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EphA2 signalling following endocytosis: role of Tiam1

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Abstract

Eph receptors and their membrane-bound ligands, the ephrins, represent a complex subfamily of receptor tyrosine kinases (RTKs). Eph/ephrin binding can lead to various and opposite cellular behaviours such as adhesion versus repulsion, or cell migration versus cell adhesion. Recently, Eph endocytosis has been identified as one of the critical steps responsible for such diversity. Eph receptors, as many RTKs, are rapidly endocytosed following ligand-mediated activation and traffic through endocytic compartments prior to degradation. However, it is becoming obvious that endocytosis controls signalling in many different manners. Here we showed that activated EphA2 are degraded in the lysosomes and that about 35% of internalized receptors are recycled back to the plasma membrane. Our study is also the first to demonstrate that EphA2 retains the capacity to signal in endosomes. In particular, activated EphA2 interacted with the Rho family GEF Tiam1 in endosomes. This association led to Tiam1 activation, which in turn increased Rac1 activity and facilitated Eph/ephrin endocytosis. Disrupting Tiam1 function with RNA interference impaired both ephrinA1-dependent Rac1 activation and ephrinA1-induced EphA2 endocytosis. In summary, our findings shed new light on the regulation of EphA2 endocytosis, intracellular trafficking and signal termination and establish Tiam1 as an important modulator of EphA2 signalling.

Keywords

EphA2; endocytosis; recycling; signalling endosomes; Tiam1

Eph receptors and their membrane-bound ligands, the ephrins, represent a large and complex subfamily of receptor tyrosine kinases (RTKs). Members of the Eph and ephrin family are integral players of signalling pathways involved during development, axon guidance, cell migration and diseases such as cancer (1, 2). A unique property of this family is that ligand binding generates bi-directional signalling (3).

Due to its complexity of signalling, Eph and ephrin family rely on finely tuned signalling network. Endocytosis has emerged as an essential process in regulating Eph signalling pathways. It plays critical roles in signalling initiation, transduction and termination. Together with the proteolytic cleavage of Eph/ephrinA complexes (4, 5), *trans*-endocytosis removes Eph/ephrin complexes from the cell surface and allows the switch between cell-adhesion and cell-repulsion (6, 7). Upon ligand binding, most of RTKs are rapidly

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internalized and trafficked through endosomes where they can be recycled back to the plasma membrane or degraded. Whether RTKs are degraded or recycled to the cell surface is crucial for the duration and intensity of their signal. With regards to Eph receptors, data about their endocytic sorting and degradation remain very scarce. Concerning Eph/ephrin intracellular trafficking and recycling, it has been reported that several Eph/ephrin complexes traffic through early endosomes (8, 9), but to the best of our knowledge, nothing to date has been investigated about Eph recycling.

A second, central aspect of RTKs endocytosis is the duration of their signals once internalized. Many evidences reveal that RTKs can signal from the endosomes prior to their degradation (10). As an example, active endosomal EGFR and PDGFR have been shown to recruit signalling molecules and determine physiological responses such as cell proliferation and migration (11, 12). Do Ephs and ephrins continue to signal into endosomes and if so, is the signal different from the one emanating from the cell surface? Up to now, only few studies suggest that EphB receptors may continue to signal into endosomes by demonstrating that they remain phosphorylated in the intracellular vesicles (13, 14).

Some Rho family GTPases are known to regulate actin cytoskeleton which in turn controls cells morphology, adhesion, motility and also receptors endocytosis and trafficking (15). Rho-like GTPases function as a molecular switch stimulating the exchange of GDP for GTP via guanine-nucleotide exchange factors (GEFs) (16). In addition to activate the Rho GTPases, GEFs also participate to downstream signalling by interacting with scaffold proteins promoting Rho GTPases/specific downstream effectors association. Many reports have shown that Eph/ephrin signalling complexes are related to Rho family. In particular, Rac1 has been linked to Eph/ephrin internalization and trafficking and its activation is required for Eph/ephrin-induced membrane ruffling and trans-endocytosis (13, 17). The molecular mechanism by which EphA2/ephrinA1 complexes regulate Rac1 activation remains to be determined. Several Rho GEFs link Ephs to the activation of Rho proteins. Ephexin 4 interacts with EphA2 to activate RhoG and mediate cell migration (18). The Vav family is involved in EphA2-mediated angiogenesis (19) and Vav2 has been shown to interact with EphA and B positively regulating Eph/ephrin endocytosis (7). Recently, Yoo et al. found that Tiam1 (T-Lymphoma invasion and metastasis protein), a Rac1-specific GEF, is required for ephrinA5/EphA8 clathrin-mediated endocytosis (8). In addition, Tiam1 has been described to mediate EphB-dependent dendritic spine development and EphA-dependent neurite outgrowth (20, 21). Thus, Tiam1 could play a central role in mediating Eph-dependent cellular behaviour.

In this work we investigated the molecular mechanisms regulating EphA2/ephrinA1 endocytosis, intracellular trafficking and signal termination in MiaPaCa-2 pancreatic carcinoma cells endogenously overexpressing EphA2. We demonstrated that in response to ephrin binding, activated EphA2 receptors internalize, traffic through endosomes and reach the lysosome for subsequent degradation, a process regulated by the ubiquitin ligase c-Cbl. We provided first insight that a part of internalized EphA2 receptors are recycled back to the plasma membrane. In addition, we demonstrated that internalized Eph/ephrin complexes remain active throughout the endocytic pathway and retain the capacity to signal from endosomes by recruiting and activating Tiam1. Silencing Tiam1 impaired both ephrinA1-dependent activation of Rac1 and EphA2 endocytosis, definitely establishing Tiam1 as an important modulator of EphA2 signalling.

Results

Ligand activated EphA2 receptors are degraded through the lysosomal pathway

EphrinA1/Fc is a soluble recombinant ephrinA1 fused to the Fc portion of human IgG. Our laboratory and others have previously shown that ephrin mimetic peptides induce receptor phosphorylation, degradation and downstream signalling as efficiently as ephrinA1 on the surface of ligand-expressing cells through juxtacrine contact (9, 22).

EphrinA1/Fc stimulation of MiaPaCa-2 cells induced degradation of total and cell surface-associated EphA2 receptors in a time-dependent manner (Fig. 1A, B). Four hours after stimulation, more than 60% of total EphA2 receptors were degraded whereas only a few receptors were degraded in cells incubated with control IgG. This was not due to a general downregulation of cellular proteins as β -actin and caveolin-1 protein levels remained unchanged (Fig. 1A).

Although many RTKs are degraded through the lysosomal pathway (23, 24), EphA2 and EphA3 receptors degradation has been shown to occur through the proteasomal pathway (22, 25, 26). To clarify the mechanisms of EphA2 degradation we incubated MiaPaCa-2 cells with a lysosomal inhibitor, bafilomycin A1 and proteasomal inhibitors, bortezomib, MG132 and lactacystin. EphA2 degradation was strongly inhibited by bafilomycin, whereas bortezomib, MG132 and lactacystin, simply delayed EphA2 degradation, suggesting that proteasomal activity may influence EphA2 degradation, which mainly occurred through the lysosomal pathway (Fig. 2A, data not shown).

