Schaupp et al., http://www.jcb.org/cgi/content/full/jcb.201305037/DC1

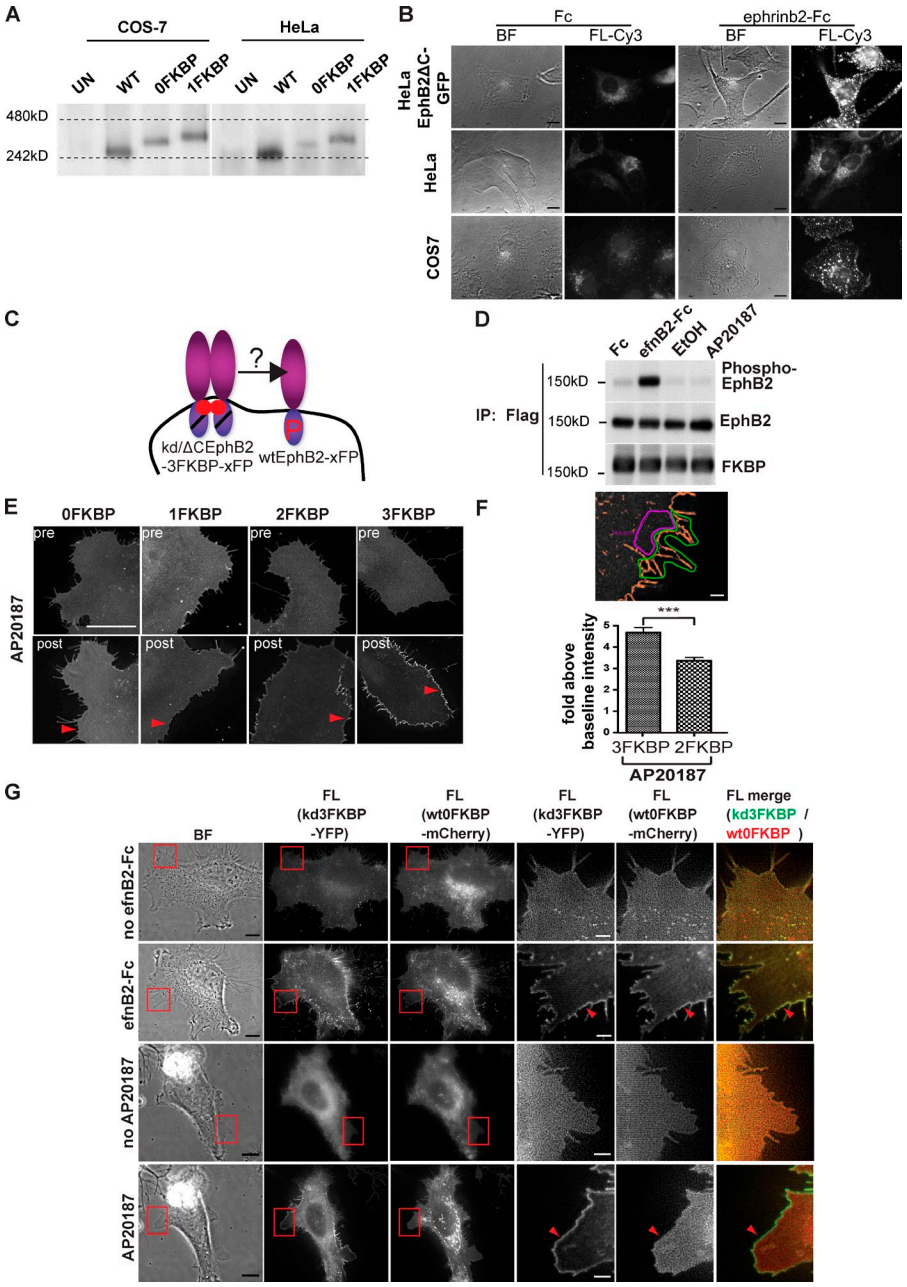


Figure S1. **Dimerizer-induced EphB2 clusters do not activate or recruit wtEphB2.** (A) Blue native PAGE of lysates of unstimulated COS-7 (left) and HeLa (right) cells either left untransfected or expressing wtEphB2 without a fluorescent tag (WT), wtEphB2-YFP (0FKBP), and wtEphB2-YFP carrying one FKBP domain (1FKBP). Western blot was performed with SAM domain–specific anti-EphB2 antibodies. Note that the migration of the different EphB2 isoforms is similar in both cell lines, despite the fact that the levels of endogenous Ephs are quite different (see B). (B) Representative BF and fluorescence images of untransfected HeLa and COS7 cells and HeLa cells stably expressing EphB2ΔC-GFP (positive control). Cells were stimulated for 30 min with 2 µg/ml of either Fc control or ephrinB2-Fc preclustered with anti-Fc-Cy3 antibody. Bars, 10 µm. Fc controls show unspecific staining for the three types of cells. Cy3 clusters seen in COS7 cells and HeLa EphB2ΔC-GFP cell line represent ephrinB2 bound to surface EphB/EphA4. Untransfected HeLa cells stimulated with either Fc or ephrinB2 show similar unspecific staining. The results confirm that HeLa cells express very low levels, whereas COS7 cells express significant levels of endogenous EphB/EphA4. (C) Cartoon depicting the experimental model to test if dimerizer-induced EphB2 clusters laterally recruit wtEphB2 lacking FKBP domains. (D) wtEphB2 does not interact with dimerizer-induced clusters. HeLa cells were transfected with wtEphB2 carrying a Flag epitope together with EphB2-3FKBP lacking kinase and SAM domains (EphB2ΔC-3FKBP), which cannot undergo tyrosine phosphorylation but should still be able to laterally recruit other Ephs via its ectodomain (as shown for EphA3 in Wimmer-Kleikamp et al. [2004]). Western blots of anti-Flag–immunoprecipitated wtEphB2 using anti-phospho-EphB2 antibodies (top), followed by anti-EphB2 antibodies (against C-terminal SAM domain to visualize wtEphB2). Anti-FKBP Western blot visualizes EphB2ΔC-3FKBP. Note that