Gaitanos et al., http://www.jcb.org/cgi/content/full/jcb.201512010/DC1

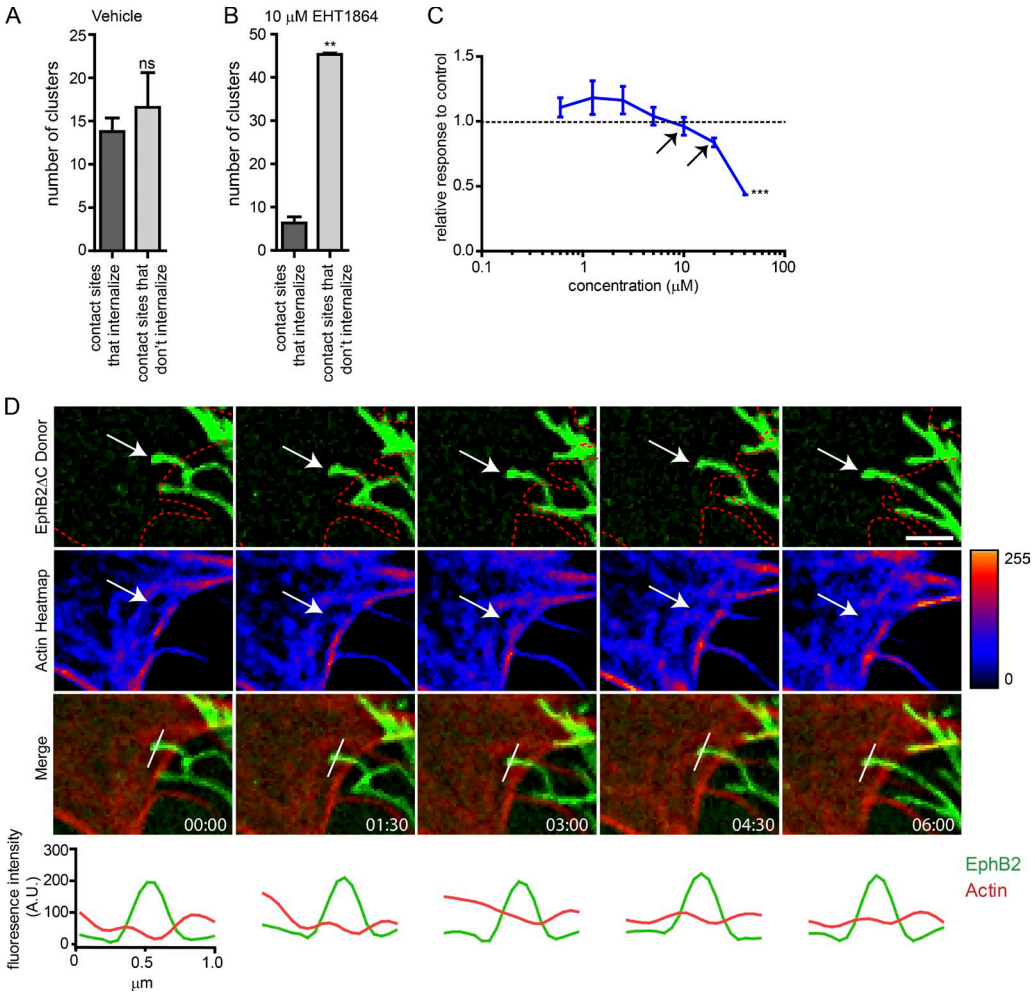


Figure S1. **The effect of Rac inhibition on actin polymerization and EphB2 trans-endocytosis.** (A) Quantification of raw data for numbers of contact sites that show internalization versus those that do not for vehicle-treated cells from experiments described in Fig. 1 (C, E, and F). Data represent mean ± SE (n = 5 independent experiments: 4–10 cells per experiment, each with multiple events recorded, ns = not significant, Student's t test. (B) Quantification of raw data for contact sites that show internalization versus those that do not for EHT1864-treated cells from experiments described in Fig. 1 (H and I). Data represent mean ± SE (n = 3 independent experiments, 9–10 cells per experiment, each with multiple events recorded). **, $P < 0.01$, Student's t test. (C) 3-(4,5-dumethylthiazol2-yl)-2,5-diphenyltetrazolium bromide reducing assay showing dose–response curve for 4-h treatment of SKN cells with EHT1864. Data are normalized to vehicle treatment, shown in log scale (n = 4 independent experiments). ***, $P < 0.001$, one-way ANOVA, Dunnett's post hoc test. Note that both 20 µM and 10 µM concentrations used in this study (marked with arrows) did not significantly reduce cell viability. (D) Full time-lapse series from Fig. 1 D of EphB2ΔC trans-endocytosis into ephrinB$^+$ SKN cells overexpressing LifeAct-mCherry treated with 10 µM EHT1864. Cells were co-cultured and images were acquired every 90 s. Top row, EphB2 channel (red outline indicates SKN cell border); second row, heatmap for fluorescence intensity from the LifeAct channel. Arrow indicates contact point. Bottom panel shows line graphs of fluorescence intensity for LifeAct (red curves) and EphB2 (green curves) measured across contact point, as indicated by a white bar in the merge row. Maximum projection of deconvolved images. Bar, 1 µm. Elapsed time shown as minutes:seconds.