Finally, to visualize EphA2 trafficking and intracellular localization after stimulation, we performed immunofluorescence studies. Confocal microscopy images and the colocalization ratio (C.R.) revealed that following stimulation, EphA2 receptors pass through early endosomes within 15 minutes and reach lysosomes within 30–60 minutes for subsequent degradation (Fig. 2B).

Activated EphA2 receptors are ubiquitinated and interact with Cbl through its TKB domain

To further investigate the molecular mechanisms leading to EphA2 degradation, we assessed receptor activation and ubiquitination. As shown in Fig. 3A (upper blot), maximal EphA2 phosphorylation was observed after 20 minutes of stimulation as well as maximal EphA2 ubiquitination (Fig. 3A middle blot).

The c-Cbl adaptor protein has emerged as a negative regulator of RTKs and other tyrosine kinases, a function dependent on its ubiquitin ligase activity following its phosphorylation (27). Many RTKs, such as EGF, PDGF or EphB1 receptors, activate c-Cbl through phosphorylation by members of the Src family (9, 28, 29). In MiaPaCa-2 cells, c-Cbl phosphorylation was detected 10 minutes after ephrinA1/Fc stimulation and increased over the time (Fig. 3A lower blot).

Next, we showed by co-immunoprecipitation that c-Cbl was recruited to EphA2 signalling complex within 10 minutes following stimulation (Fig. 3B). This interaction was dependent on EphA2 phosphorylation status, as no EphA2/Cbl complex was observed when EphA2 phosphorylation was impaired (data not shown). EphA2/Cbl interaction was confirmed by confocal microscopy and glutathione S-transferase (GST) pull-down assay. Before stimulation, EphA2 localized at the plasma membrane, whereas Cbl was mainly cytoplasmic. However, after 30 minutes of ligand stimulation, EphA2 and Cbl colocalized into the cytoplasm (Fig. 3C).

Cbl has been shown to bind to activated RTKs through its tyrosine kinase binding domain (TKB) (30). A GST pull-down assay demonstrated that EphA2 receptors in lysates of stimulated cells strongly bound to GST-Cbl-N whereas no binding was observed with GST control or with GST-Cbl-N-G306E (Cbl-N with a point mutation in the TKB domain). No binding was also observed with unstimulated lysates (Fig. 3D).

Collectively, these results indicate that activated EphA2 recruits Cbl and induces its phosphorylation, which probably leads to EphA2 ubiquitination and downregulation through the lysosomal pathway.

Part of internalized EphA2 receptors is recycled back to the plasma membrane via both the early and the late recycling pathways

Once internalized, RTKs may turn uncoupled from their ligand due to acidic intracellular compartments and recycle back to the cell surface, or continue through the endocytic pathway to be degraded (31). To investigate the ability of EphA2 receptors to recycle back to the plasma membrane after endocytosis, we adapted the protocol used by Turvy and Blum (32). This method allowed us to directly quantify cell-surface expression, endocytosis and recycling of EphA2 (Protocol in Fig. 4A).

To block EphA2 degradation and observe its accumulation in the endocytic pathway, cells were incubated with bafilomycin, an inhibitor of endosomal acidification and thus of lysosomal degradation. Using capture-ELISA to quantify internalized biotinylated EphA2, we found that 30 minutes after ligand stimulation, about 40% of the initial amount of EphA2 was internalized, with the caveats that at this time point, some parts are already recycled back to the plasma membrane (Fig. 4B). EphA2 receptors accumulation in endosomes increased when cells were incubated with bafilomycin. The fact that bafilomycin prevents receptors degradation could explain the accumulation during stimulation times of 15 to 60 min. Before receptors reach lysosomes at the 5 and 10 min time points, bafilomycin also affects EphA2 endocytosis. Based on the literature (33), one possibility is that bafilomycin affects the recycling rate of EphA2, however more evidences should be provided to confirm this hypothesis. To demonstrate EphA2 recycling, cells were incubated one more time at 37°C for different periods of time (Fig. 4A). Capture-ELISA showed that a recycling time of 15–30 minutes allow 28 to 35% of the internalized EphA2 to recycle, meaning that 10 to 12% of total surface EphA2 receptors are recycled after stimulation. The longer the cells are incubated at 37°C, the more EphA2 receptors are recycled back to the plasma membrane. Primaquine, an inhibitor of endosomal recycling, blocked transferrin recycling in our cells (data not shown) and indeed blocked EphA2 recycling (Fig. 4C). The same experiments were performed with PC-3 cells, a human prostate cancer cell line overexpressing EphA2, and confirmed that the percentages of internalized and recycled EphA2 were comparable (data not shown). To validate our experiments, biotinylated proteins were isolated with streptavidin agarose beads. EphA2 immunoblotting confirmed the following findings: cell-surface EphA2 receptors were correctly biotinylated (“total surface”), the stripping step efficiently removed biotin from cell-surface EphA2 (“strip”), the longer the cells were incubated at 37°C the more EphA2 were endocytosed (“internalized”) and finally, less biotinylated EphA2 remained after a second incubation at 37°C and a reducing wash, suggesting that a part of EphA2 receptors are recycled back to the plasma membrane (“post-recycling”) (Fig. 4D).

Rab4 and Rab11 belong to the Rab family, a part of the Ras superfamily of small GTPases. They both function as regulators of the recycling pathway. Rab4 is localized to early and recycling endosomes, and has been shown to sort recycling receptors directly from early endosomes to the plasma membrane in a “fast recycling” route (34, 35). Rab11 can regulate the receptors traffic from the Golgi apparatus to the plasma membrane and control the “slow

recycling” route through the perinuclear recycling endosomes (36). To further characterize EphA2 recycling pathways, we performed co-immunoprecipitation and immunofluorescence experiments with Rab4 and Rab11. As shown in Fig. 5A, a swift EphA2/Rab4 interaction was observed after 5 minutes of ligand stimulation, whereas EphA2 and Rab11 immunoprecipitated at a later time-point, 15–30 minutes following stimulation. Identical results were obtained with co-immunoprecipitation using PC-3 cells, a prostate cancer cell line endogenously expressing EphA2 (Fig. 5B). Confocal microscopy images confirmed that Rab4 and EphA2 colocalized 5 and 15 minutes after ligand stimulation, whereas Rab11 and EphA2 colocalized 15, 30 and 60 minutes after ligand stimulation (Fig. 5C). Studies measuring the recycling rates of the canonical recycling receptor, the transferrin receptor, confirmed the kinetic of the ‘fast’ recycling pathway ($t_{1/2} \sim 5$ min) and the ‘slow’ recycling pathway ($t_{1/2} \sim 15$ –30 min) (37). In addition, the pathway followed by a part of EphA2 is similar to the one taken by transferrin, as demonstrated by labelled transferrin / EphA2 colocalization experiments (data not shown).

