine serum albumin, 4% donkey and/or 4% goat serum (Jackson Immuno Research, West Grove, PA). Primary antibodies for surface labelling were applied for 60 min at room temperature. Cells were washed three times for 5 min with D-PBS and then permeabilized for 5 min with ice cold 0.1% Triton X-100 in D-PBS at 4 °C. After washing, blocking was done for 30 min at room temperature or overnight at 4 °C. Primary antibodies for total stainings were applied for 60 min at room temperature. After washing, secondary antibodies were incubated for 30 min at room temperature. After washing, samples were mounted using the ProLong antifade kit (Molecular Probes, Eugene, OR). Images were acquired using an epifluorescence microscope (Zeiss, Gõttingen, Germany) equipped with a digital camera (SpotRT; Diagnostic Instruments, Sterling Heights, MI). All fluorescence images of fixed cells (except in Fig. 1) are maximum projections of 2–10 focal planes generated by the MetaMorph software (Visitron, München, Germany).

Antibodies. Primary antibodies. HA-ephrinB1: 8 μg ml^{-1} mouse anti-HA (12CA5, Roche, Basel, Switzerland) (surface ephrinB1 in Fig. 1a–d, e; Fig. 3i–k, m–o, Fig. 5i–p; total ephrinB1 in Fig. 3a–d, g; Fig. 4d, f, h; Fig. 5a–h). Surface ephrinB1: 0.4 μg ml^{-1} goat anti-ephrinB1 (AF473; R&D, Minneapolis, MN) (Fig. 3c, d, f; Fig. 5a–h). Total ephrinB1: 1 μg ml^{-1} rabbit anti-ephrinB1 (C-18, Santa Cruz Biotechnologies, Santa Cruz, CA) (Fig. 1a–d, ; Fig. 3i, j, l–n, p; Fig. 4d, e, h; Fig. 5i–p). Surface EphB2: 4 μg ml^{-1} goat anti-EphB2 (AF467, R&D) (Fig. 1g–j). Total EphB2: 1:2000 rabbit anti-(SAM)EphB2 (ref. 9; Fig. 1g–j and Fig. 3a, b and h). Surface and total anti-Fc: 7.5 μg ml^{-1} Fluorescein isothiocyanate (FITC)-conjugated or Texas-red-conjugated, goat anti-human Fc_γ (Jackson ImmunoResearch) (Fig. 1e–f, k–l; Fig. 2). Secondary Antibodies. Donkey anti-mouse-Cy3, -Cy5; anti-rabbit-Cy3, -Cy5 (Jackson ImmunoResearch): 3.75 μg ml^{-1}; goat anti-mouse–Alexa488, donkey anti-rabbit–Alexa488, donkey anti-goat–Alexa488 (Molecular Probes): 10 μg ml^{-1}. Colour codes in Figs 1 and 3–5 are standardized for surface and total labelling

and do not correspond to the dye conjugations of the secondary antibodies.

Time-lapse recordings. Live cell imaging was performed at a Zeiss Axiovert 200M microscope equipped with a temperature-controlled carbon dioxide-incubation chamber (EMBL, Heidelberg, Germany) set to 37 °C, 65% humidity, 5% carbon dioxide (neurons) or 7% carbon dioxide (HeLa) and an AttoArc2 System (Zeiss) set to 20%. Images were acquired with a Coolsnap-fx camera (Photometrics, Roper Scientific, Tucson, AZ). Sequential couples of phase contrast and YFP images were acquired at a rate of 1 frame per min. In the case of HeLa cell experiments one CFP image was also taken every 10 min.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank A. Weiss, T. Schwickert, L. Peters and C. König for experimental help. We also thank M. Osterfield, J.G. Flanagan, M. Dalva and M.E. Greenberg for providing reagents, and I.C. Grunwald, G.A. Wilkinson, F. Helmbacher and F. Bradke for critically reading the manuscript. We thank M. Zerial for helpful discussions. This work was funded in part by grants from the Deutsche Forschungsgemeinschaft.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Received 4 April 2003; accepted 19 August 2003
Published online at http://www.nature.com/naturecellbiology

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