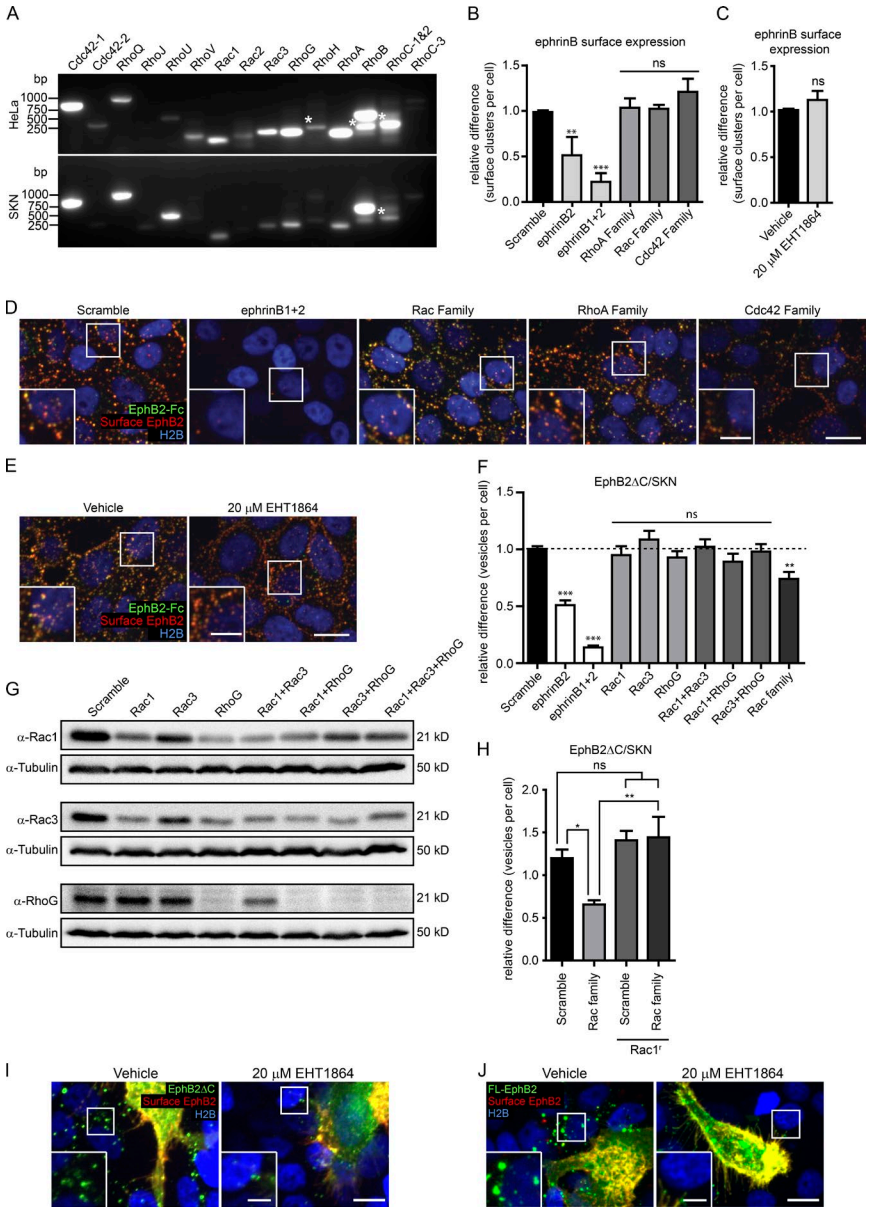


Figure S2. **Rac is required for EphB2ΔC trans-endocytosis into ephrinB⁺ cells.** (A) RT-PCR expression profile for RhoA, Rac1, and Cdc42 subfamilies of GTPases in SKN and HeLa cells. Numbers indicate different isoforms where relevant. An asterisk is positioned to the top left of nonspecific bands. All negative probes were redesigned and their expression profile repeated (not depicted). Quantification and representative images of surface expression of ephrins after Rho family GTPase knockdowns (B and D) or EHT1864 treatment (C and E). Cells were fixed without permeabilization 5 min after soluble Fc-fusion addition and immunostained against Fc. Surface clusters were detected using CellProfiler. Results shown as mean ± SE (n = 3 independent experiments, data normalized to median scramble value per experiment). Ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, repeated measures one-way ANOVA with Dunnett's post hoc test. Bars: 10 µm; (inset) 5 µm. (F) Extended quantification for Fig. 2 C, including knockdown of all Rac1 subfamily combinations. Results shown as mean ± SE (n = 8 independent experiments, >125 responder cells per condition per experiment, data normalized to median scramble value per experiment); statistics are as described in B. (G) Western blot of Rac1-like family knockdown combinations in SKN cell after 72-h siRNA treatment. The final concentration of siRNAs exposed to cells is always balanced to 20 nM. Antibodies were used to blot endogenous proteins. Note cross-reactivity between Rac1 and Rac3 antibodies with other members of the Rac1 subfamily. (H) Quantification of the rescue of the reduction in EphB2ΔC trans-endocytosis in SKN cells after Rac subfamily depletion. SKN responder cells were treated with either scramble siRNA oligos or triple depletion