wtEphB2 is not autophosphorylated in AP20187 dimerizer-stimulated cells, only after ephrinB2-Fc stimulation. (E) EphB2 accumulation at cell edges correlates with the number of inserted FKBP domains. HeLa cells expressing YFP-tagged kdEphB2-[0–3]FKBP isoforms were imaged in high resolution before (pre) and after (post) stimulation (10 min) with AP20187 dimerizer (250 nM). Arrowheads indicate sites of receptor accumulation at cell edges correlating with the number of FKBP domains. Bar, 10 µm. (F) Quantification of receptor accumulation at cell edges. The approach is shown as a screenshot from the analysis procedure in MetaMorph software (kdEphB2-2FKBP/AP20187). In fluorescence thresholded images two regions were defined, one to outline the cell edge where accumulation occurs and one to define a region close by to correct for receptor membrane density (baseline intensity). Images for analysis: maximum projection from 6× 0.2-µm z-stacks; background correction. Data represent mean fold above baseline intensity ± SEM of $n = 15$ and 14 region pairs from five cells for 3FKBP/AP20187 and 2FKBP/AP20187; ***, $P < 0.001$; one-way ANOVA with post hoc Bonferroni test. Bar, 0.5 µm. (G) Dimerizer-induced EphB2 clusters do not recruit wtEphB2. HeLa cells were cotransfected with kdEphB2-3FKBP (tagged with EYFP) and wtEphB2-mCherry. Before stimulation with ephrinB2-Fc or AP20187, both receptor isoforms are evenly distributed in the cell (first and third rows). Upon stimulation with ephrinB2-Fc, both receptor variants cluster/accumulate at cell edges (second row, arrowheads). Upon stimulation ($t = 30$ min) with dimerizer AP20187, only kdEphB2-3FKBP accumulates at cell edges (bottom, red arrowheads; merge, green channel), whereas EphB2-mCherry remains evenly distributed (merge, red channel). BF and fluorescence images in the first three columns are low power magnifications. Red boxes indicate areas of higher magnifications. Image acquisition and processing: 6× 0.2-µm z-stacked epifluorescence images; optical density correction; adaptive-blind psf deconvolution; sum projection. Bars: (left) 10 µm; (right) 2 µm.