Overall, these results suggest that following stimulation, a part of EphA2 receptors are recycled back to the cell surface *via* both the early and late recycling routes.

EphA2 receptors remain ligand-associated and phosphorylated in early endosomes

Since the emergence of the signalling endosomes concept coming from neuronal studies (38, 39), numerous examples show the signalling capacity of receptors localized to endosomes (11, 12). Once internalized, a receptor can remain active if it stays ligand-coupled, phosphorylated and transduces downstream signalling. Although it has been shown that internally Eph receptors can be tyrosine phosphorylated (13, 14), it is not known how long they stay active and whether they remain associated with their ligands.

Internalized receptor/ligand complexes progressively encounter more acidic environments once they penetrate and advance into the endocytic pathway. In addition, receptor-ligand associations are pH-sensitive. To test at which pH ephrinA1 dissociates from EphA2 receptors, two different methods were applied: one using biotinylated ephrinA1/Fc and capture-ELISA (Fig. 6A) and one using immunofluorescence (Fig. 6B). As shown in Fig. 6A and C, 50% of receptor/ligand complexes were dissociated at a pH slightly lower than 5.5. The internal pH of endosomes decreases the closer they get to lysosomes: early sorting endosomes have a pH of 5.8 – 6.3, late endosomes a pH of 5 – 6 and lysosomes a pH of 5 – 5.5 (40, 41). In aggregate, our findings suggest that EphA2 and ephrinA1 dissociate when they reach late endosomes and lysosomes, and thus that the bulk of internalized EphA2 receptors remain ligand-associated within the early endosomes. Immunofluorescence experiments confirmed these results, showing a strong colocalization ratio of EphA2 and ephrinA1 within the early endosomes, 15 minutes after stimulation (Fig. 6D).

To confirm the presence of active EphA2 in early endosomes, we assessed its phosphorylation status throughout the whole internalization process (Fig. 6E). Using an antibody specifically recognizing phospho-EphA2 (Y594), we demonstrated that 5 minutes after stimulation, EphA2 was phosphorylated and starts to be internalized. Fifteen minutes after stimulation, most of phospho-EphA2 has reached the early endosomes and 60 minutes after stimulation most of phospho-EphA2 receptors were degraded.

Taken together, our results from Fig. 6 indicate that most of the internalized EphA2 receptors remain ligand-associated and phosphorylated in the early endosomes, suggesting that internalized EphA2 receptors could retain the capacity to transduce downstream signalling.

EphA2 receptors associate with Tiam1 in the early endosomes

At this point, the most interesting question relates to the potential effects of endosomes-based EphA2 signalling. Signalling from endosomes could be functionally distinct from those emanating from the cell surface, or they could just be an extension of the signal initiated at the plasma membrane (42).

To examine the ability of EphA2 receptors to associate with specific molecules after endocytosis, we adapted the technique developed by Burke et al. to separate internal from cell-surface proteins (43). Cells were incubated with ephrinA1/Fc prior to cell-surface biotinylation. As shown in Fig. 7B, biotinylated proteins were precipitated using streptavidin agarose, allowing the separation of non-biotinylated, internalized proteins (supernatant) from biotinylated cell surface proteins (beads). As we have previously shown, internalized EphA2 remained phosphorylated for a considerable length of time. Total EphA2 and tyrosine phosphorylated EphA2 levels decreased at the same time, meaning that receptor dephosphorylation did not precede receptor degradation (Fig. 7C, upper blots). Moreover, the ratio between phosphorylated EphA2 and total EphA2 was higher in the internalized fraction than in the cell surface fraction, showing that phosphorylated receptors were internalized within 30 minutes (Fig. 7C–E). Tiam1, a GEF specific for Rac1, has previously been reported to be involved in EphA8 endocytosis (8). Although Tiam1 is mainly cytoplasmic, it has been shown to be recruited to early endosomes in Rab5 overexpressing cells (44). Therefore we hypothesized that following stimulation; EphA2 and Tiam1 could associate into endocytic vesicles and early endosomes. Immunoprecipitation experiments first revealed that Tiam1 is recruited to EphA2 complex 2–5 minutes after stimulation in MiaPaCa-2 and PC-3 cells (Fig. 7A). Then, co-precipitation experiments demonstrated that internalized EphA2 associated with Tiam1 5–15 min after stimulation, but no Tiam1 associates with cell-surface EphA2 (Fig. 7C, D). The Tiam1 blot of the entire biotinylated fraction (prior to the step of precipitation) revealed that no Tiam1 was present in the surface protein fraction in contrast to EphA2 (Fig. 7D lower part)

To visualize EphA2-Tiam1 interaction we performed immunofluorescence studies. Confocal microscopy images confirmed that EphA2 and Tiam1 associate following ephrinA1/Fc stimulation. In addition, more Tiam1 were found in early endosomes after ephrinA1/Fc treatment (Fig. 7F).

Taken together, these results demonstrate that EphA2 remains phosphorylated during the endocytic pathway. For technical reasons (use of EEA1 for co localization) our experiments indeed prove the interactions in early endosomes; however they cannot rule out that it could also take place at an earlier time point, i.e. in (coated or uncoated) endocytic vesicles already.

Therefore, endosomes could be important sites for EphA2-initiated signal transduction via Tiam1.

Characterization of Tiam1 activation

To further characterize EphA2-Tiam1 interaction, cells were transiently transfected with EphA2 c-myc tagged mutants plasmids and their ability to interact with Tiam1 under ephrinA1/Fc stimulation was tested. We confirmed that kinase dead (KD) EphA2 mutant and the non phosphorylable double-mutant retaining a kinase activity (EE) were not phosphorylated by ephrinA1/Fc stimulation. We found that Tiam1 precipitates with wild type (WT) EphA2 but co-immunoprecipitation with EphA2 mutants lacking kinase activity and/or phosphorylated kinase was significantly decreased (Fig. 8A). Since the mutated tyrosines are localized in the juxtamembrane of EphA2 these results suggest that Tiam1 may directly bind the phosphorylated tyrosines in the juxtamembrane region of EphA2. The lack

of Tiam1 binding to EphA2 mutants cannot be due to their inability to internalize as their internalization process is as good as for the wild type (data not shown). Knowing that Tiam1 and EphA2 interact in a phosphorylation-dependent manner we investigated whether this interaction triggers Tiam1 activation. Using an active Rac-GEF assay kit we succeeded at pulling down active Tiam1 in 2, 5 and 10 minutes stimulated cell lysates, but not in unstimulated lysates. Cells stimulated with hydrogen peroxide and sodium orthovanadate were used as a positive control of Tiam1 activity (45) (Fig. 8B). To further confirm that ephrinA1/Fc induces Tiam1 activation, we performed co-immunoprecipitation assay between Tiam1-HA and endogenous Rac1. We found that the Rac1/Tiam1 interaction was maximum 2 minutes after stimulation and diminished in later time points, suggesting that Tiam1 is activated at these time points and facilitates Rac1 activation (Fig. 8C).