of Rac1, Rac3, and RhoG for 72 h and transfected with an siRNA-resistant mutant of Rac1 (Rac1^r) for 48 h. Co-culture with EphB2ΔC-GFP/Flag–positive cells was performed for 80 min. Cells were fixed and probed against Flag before permeabilization with 0.1% Triton and staining with anti-Rac. Number of internalized vesicles was determined in Rac1-overexpressing versus nonoverexpressing SKN cells in the vicinity of EphB2ΔC-GFP/Flag–positive cells within the same image. Results are shown as mean ± SE (n = 4 independent experiments, >16 responder cells per condition per experiment, data normalized to median of nonoverexpressing, scramble-treated cells). ns, not significant; *, $P < 0.05$; **, $P < 0.01$, repeated measures one-way ANOVA, with post-hoc Bonferroni test. (I) Representative images of Fig. 2 D showing the effect of Rac inhibitor EHT1864 versus vehicle control on EphB2ΔC trans-endocytosis into ephrinB⁺ SKN cells. SKN cells were treated for 4 h with either vehicle or EHT1864 at the indicated concentrations. Cells were fixed without permeabilization and probed against Flag (surface EphB2ΔC, shown in red or yellow in merge). Internalized vesicles appear as green puncta (total EphB2ΔC signal) within the vicinity of the SKN nuclei (H2B channel, shown in blue). Bars: 20 µm; (inset) 5 µm. (J) Representative images for Fig. 2 E showing the effect of Rac inhibitor EHT1864 versus vehicle control on EphB2FL trans-endocytosis into ephrinB⁺ SKN cells. SKN cells were treated for 4 h with either vehicle or EHT1864 at the indicated concentrations. Cells were fixed without permeabilization and probed against Flag (surface EphB2, shown in red or yellow in merge). Internalized vesicles appear as green puncta (total EphB2 signal) within the vicinity of the SKN nuclei (H2B channel, shown in blue). Bars: 20 µm; (inset) 5 µm.