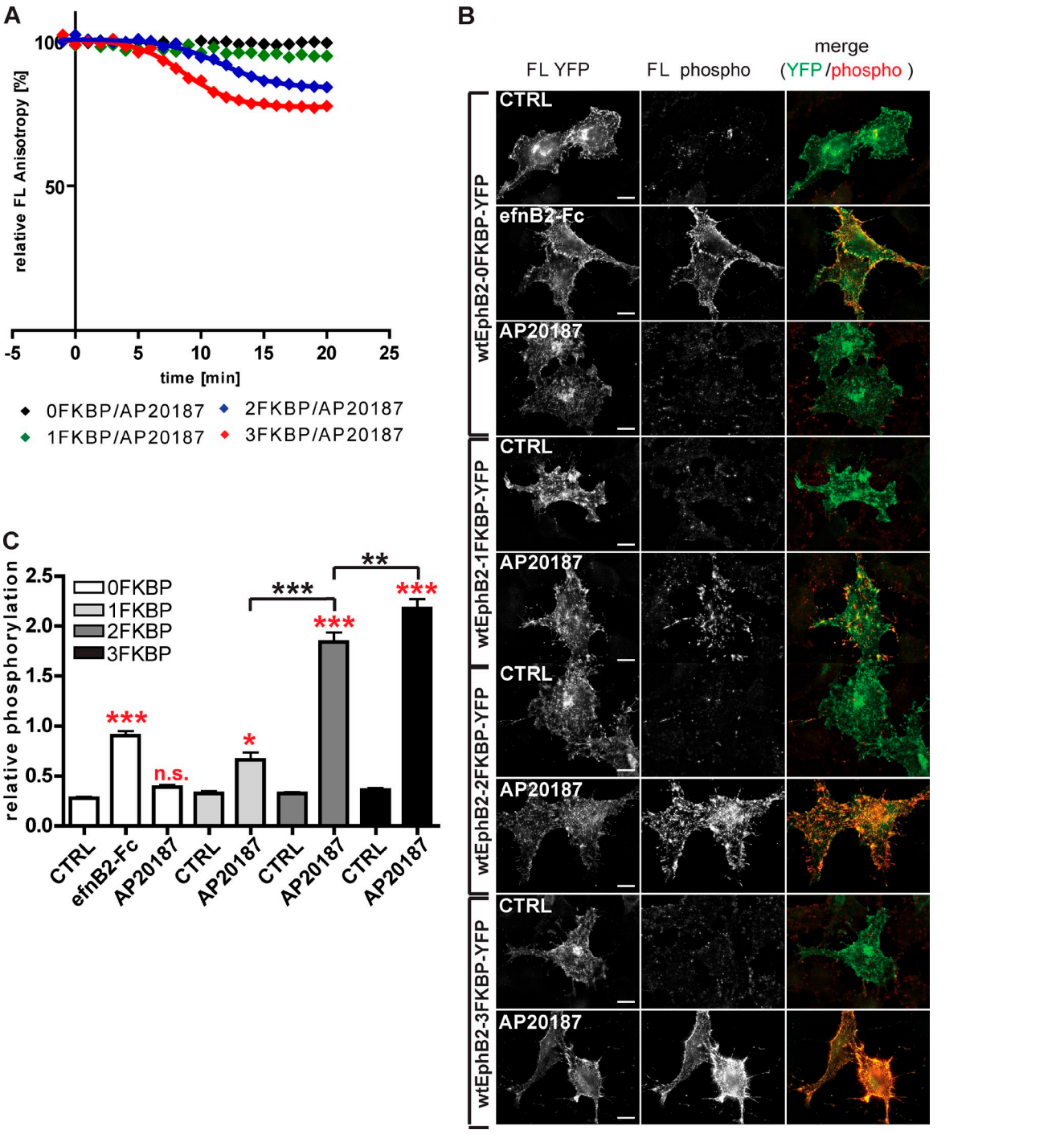


Figure S2. **Analysis of EphB2 clusters by fluorescence anisotropy and autophosphorylation.** (A) Dimerizer-induced EphB2 clustering kinetics visualized by time-lapse fluorescence anisotropy. The indicated FKBP isoforms were transiently transfected in COS-7 cells. Fluorescence anisotropy recordings show a sigmoidal decrease after AP20187 stimulation over time, reaching steady-state after 20 min. Fluorescence anisotropy is indicated in percentage relative to unstimulated condition, normalized to 0FKBP/AP20187 control. Curves are representative examples of time-lapse anisotropy measurements from single cells. In total, $n = 57$, 28, 22, and 20 cells were analyzed from $n = 3$ independent experiments for 3FKBP, 2FKBP, 1FKBP, and 0FKBP. (B) Representative fluorescence images of HeLa cells expressing different FKBP isoforms of wtEphB2 used for quantification in C. Cells were stimulated with ethanol (CTRL), 250 nM AP20187, or 2 µg/ml of preclustered ephrinB2-Fc for 30 min. After fixation and permeabilization, cells were stained with anti-phospho-EphB2 antibodies. Total EphB2 protein levels are visualized by YFP fluorescence and were used to normalize the phospho-EphB2 signal. Image processing: 12× 0.2-µm z-stacked epifluorescence images; polynomial optical density correction; adaptive-blind psf deconvolution; maximum projection; equal scaling over all YFP fluorescence and