Having established that Tiam1 is activated following stimulation, we next sought to determine the mechanisms leading to this activation. Since several studies have previously demonstrated that Tiam1 activity can be modulated by tyrosine phosphorylation (20, 21), we examined Tiam1 tyrosine phosphorylation status in cells treated with ephrinA1/Fc. Using endogenous Tiam1 immunoprecipitation and anti-phosphotyrosine immunoblots we were not able to demonstrate Tiam1 phosphorylation following ephrinA1/Fc stimulation. However, when we used the specific phospho Tiam1 (Y384) antibody in Tiam-HA transfected cells we observed a weak Tiam1 phosphorylation 2, 5 and 10 minutes after ephrinA1/Fc stimulation (Fig. 8D).

Given the weakness of the phosphorylation, we sought to determine whether Tiam1 is activated by another mechanism. An interesting pathway leading to Tiam1 activation is the one mediated by Ras. It has been reported that Ras can directly bind to the Ras-binding domain (RBD) of Tiam1 and facilitate its activation (46, 47). Therefore, in order to evaluate the role of Ras in Tiam1 activation mediated by ephrinA1/Fc, we performed co-immunoprecipitation assays between Ras and Tiam1. Using this method we did not detect any Ras and Tiam1 association in response to ephrinA1/Fc (Fig. 8E.). These results suggest that Tiam1 is activated by a mechanism independent of the Ras pathway.

Taken together, our data demonstrate that only a small part of Tiam1 is activated by phosphorylation - how Tiam1 is fully activated following ephrinA1/Fc stimulation is not completely determined. According to the literature (48), the activation of Tiam1 is complex and involves a myriad of potential mechanisms such as phosphorylation, phosphoinositide binding, modulation of cellular levels and/or interaction with other proteins. However, in the majority of the cases, the precise stimuli leading to Tiam1 activation and determining Tiam1-mediated cellular functions remain to be defined.

Tiam1 is required for Rac1 activation induced by ephrinA1/Fc

Previous studies have reported that ephrinA1/Fc stimulation increases or decreases Rac1 GTPase activation and that Rac1 activity is required for endocytosis of Eph proteins (13, 49). We thus investigated whether Eph/ephrin signalling-mediated Rac1 activation is affected by Tiam1 downregulation.

First, we performed RNA interference experiments. As shown in Fig. 8F, Tiam1 protein expression was reduced by 62% by Tiam1 siRNA compared with scrambled siRNA. As shown in Fig. 8G, upon ephrinA1/Fc stimulation, we detected a transient activation of Rac1 GTPase, with a peak at 2–5 min. In Tiam1 knock down cells, Rac1 activation was significantly decreased 2 and 5 min after stimulation. In contrast, ephrinA1/Fc stimulation decreased RhoA activity. Tiam1 knock down seems to attenuate ephrinA1-induced RhoA-GTP decrease; however the results are not significant, suggesting that another GEF could be involved in this process. EphrinA1/Fc stimulation increased Cdc42 GTPases level in wild

type cells, whereas GTP-bound-Cdc42 level seems reduced in Tiam1 knock down cells. However this result is not statistically significant. Taken together, these results strongly suggest that Tiam1 specifically mediates ephrinA1-dependent Rac1 activation and probably ephrinA1-dependent Cdc42 activation (Fig. 8G and 8H) (* $p < 0.05$).

Tiam1 is required for ephrinA1/Fc-induced endocytosis of EphA2 receptors

Next, we sought to determine the biological effects of Tiam1/EphA2 association. As it is known that Tiam1 is involved in Rac1 activity and EphA8/ephrinA5 endocytosis (8), we investigated whether Tiam1 would play a role in ephrinA1-induced EphA2 endocytosis.

Cells were transfected with Tiam1 siRNA or scrambled siRNA and subjected to a biotinylation endocytosis assay, as described in Fig. 3A. Using capture-ELISA to measure the amount of internalized EphA2, we found that EphA2 endocytosis was significantly reduced in Tiam1 siRNA transfected cells (Fig. 8I) (~40%, * $P < 0.05$; ** $P < 0.01$). These data suggest that Tiam1 is required for ephrinA1-induced EphA2 endocytosis.

Discussion

In this study, we confirmed in MiaPaCa-2 and PC-3 cells endogenously overexpressing EphA2 that upon ephrinA1/Fc stimulation, activated EphA2 recruits Cbl and induces its phosphorylation, which in turn leads to EphA2 ubiquitination and lysosomal degradation. EphA2 internalization is the first step in receptors degradation; however, internalization is not necessarily an attenuation process. Our findings suggest that a part of internalized EphA2 is recycled back to the plasma membrane, increasing receptors half-life. In addition, as demonstrated for other RTKs, but not to date for Eph receptors, we present evidence that internalized ephrinA1/EphA2 complexes are still engaged in active signalling. We demonstrated that active EphA2 reaches endosomes as early as 5 minutes following stimulation. In those endocytic compartments, EphA2 induces the activation and recruitment of a Rho GEF, Tiam1, leading to increase Rac1 activity. We also observed that disrupting Tiam1 function with RNA interference impaired ephrinA1-dependent Rac1 activation, suggesting that Tiam1 is an important GEF downstream of Eph receptors. Our data also indicate that Tiam1 is required for ephrinA1-induced EphA2 endocytosis. Taken together, our observations clarify the molecular mechanisms regulating Eph endocytosis, intracellular trafficking and signal termination (Model Fig. 9).

Degradation and recycling pathways of Eph receptors

The lysosomal pathway is the preferred route of degradation for most of the RTKs (23, 24). This is also true for EphA receptors as demonstrated by Kajiho et al. and previous work from our lab (9, 50). EphA2 and EphA3 are degraded through the proteasomal pathway (22, 25, 26). We and Kajiho have also found a participation, albeit weak, of the proteasome in the process of EphA degradation, thus the proportion of the proteasomal involvement may depend on receptor or/and on cell type (50).

Classically, upon ligand binding, RTKs are rapidly internalized and trafficked to endosomes. From endosomes, receptors are either transported to lysosomes for degradation, to trans-Golgi network or to recycling endosomal compartments that return to the plasma membrane. Receptors fate is regulated by ubiquitination, which in turn is dependent on ligand binding. Ligand dissociation in the mildly low pH of early endosomes results in loss of ubiquitin signal and promotes EGFR recycling to the cell surface (51). In contrast, PDGF receptors have been shown to be efficiently targeted to lysosomes independently of ligand binding (52). What about Eph receptors? There has been to date, only one study suggesting that Eph receptors can be recycled. VAB-1, the Eph receptor in *C. elegans*, was shown to transit to

the endocytic-recycling compartment independently of ligand binding (53). Our results demonstrate that Eph receptors can be recycled back to the plasma membrane. However, compared to other receptors such as EGFR (TGF- α or Epi stimulated), met receptors or transferrin receptors which are almost 100% recycled, only 35% of internalized EphA2 receptors take this pathway (54, 55). Our data show that ephrinA1/Fc binding to EphA2 is relatively stable at the mildly acidic pH found in early endosomes, thus EphA2 is continuously ubiquitinated by c-Cbl and transported to lysosomes. This result could explain why only a small part of Eph receptors can be recycled back to the plasma membrane.