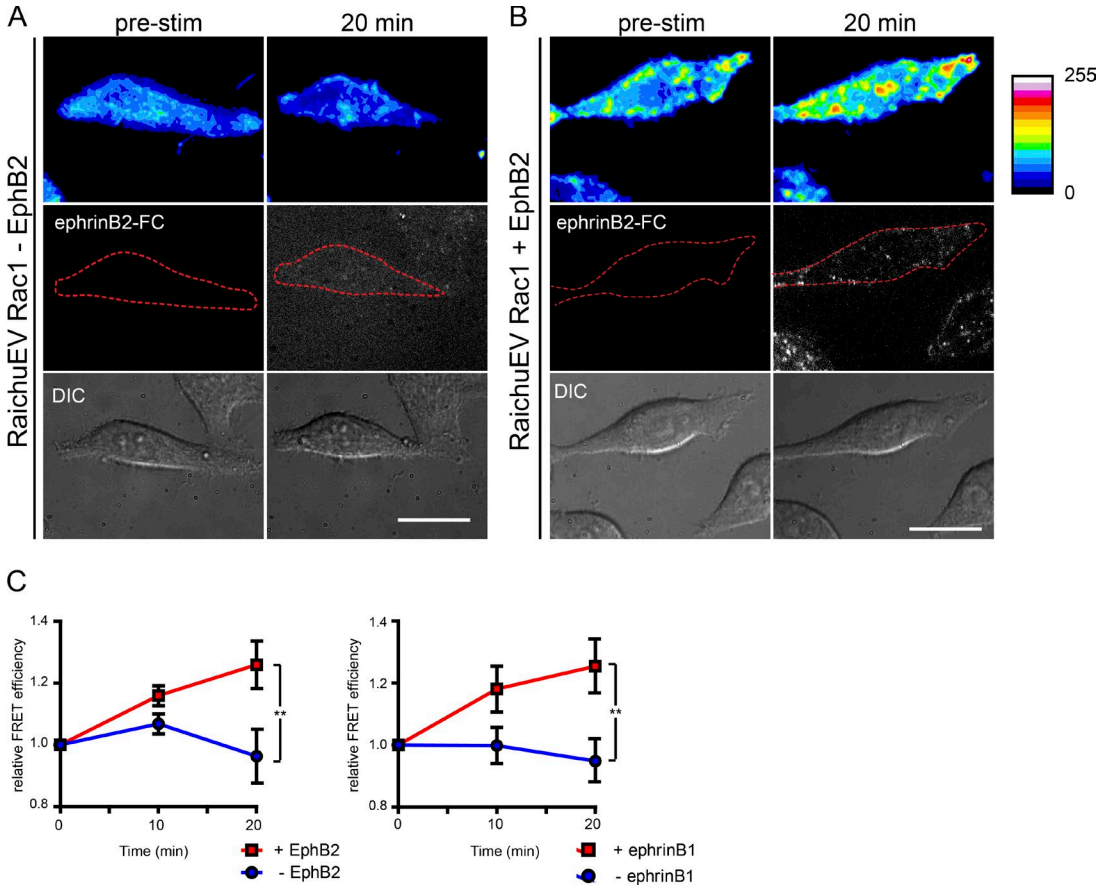


Figure S3. **EphB2 and ephrinB1 trans-endocytosis correlates with elevated Rac activity.** (A and B) Representative images of Fig. 4 (K and M) showing Rac activation using RaichuEV-Rac1 FRET biosensor either in wild-type (A) or EphB2$^+$ (B) HeLa cells after stimulation by ephrinB2-Fc. Cells were imaged before and 20 min after fluorescently labeled, preclustered ephrinB2-Fc stimulation. (B) Included is the prestimulation image in addition to the 20-min time point shown in Fig. 4 M. Top panels show pseudocolor intensity code. Middle panel shows accumulation of ephrinB2-Fc, and the cell boundary is outlined by dashed red border. Bottom panel shows the differential interference contrast image. Bars, 10 µm. (C) Time course for Fig. 4 (H–K and M) and A and B showing FRET activity in wild-type and EphB2$^+$ HeLa cells stimulated with ephrinB2-Fc (left) or wild-type and ephrinB1$^+$ HeLa cells stimulated by EphB2-Fc (right). Results are shown as mean ± SE (n = 15–20 cells from three independent experiments, data normalized to prestimulation time point per cell). **, $P < 0.01$, Student's t test.