phosphofluorescence images. Bars, 5 µm. (C) Quantification of EphB2 activation by immunostaining of individual transfected cells. Graph displays mean ratio ± SEM of phosphorylated versus total EphB2 protein (YFP tag) over $n = 25–46$ HeLa cells (fluorescence intensity total protein <2,000 a.u.) per condition tested as indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant; one-way ANOVA with post hoc Tukey-Kramer test; asterisks in red represent significance level to Fc/EtOH control stimulation of each dataset.

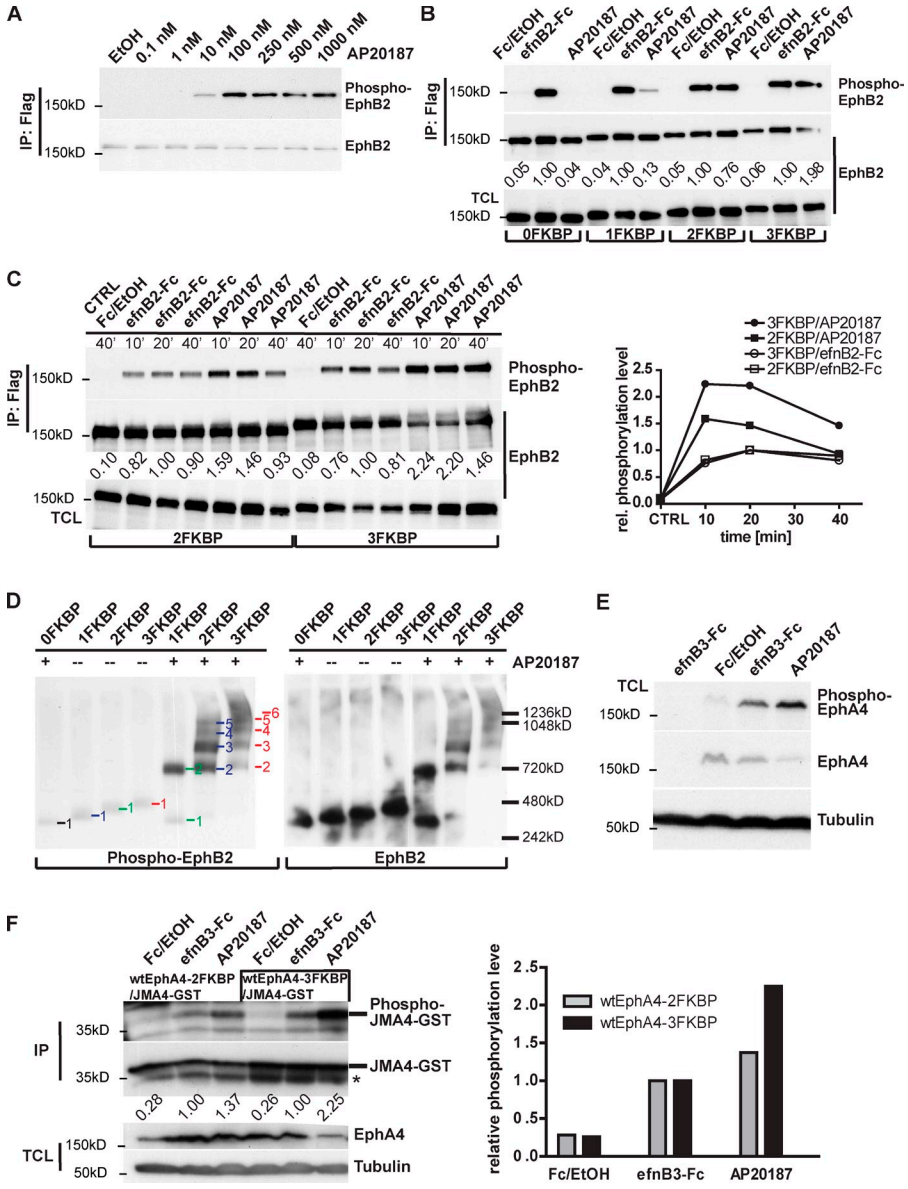


Figure S3. **Dimerizer-induced Eph kinase activation and exogenous substrate phosphorylation.** (A) Western blot of dimerizer-induced EphB2-3FKBP autophosphorylation using the indicated doses of AP20187. EphB2 was immunoprecipitated using the Flag epitope tag and immunoblotted with anti-phospho-EphB2 antibodies. Blot was stripped and reprobed with anti-Flag antibodies. (B) Representative Western blot of anti-Flag–immunoprecipitated EphB2-FKBP isoforms (as indicated) using anti-phospho-EphB2 antibodies; blots were stripped and reblotted for total EphB2 protein levels. For comparison, EphB2 levels in total cell lysates (TCL) are also shown. Autophosphorylation analysis of cells stimulated with Fc/EtOH control or AP20187 (250 nM; *t* = 20 min) indicates a positive correlation between the fraction of autophosphorylated EphB2 and the number of FKBP domains