Many Rab GTPases including Rab4, Rab5, Rab7, Rab11 and Rab21, have been identified as critical proteins that regulate RTKs endocytosis and recycling and thereafter RTKs signalling (54, 56, 57). To further characterize EphA2 recycling pathways we demonstrated that active EphA2 colocalized and interacted with Rab4 and Rab11 (Fig.5). Although several RTKs such as EGFR (54, 58), PDGFR (57, 59, 60), VEGFR (61) and MET RTKs (55), have been shown to colocalized with Rab4 and Rab11 in different cell lines, studies demonstrating that cargos interacted with Rab proteins are scarce. To our knowledge, the only study showing such an interaction demonstrated that Rab21 interacted with EGFR and enhanced its degradation (56). What could be the role of EphA2/ Rab4 and EphA2/Rab11 interactions? It is well known that Rab4 and Rab11 coordinate vesicles trafficking during recycling (62). We envision that Rab4 and Rab11 interacted with activate EphA2 to regulate its trafficking and signalling, as it was demonstrated for Rab21 and EGFR (56). However, more evidence should be provided to define the specific role of EphA2/Rabs interactions, which is beyond the scope of this study.

Sustained Eph receptor signalling within the endosomal compartment

The ability of a receptor to signal after endocytosis is important to ensure the sufficient duration and amplitude of signalling. However, this capacity requires receptors to remain active in endosomes. It has been reported that phosphorylated EphBs are internalized (13, 14). In this work, we have now clearly demonstrated that EphA2 remains ligand bound, phosphorylated and active until late stages of endosomal trafficking. A crucial question is whether these internal active Ephs still transduce signals from endosomes. Our demonstration by fluorescence microscopy and proteins isolation that EphA2 and Tiam1 associate in endosomes but not at the plasma membrane, leading to Tiam1 and Rac1 activation, strongly suggest that EphA2 indeed is able to transduce specific signals from the endosomal membrane. It has previously been proposed that following RTKs activation, Rac1 could be activated in early endosomes through Tiam1 (44). While Tiam1 is mainly cytoplasmic and has been shown to translocate to the plasma membrane upon cellular activation, we proposed that active EphA2 recruits soluble Tiam1 to the endosomes (early endosomes and even before to endocytic vesicles). Whether soluble Rac1 is also recruited to the endosomes or whether Rac1 is activated at the plasma membrane remain to be elucidated.

Regulation of Tiam1 activation

Only few studies have shown an interaction between Eph receptors (including EphA2, EphB2, EphA4 and EphA8) and Tiam1 (8, 20, 21). In all the reports, the cytoplasmic region of Eph receptors is required for Tiam1 association. Although the precise region is not clearly established, one group found that the juxtamembrane region of EphA8 may be involved in Tiam1 association (8). In accordance with this observation, we showed that EphA2 tyrosine phosphorylation in the juxtamembrane region is necessary for its association with Tiam1.

How is Tiam1 activated by Eph receptors? Previous studies suggest that phosphorylation of Tiam1 (probably by Src) is an efficient mechanism to trigger its activation (20, 21, 45, 63).

However, the robustness of Tiam1 phosphorylation was very much cell- and context dependent. For example, although downregulation of Tiam1 significantly inhibited nerve growth factor (NGF)-dependent Rac1 activation and neurite outgrowth, Tiam1 phosphorylation was not observed in response to NGF whereas Tiam1 is phosphorylated by TrkB following BDNF stimulation (46). In our system, we were able to detect phosphorylation of Tiam1 only when it was overexpressed in MiaPaca2 cells. This observation could be explained by the low amount of phosphorylated Tiam1 compared to total Tiam1, or by the fact that Tiam1 may not be significantly phosphorylated.

Several groups have demonstrated that active Ras can cooperate with Tiam1 to activate Rac1 in a mechanism independent of PI3K (46, 64). Our findings suggest that Ras is not involved in Tiam1 activation. These results open the possibility that ephrinA1/Fc activates Tiam1 through different intracellular mechanisms.

Binding of lipids to the PH domains of Tiam1 is also a mechanism regulating its activity. Interestingly, it has been reported that PI3K products enhanced Tiam1 activity, while constitutively active PI3K increased the amount of active Rac1 (65). Given that active EphA2 recruits the p85 subunit of PI3K, increases PIP3 levels and induces Rac1 activation (19, 66), ephrinA1/Fc stimulation could activate Tiam1 through an indirect PI3K-dependent mechanism, as it has been described for Vav activation (19). However, the exact mechanism dependent on Tiam-EphA2 association and leading to direct Tiam1 activation remain to be elucidated. The current literature suggests that the modes of regulation of Tiam1 could involve both intra- and inter-molecular interactions. In general, however, the precise biochemical mechanisms of Tiam1 activation remain poorly understood.

Role of Tiam1 in ephrinA1-dependent Rac1 activation and endocytosis

Although it has been shown that ephrin-dependent endocytosis requires Rac1 activation (17), the precise mechanisms remain to be defined. Using RNA interference we demonstrated that Tiam1 is required for ephrinA1-induced Rac1 and probably also Cdc42 activation. In the case of RhoA, ephrinA1 stimulation decreases its activation which is not significantly affected by silencing Tiam1. Those data suggest that ligand stimulated EphA receptor activates Tiam1, which in turn leads to Rac1 activation. Although Tiam1 can act directly as an exchange factor, it can also interact with different membrane and intracellular proteins, which control its GEF activity and determine the outcome signalling of Tiam1-mediated Rac1 activation. This may reconcile the different findings demonstrating that ephrins can increase or decrease GTPases activity depending on the recruited GEFs (19, 67–69).

Other GEFs (including Vav2, ephexin, Kalirin) have been reported to mediate ephrinA1-dependent Rac1 activation (70) and cellular functions. Why are several Rac1-specific GEFs required to regulate Eph-dependent biological responses? One explanation could be that different GEFs may regulate specific biological functions (7, 19). In our model, Tiam1 mediated ephrin-induced endocytosis in cancer cells. Given the large numbers of Rho GEFs and Rho GTPases, the ability of many GEFs to activate several Rho GTPases and the potential of RTKs to interact with multiple GEFs, we cannot conclude that Tiam1 is the lonely scaffold protein responsible for ephrin-induced Eph endocytosis. However, we can for sure affirm that Tiam1 is associated with the Eph endocytosis process.