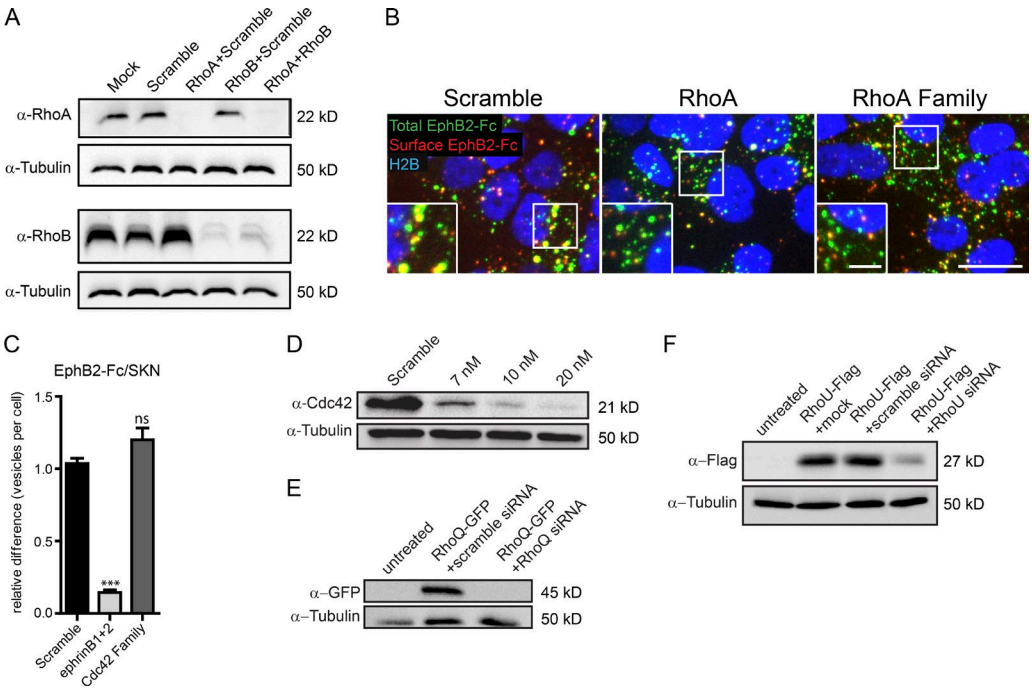


Figure S4. **The role of RhoA and Cdc42 GTPase families on EphB2-Fc endocytosis.** (A) Western blot showing RhoA subfamily knockdown efficiency in SKN cells after 72 h siRNA treatment. Final concentration of siRNAs set to 20 nM, with single target pools comprising 10 nM specific oligo and 10 nM scramble. Antibodies probed against endogenous protein. (B) Representative images of Fig. 5 D showing the effects of siRNA depletion of RhoA subfamily on EphB2-Fc endocytosis in ephrinB$^+$ SKN cells. SKN-H2B-RFP cells were treated with indicated siRNAs before stimulation with fluorescently labeled, preclustered EphB2-Fc, fixed without permeabilization, and stained against Fc (surface EphB2, red or yellow in the merge). Internalized vesicles appear as green puncta (total EphB2-Fc) within the vicinity of the SKN nuclei (H2B channel, blue). Bars: 10 µm; (inset) 2.5 µm. (C) Quantification showing the effect of Cdc42 subfamily depletion on EphB2-Fc endocytosis in ephrinB$^+$ SKN cells. SKN cells were treated with indicated siRNAs before stimulation with fluorescently labeled, preclustered EphB2-Fc, fixed without permeabilization, and stained against Fc (surface EphB2). Images analyzed with CellProfiler. Results are shown as mean ± SE (n = 6 independent experiments, >624 cells per condition per experiment, data normalized to median scramble value per experiment). ns, not significant; ***, $P < 0.001$, repeated measures one-way ANOVA with Dunnett's post hoc test. (D) Western blot showing Cdc42 knockdown efficiency in SKN cells after 72 h siRNA treatment. Antibodies probed against endogenous protein. (E and F) Western blot showing RhoQ and RhoU knockdown efficiency in SKN (E) and Hek293 (F) cells. Cells overexpressing tagged RhoQ (GFP; E) or RhoU (Flag; F) constructs were treated with siRNA for 48 h before harvest. Antibodies were probed against tagged proteins.

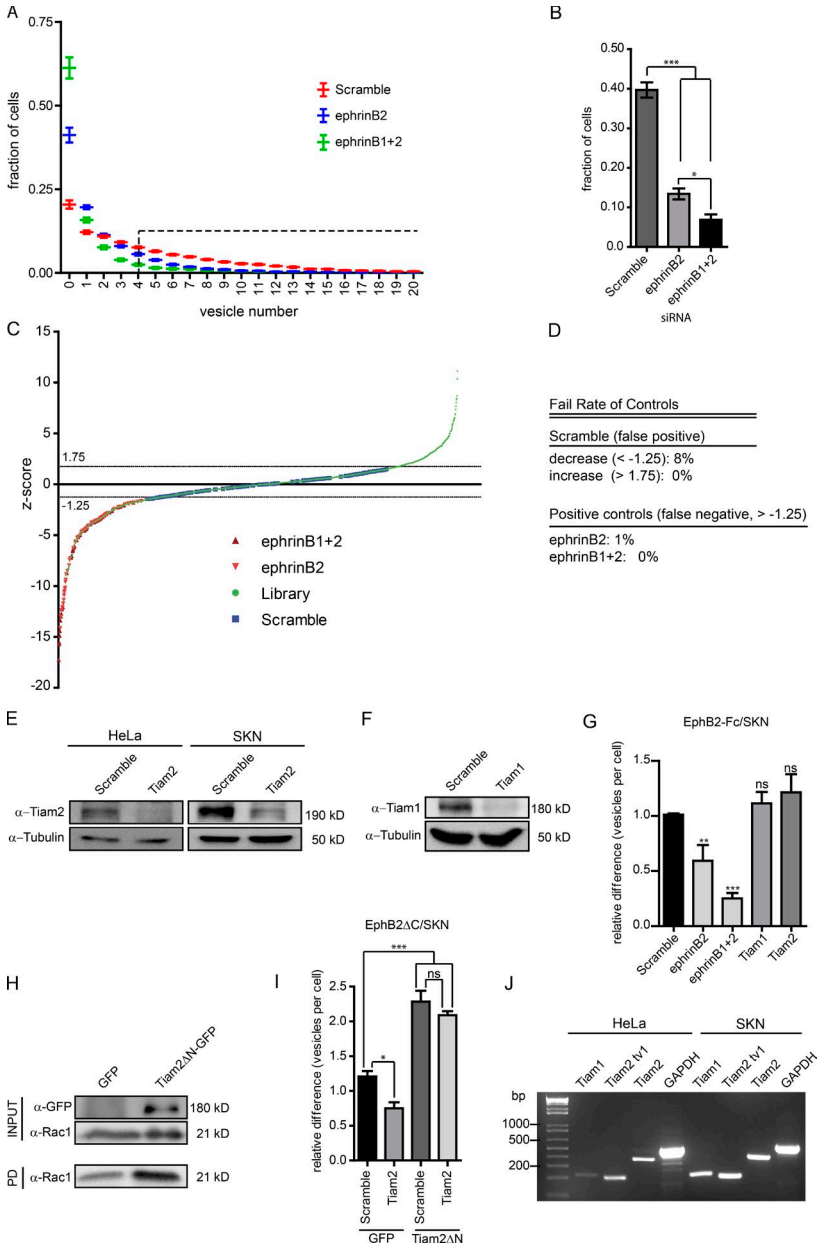
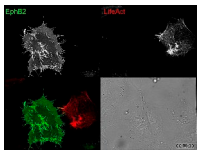
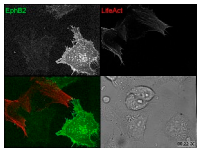