Mechanisms leading to Tiam1-mediated EphA2 endocytosis

It is well established that actin polymerization and rearrangement depend on different Rho GTPase including Cdc42, Rho and Rac (71, 72), that actin plays a crucial role in endocytosis (73) and Rac1 is involved in endocytosis mediated by ephrinA2 during growth cone collapse

(17). Thus, it is clear that Rac1 regulates endocytosis through actin modulation. Here, we demonstrated that Tiam1 is activated in endosomes promoting Rac1 activation and endocytosis. However, an important question arises as to the localization of such activation. Based on previously published studies, two possible mechanisms could be envisaged. Palamidessi et al proposed that Rac1 and Tiam1 are simultaneously recruited to early endosomes by an unknown mechanism. From the endosomal membrane, Tiam1 activates Rac1 and thereafter Rac1 is targeted to the plasma membrane in an Arf6-dependent manner to ensure actin dynamics and thus promote cell migration (44). Similarly, following activation by EphA2/Tiam1 complex Rac1 could be recycled to specialized plasma membrane area where it could promote actin reorganization and thus endocytosis. Another more probable mechanism is that Tiam1 activates Rac1 directly at the plasma membrane. This hypothesis is supported by a study showing that Tiam1 interacts with the Arp2/3 complex at the site of actin polymerization such as membrane ruffles and epithelial cell-cell contacts (74). In addition, it has been reported that Tiam1 is mainly cytoplasmic and can translocate to the plasma membrane upon cellular activation (75). This suggest that once activated in endosomes, Tiam1 could be recycled or translocated to the plasma membrane, where it can activate Rac1, thus leading to subsequent actin polymerization and Eph endocytosis.

In conclusion, our results shed new light on two important aspects of EphA2 receptors' fate following ligand stimulation. First, they definitely establish that EphA2 receptors can still actively signal while positioned in the endosomal compartment. Second, they highlight the importance of EphA2-Tiam1 interactions in this process. Tiam1 may convert a repulsive response to an adhesive interaction and vice-versa, modulating cellular responses. Being involved in Eph-dependent neurite outgrowth, spine development and endocytosis, Tiam1 is an important regulator of Eph-mediated cellular functions, suggesting that these two proteins could interplay in cellular migration, invasion and adhesion, all processes where they have been shown to play a role by their own. In addition, because endocytosis, trafficking, recycling and degradation of Eph receptors are key regulators of the duration, intensity and specificity of the signalling response, Tiam1 could be one of the main actors responsible for the oncogenic properties of EphA2 receptors.

Materials and Methods

Antibodies and reagents

Rabbit polyclonal antibodies against EphA2, ephrinA1, c-Cbl, ubiquitin, caveolin-1, c-myc (A14), Tiam-1 (C-16), monoclonal β -Actin, c-myc (9E10), and anti-phosphotyrosine antibody PY20 were from Santa Cruz Biotechnology. Monoclonal anti-Ras (K-, H-, N-), clone 9A11.2, polyclonal anti-Tiam1 and anti-phosphotyrosine HRP (clone 4G10) were from Millipore. Monoclonal anti-EphA2 (clone D7) was from Sigma-Aldrich and anti-EEA1 (Early Endosomal Antigen 1) from BD Biosciences. Rabbit polyclonal antibodies against Phospho-EphA2 (Y594), Rab4 and Rab11 were from Cell Signaling Technology. Polyclonal anti-Tiam1 phospho Y384 was from Abcam and monoclonal anti-HA from Roche Diagnostics. HRP- goat anti-mouse or anti-rabbit secondary antibodies, control rabbit or mouse IgG were from Santa Cruz. For immunofluorescence, Alexa Fluor-conjugated antibodies and Lysotracker® were from Invitrogen AG.

The soluble recombinant mouse ephrinA1/Fc and the biotinylated ephrinA1/Fc were from R&D systems.

The lysosomal inhibitor, bafilomycin A1 and the proteasome inhibitor MG132 were from Calbiochem. The proteasome inhibitor bortezomib (Velcade®) was from Janssen-Cilag. The inhibitor of vesicular transport, brefeldin A (BFA) was from Sigma-Aldrich.

ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt), EZ-Link Sulfo-NHS-SS-Biotin, EZ-Link Sulfo-NHS-LC-Biotin and the active Rac1 Pull-down kit were purchased from Thermo Scientific. The active Rac-GEF assay kit was from Cell Biolabs.

Cell Culture

MiaPaCa-2 cells, a human pancreatic tumor cell line, and PC-3 cells, a human prostate cancer cell line were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI-1640) respectively, supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C in humidified atmosphere containing 5% CO₂.

RNA interference and transient transfections

The Ambion® Silencer® Tiam siRNA and control siRNA (Life Technologies) were used at a final concentration of 15 nM and reverse transfected using Lullaby® siRNA transfection reagent (Oz Biosciences) for 48 hours.

Tiam-HA plasmid was a kind gift of A. Malliri (Paterson Institute for Cancer Research, Manchester, UK). Transient transfection of EphA2-c-myc mutants (76) and Tiam-HA was performed using 2–4 µg of plasmids and Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours after transfection.

Immunoprecipitation and Immunoblotting

Cells were serum-starved for 24 hours, then stimulated with 1 µg/mL of ephrinA1/Fc or control IgG protein and incubated at 37°C for the indicated times. In some experiments, prior to stimulation, cells were preincubated 1 hour with inhibitors. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 120 mM NaCl, 1 mM EDTA pH 8, 6 mM EGTA pH 8.5, 1% Nonidet P-40, 20 mM NaF, 10 mM Sodium pyrophosphate, 0.1% Sodium Dodecyl Sulfate, 2 mM sodium orthovanadate and protease inhibitor cocktail (Roche Diagnostics)). For co-immunoprecipitations, lysates were incubated overnight with the appropriate antibody and 1 hour with protein A/G Plus agarose (Santa Cruz). The resulting proteins were resolved on SDS-PAGE and subjected to immunoblot. To confirm equal loading, membranes were stripped and reprobed with the appropriate antibody.

Immunofluorescence and confocal microscopy

Cells were grown on glass coverslips overnight. When indicated, cells were preincubated 30 min with 20 µM of BFA. After stimulation, cells were fixed for 10 minutes at room temperature in 4% sucrose, 2% paraformaldehyde (PFA) or 5 minutes at – 20°C in acetone; background staining was blocked by 1 hour incubation in PBS, 1% BSA, 5% normal serum, Triton X100 0.2 %. Primary antibodies were diluted in PBS, 1% BSA and applied overnight at 4°C. Alexa Fluor antibodies were used to detect primary antibodies. Cells were mounted in Vectashield with DAPI (Vector Laboratories). Confocal images were recorded using a Zeiss LSM510 confocal microscope (Carl Zeiss Microscopy) and analysed with the Imaris software.

Capture - ELISA analysis

Maxisorb 96-wells plates were coated overnight at 4°C with 1 µg/mL ephrinA1/Fc or EphA2 antibodies and blocked in PBS containing 0.05% Tween-20 (PBS-T) with 5% FCS for 1 hour. Wells were incubated with 0.05 mg/mL of biotin-labelled cell lysates for 2 hours at 4°C. Unbound material was washed with PBS-T and Avidin-HRP-conjugated was added

for 30 minutes. After extensive washing biotinylated proteins were detected by addition of ABTS substrate. The optical density was read at 405 nm 20 minutes after substrate addition.