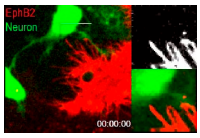
Figure S5. **Control data for the siRNA screen of Rho GEFs and GAPs and the role of Tiams in EphB2ΔC trans-endocytosis.** (A) Frequency distribution of vesicles per cell for control conditions (scramble, ephrinB2, and ephrinB1+2 knockdowns). Data were taken from all 26 plates, with 4 scramble, 3 ephrinB2, and 2 ephrinB1+2 wells per plate. Values included in the cutoff of 40% lie within dotted box. (B) Significance between control conditions at 40% cutoff. Data were taken from all 26 plates, outlined by the dotted box in A. Results are shown as mean ± SE; *, $P < 0.05$; ***, $P < 0.001$, repeated measures one-way ANOVA with Dunnett's post hoc test. (C) Z-scores for all conditions in all 26 plates were calculated for each plate set at a cutoff ($X \leq$ number of vesicles) set to contain 40% of scramble cells from the mean value for each individual plate. Hits were considered as positive regulators of Eph–ephrin endocytosis when below −1.25 and negative regulators when above 1.75. (D) Table of failure rates for the positive (ephrinB1 and ephrinB1+2) and negative (Scramble) controls. Note that no positive control increased endocytosis. (E) Western blot showing Tiam2 knockdown efficiency in SKN and HeLa cells after 72- and 48-h siRNA treatment, respectively. Antibodies probed against endogenous protein. (F) Western blot showing Tiam1 knockdown efficiency in SKN cells after 72-h siRNA treatment. Antibodies probed against endogenous protein. (G) Quantification showing the effect of Tiam1 and Tiam2 depletion on EphB2-Fc endocytosis in ephrinB$^+$ SKN. SKN cells were treated with indicated siRNAs before stimulation with fluorescently labeled, preclustered EphB2-Fc, fixed without permeabilization, and stained against Fc (surface EphB2). Images were analyzed with CellProfiler. Results are shown as mean ± SE ($n = 4$ independent experiments, >472 cells per condition per experiment, data normalized to median scramble value per experiment). ns, not significant; **, $P < 0.01$; ***, $P < 0.001$, repeated measures one-way ANOVA with Dunnett's post hoc test. (H) Pull-down of active Rac1 after Tiam2 and Rac1 overexpression in Hek293 cells. Representative Western blots showing active Rac1 pulled down (bottom panel) with PAK-containing beads from cells transfected with either GFP (left lane) or Tiam2ΔN-GFP/HA (right lane). Total Rac1 and Tiam2ΔN input levels shown in top panels. (I) Quantification for the rescue of the reduction in EphB2ΔC trans-endocytosis in SKN cells after Tiam2 depletion. SKN responder cells were treated with siRNA for 72 h and transfected with either GFP or Tiam2ΔN-GFP/HA for 48 h before co-culture with EphB2ΔC-mCherry/Flag$^+$ cells for 80 min. Cells were fixed and probed against Flag without permeabilization. Results shown as mean ± SE ($n = 4$ independent experiments, 31–90 responder cells per condition per experiment, data normalized to median of GFP-expressing, scramble-treated cells). ns, not significant; *, $P < 0.05$; ***, $P < 0.001$, repeated measures one-way ANOVA with Dunnett's post hoc test. (J) RT-PCR expression profile for Tiam1 and Tiam2 in HeLa and SKN cells. Tiam2 were probed against transcript variant 1 only (Tiam2 tv1) or all Tiam2 transcript variants (Tiam2).



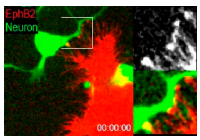
Video 1. **F-actin is enriched at sites of EphB2 trans-endocytosis into SKN cells.** Representative movie from Fig. 1 (C and E–G) showing EphB2ΔC-GFP (green) trans-endocytosis into ephrinB$^+$ SKN cell overexpressing LifeAct-mCherry (red). Cells were co-cultured and imaged live once contact occurred. EphB2ΔC-GFP–positive vesicles (green and yellow puncta) can be seen in the SKN cell. Time stamp shows hours:minutes:seconds.



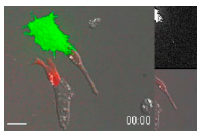
Video 2. **Rac inhibition blocks F-actin enrichment at sites of EphB2 contact with SKN cells.** Representative movie from Fig. 1 (D, H, and I) and Fig. S1 B showing EphB2ΔC-GFP (green) trans-endocytosis into ephrinB$^+$ SKN cell overexpressing LifeAct-mCherry (red) exposed to 10 µM EHT1864. Cells were co-cultured and imaged live once contact occurred. Time stamp shows hours:minutes:seconds.



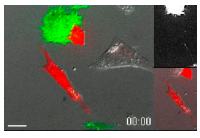
Video 3. **EphB2ΔC trans-endocytosis into primary neurons.** Representative movie for Fig. 3 (A and B) showing EphB2ΔC trans-endocytosis into embryonic day 15.5 mouse cortical neurons in vehicle control condition. Neurons were stained with CellTracker green, co-cultured with EphB2ΔC-mCherry–positive HeLa cells, and imaged live. Time stamp shows hours:minutes:seconds.



Video 4. **Rac inhibition blocks EphB2ΔC trans-endocytosis into primary neurons.** Representative movie for Fig. 3 (A and B) showing prolonged contact between EphB2ΔC$^+$ cells and embryonic day 15.5 mouse cortical neurons after 10 µM EHT1864 treatment. Neurons were stained with CellTracker green, co-cultured with EphB2ΔC-mCherry–positive HeLa cells, and imaged live. Time stamp shows hours:minutes:seconds.



Video 5. **EphB2ΔC-triggered cell repulsion in SKN cells.** Representative movie for Fig. 8 (A and B) showing EphB2-triggered cell repulsion in ephrinB$^+$ SKN cells in the vehicle control condition. SKN cells were transfected with mCherry, co-cultured with EphB2ΔC-GFP–positive HeLa cells, and imaged live. Time stamp shows hours:minutes. Bar, 20 µm.



Video 6. **Rac inhibition blocks EphB2ΔC-triggered cell repulsion.** Representative movie for Fig. 8 (A and B) showing prolonged contact between EphB2$^+$ cells and ephrinB$^+$ SKN cells after 10-µM EHT1864 treatment. SKN cells were transfected with mCherry, co-cultured with EphB2ΔC-GFP–positive HeLa cells, and imaged live. Time stamp shows hours:minutes. Bar, 20 µm.

Provided online is Table S1, showing results from the Rho GEF and GAP screen and the entire data set for the siRNA screen from Figs. 6 and S4. Included in the table are the siRNA sequences for each gene, the average z-score from the two runs, and the combined gene average. GTPase specificity is also labeled.