GST fusion protein pull-down

The vectors encoding GST, GST-cbl-N (encoding amino acids 1–357 of human cbl) and GST-cbl-N/G306E (G306E) (amino acids 1–357 of human cbl with a G306E point mutation) were a gift from Hamid Band (ENHRI, Evanston, IL, USA).

GST fusion proteins were expressed and purified as described (9). Cell lysates were prepared as described above. Binding reactions were carried out with 20 µg of purified GST fusion protein and 500 µg of cells lysates for 2 hours at 4°C. Beads were washed, bound proteins were eluted in SDS sample buffer and analysed by SDS-PAGE. Membranes were blotted for anti-EphA2, equal loading was ensured by Ponceau staining.

Cell-surface biotinylation, EphA2 internalization and recycling assays

Cell-surface biotinylation and recycling assays were adapted from (32) (Fig. 4A). Cells were pre-incubated at 37°C with 0.5 µM of bafilomycin or DMSO for 1 hour. Cell membrane proteins were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce) according to manufacturer's instructions; the percentage of surface receptor biotinylation is close to 100%. Control samples (total biotinylation and stripping control) were incubated at 4°C while other samples were incubated at 37°C with ephrinA1/Fc for the indicated times. Cell surface biotin was stripped at 4°C using a reducing solution (50 mM glutathione (GSH), 100 mM NaCl, 1 mM EDTA, 75 mM NaOH, 1% (w/v) BSA, pH 8.7) and the reaction was quenched in 120 mM iodoacetamide. To demonstrate receptor recycling, cells were subjected to an additional incubation at 37°C for different times prior to reducing washes. Remaining biotinylated endocytosed receptors were quantified by ELISA. The percentages of internalized or recycled receptors were calculated as followed: internalized EphA2 = (EphA2 level after first incubation at 37°C) – (EphA2 level after stripping step without incubation at 37°C)/(total surface EphA2); recycled EphA2 = 100 – [(EphA2 level after re-incubation at 37°C) – (EphA2 levels after stripping step without incubation at 37°C)/(EphA2 level after first incubation at 37°C) – (EphA2 levels after stripping step without incubation at 37°C) x 100]. For Western blot, biotinylated proteins were isolated with streptavidin agarose beads (Pierce), washed and released by heating 3 minutes at 95°C in Laemmli buffer.

Separation of internal from surface EphA2

Stimulated cells were biotinylated 1 hour on ice with 0.35 mg/mL of EZ-Link Sulfo-NHS-LC-Biotin, washed and lysed in RIPA buffer. Biotinylated receptors were pulled-down with streptavidin agarose; proteins from the supernatant (internalized proteins) and from the agarose beads (biotinylated cell surface proteins) were precipitated with anti-EphA2 or anti-Tiam1, isolated using magnetic beads (Millipore) or protein A/G beads and subjected to immunoblot (Fig. 7A).

pH sensitivity of ephrinA1 binding

Cells were incubated with 0.5 µg/mL of ephrinA1/Fc biotinylated for 30 minutes on ice. Ligand was removed by washes with ice-cold buffers of varying pH (50 mM acetic acid, 50 mM NaCl, pH 2.5 – 6). After acid wash, the percentage of ligands dissociated from the receptors was determined by ELISA.

Active Rac-GEF and Rac1 Pull-Down assay

Pull-down assay using nucleotide-free Rac1 G15A agarose beads which selectively isolate active form of Tiam1, pull-down assay using Pak1-PBD agarose beads which binds to active state of Rac1 or Cdc42 and pull down assay using Rhotekin-RBD were performed following the manufacturer's instructions (Cell Biolabs).

The levels of Rac-GTP, RhoA-GTP and Cdc42-GTP were measured by the band intensity quantification using Image J software and normalized to the amount of total Rac1, total RhoA and total Cdc42 respectively, present in cell lysates.

Statistical analysis

All values are presented as mean values $\pm$ standard deviation (s.d.). Student's *t*-test using GraphPad software was performed for statistical analyses. P-values are indicated in the figure legends and differences were considered statistically significant when $P < 0.05$. Results are representative of at least three independent experiments.

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Synopsis

Eph receptors, a complex subfamily of receptor tyrosine kinases, are rapidly endocytosed following ligand-mediated activation and traffic through endocytic compartments prior to degradation. Here, we depict the trafficking of the angiogenic and tumorigenic EphA2 receptors and show that a part of them are recycled back to the plasma membrane. Our study also demonstrates for the first time that EphA2 retains the capacity to signal in endosomes. In particular, our findings establish Tiam1, a Rac1-specific GEF, as an important modulator of EphA2 signalling.

A **B**

Figure 1. EphA2 receptors are degraded following ephrinA1/Fc stimulation

(A) MiaPaCa-2 cells were stimulated with ephrinA1/Fc or control IgG for the indicated times. Cell lysates were immunoblotted with EphA2, β-Actin and caveolin-1 antibodies. Bands quantification was performed using Image J software; percentages of remaining EphA2 were calculated relatively to unstimulated sample. (B) Biotinylated cell-surface EphA2 were stimulated with ephrinA1/Fc or IgG. Remaining cell-surface EphA2 were quantified by capture-ELISA.

Figure 2. EphA2 receptors are degraded through the lysosomal pathway
(A) Cells were pre-treated with 0,5 μM bafilomycin, 2 μM bortezomib or 20 μM MG132. Cell lysates were immunoblotted with EphA2 and β-Actin. **(B)** EphA2 trafficking was followed by confocal microscopy. Simulated cells were stained for EphA2 (green) and EEA1 (red) (upper panel) or incubated with a Lysotracker® (LT) (Red) (lower panel). Images were analysed by Imaris software and the colocalization ratio (C.R.) quantified with the Pearson's correlation coefficient. Scale bars: 50 μm.

Figure 3. EphA2 receptors signal termination

(A) Proteins were immunoprecipitated (IP) with anti-EphA2, immunoblotted with phosphotyrosine antibody (upper panel) and anti-ubiquitin (middle panel). Proteins were immunoprecipitated with anti-Cbl and immunoblotted with phosphotyrosine antibody (lower panel) and anti-Cbl. (B) Proteins were immunoprecipitated with anti-Cbl and immunoblotted with anti-EphA2. (C) Cells were preincubated 30 min with 20 μ M of BFA, fixed with a cold solution of acetone/methanol and stained for EphA2 (red) and Cbl (green). Images were recorded by confocal microscopy. Scale bar: 50 μ m. (D) GST fusion proteins were incubated with cell lysates stimulated with IgG (left) or ephrinA1/Fc (right). The precipitated protein complex was subjected to Western blot analysis with EphA2 antibody. The blot was stained with Ponceau S (lower panel) to ensure equal loading.

Figure 4. A part of EphA2 is recycled back to the plasma membrane

(A) Schema of biotinylation endocytosis / recycling assays (see Materials and Methods). (B) Cells were pre-incubated with bafilomycin or DMSO. The amount of biotinylated, internalized EphA2 was measured by capture-ELISA. (C) To demonstrate EphA2 recycling, cells were treated with 0.6 mM of primaquine. After reducing washes, cells were subjected to an additional incubation at 37°C for the indicated times. The percentage of remaining biotinylated, internalized EphA2 was measured by capture-ELISA. The percentage of recycled receptors was calculated as described in Materials and Methods. (D) Biotinylated proteins were isolated using streptavidin agarose beads and immunoblotted with anti-EphA2. Bands quantification was performed using Image J, percentages of biotinylated EphA2 were calculated relatively to biotinylated untreated sample.

Figure 5. Activated EphA2 receptors interact and colocalize with proteins involved in the endocytic recycling pathway: Rab4 and Rab11

(A) Stimulated lysates of MiaPaCa-2 cells were immunoprecipitated with anti-Rab11 or anti-Rab4 and immunoblotted with anti-EphA2. (B) Stimulated lysates of PC-3 cells were immunoprecipitated with anti-Rab11 or anti-Rab4 and immunoblotted with anti-EphA2. (C) Confocal sections of MiaPaCa-2 cells stained for EphA2 (green) and Rab4 (red-left) or Rab11 (red-right). Scale bars: 50 μ m.

Figure 6. EphA2 receptors remain associated with ephrinA1/Fc and phosphorylated in early endosomes

(A–C) pH sensitivity of ligand binding. (A) Cells were incubated with 0.5 µg/mL of biotinylated ephrinA1/Fc on ice, and then washed with buffers of various pH. The percentage of ephrinA1/EphA2 complexes was determined by capture-ELISA. (B) Cells were incubated with ephrinA1/Fc on ice for 1 hour, followed by washes with buffers of various pH. Cells were stained for ephrinA1 (red) and DAPI (blue). Images were analysed by confocal microscopy. Scale bar: 50µm. (C) The data in figure B were quantified using Image J, 30 cells were counted for each condition. (D) Confocal sections of MiaPaCa-2 stained for EphA2 (green) and ephrinA1 (red). The images were analysed by Imaris and the colocalization ratio (C.R.) quantified with the Pearson's correlation coefficient. Scale bars: 10 µm. (E) Confocal sections of MiaPaCa-2 stained for Phospho-EphA2 (green) and EEA-1 (red). Scale bars: 20 µm.

Figure 7. EphA2 interacts with Tiam1 in early endosomes

(A) Stimulated total lysates of MiaPaCa-2 and PC-3 cells were immunoprecipitated with anti-Tiam1 and immunoblotted with anti-EphA2 (B) Separation of internalized from surface-associated EphA2 (Materials and Methods). (C) Internalized and (D) cell-surface proteins were immunoprecipitated with anti-EphA2 and immunoblotted with phosphotyrosine or EphA2 antibodies. Internalized and cell-surface proteins were immunoprecipitated with anti-Tiam1 and immunoblotted with EphA2 or Tiam1 antibodies. Controls represent Western Blot of all cell lysates. The entire biotinylated cell surface protein fraction was immunoblotted with Tiam1 and EphA2 antibody prior to

immunoprecipitation (lower part) **(E)** The histogram represents the ratio between phosphorylated EphA2 and total EphA2 amount. **(F)** Confocal sections of MiaPaCa-2 preincubated 30 min with 20 μ M of BFA and stained for EphA2 or EEA-1 (red) and Tiam1 (green). Upper panel cells were fixed with a cold solution of acetone/methanol, lower panel; cells were fixed with a solution of PFA. Scale bars: 50 μ m.

Figure 8. EphrinA1/Fc stimulation induces Tiam1 activity leading to Rac1 activation and EphA2 internalization. (A–E) Characterization of Tiam1 activation

(A) Tiam1 interacts with EphA2 in a phosphorylation-dependent manner. Cells were transfected with c-myc tagged WT, Y593F and Y587/593EE EphA2 mutants. Cell lysates were immunoblotted with c-myc to ensure similar transfection efficiency. C-myc immunoprecipitates were immunoblotted with anti-p-Tyr and anti-c-myc. Tiam1 immunoprecipitates were immunoblotted with anti-c-myc and reprobed with anti-Tiam1 to ensure equal loading. (B) Tiam1 activity measured in 2.5 mg of cell lysates after ephrinA1/Fc or 50 μ M H₂O₂ and 50 μ M orthovanadate stimulation. The lower blot shows endogenous Tiam1 levels in the total lysates. (C) Cell lysates of Tiam1-HA transfected cells were

immunoprecipitated with anti-HA and immunoblotted with anti-Rac1. **(D)** Cell lysates of Tiam1-HA transfected cells were immunoprecipitated with anti-HA and immunoblotted with phospho Tiam1 (Y384) antibody and reprobed with anti-HA. **(E)** Tiam1 does not interact with Ras after ephrinA1/Fc stimulation. Cell lysates of Tiam1-HA transfected cells were immunoprecipitated with anti-HA and immunoblotted with anti-Ras and anti-HA. **(F–I) Tiam1 is required for Rac1 activation and EphA2 endocytosis induced by ephrinA1/Fc.** **(F)** Endogenous level of Tiam1 protein in MiaPaCa-2 from 3 independent experiments were analysed by Western blot 48 hours post transfection with scrambled or Tiam1 siRNA. Tiam1 expression was calculated relatively to scrambled transfected cells and normalized to the levels of β -Actin. **(G)** Cells were transfected with scrambled or Tiam1 siRNA. Activated Rac1, Cdc42 and RhoA GTPases in response to ephrinA1/Fc stimulation were measured by Pak-PBD and Rhotekin-RBD pull-down assays, respectively. Total level of Rac, Cdc42 and RhoA proteins were assay by Western Blot analysis (lower blot). **(H)** Quantification of Rac1, Cdc42 and RhoA activity (from Fig. 8F) after ephrinA1/Fc stimulation in scrambled or Tiam1 SiRNA transfected MiaPaCa-2 cells. Results represent the mean $\pm$ s.d. of 3 independent experiments (* $P < 0.05$ Student's t test). **(I)** Endocytosis assay as described in Fig. 4A. Cells were transfected with scrambled or Tiam1 siRNA. The level of internalized biotinylated EphA2 was measured by capture-ELISA. Tiam1 knockdown cells showed a significant decrease in EphA2 endocytosis (* $P < 0.05$, ** $P < 0.01$, Student's test).

Figure 9. Proposed model of EphA2 endocytic trafficking

The model of EphA2 endocytic trafficking is deduced from the current study and integrates results from other studies mentioned in the paper. Following ephrinA1/Fc binding, EphA2 is tyrosine phosphorylated. Activated EphA2 recruits and phosphorylates Cbl, which in turn leads to EphA2 ubiquitination. EphA2/ephrinA1 complex is internalized by clathrin or caveolae pathways and reaches early endosomes where it can be sorted for lysosomal degradation, dissociated from ephrins and recycled back to the plasma membrane, or signal from endosomes. Activated EphA2 in early endosomes recruits and activates Tiam1. Activated Tiam1 could be translocated to the plasma membrane, where it might increase Rac1-GTP levels and facilitate Eph-endocytosis.