relative to a positive control stimulation with 2 µg/ml of preclustered ephrinB2-Fc (quantification is indicated as fold change of ephrinB2-Fc stimulation; similar results were obtained in three to four separate experiments). (C) Western blot of representative time course of activation of EphB2 carrying 2 or 3FKBP domains. Experiment and quantifications were done as in B. Preclustered ephrinB2-Fc (2 µg/ml) was used to normalize the EphB2 autophosphorylation levels (stimulation with ephrinB2-Fc at 20 min was set at 1.0). Note that dimerizer-induced phosphorylation levels of the 3FKBP construct were higher than the 2FKBP construct over all time points. The experiment was repeated three times with the same outcome. (D) Western blots of blue native PAGE experiment separating different EphB2 cluster species. Left blot shows phospho-EphB2–containing oligomers (same as in Fig. 3 A); blot was stripped and reblotted for total EphB2 protein (right blot). EphB2 oligomers are indicated with numbers (1, monomers; 2, dimers; etc.). Optical density of total EphB2 protein bands was used for normalization to generate cumulative and individual phosphorylation levels of cluster species (see Fig. 3, B and C) and relative abundance of cluster species (Fig. 3 D). (E) Western blot analysis of total lysates from wtEphA4-3FKBP-YFP–transfected HeLa cells to test for EphA4 autophosphorylation upon stimulation (40 min) with control, ephrinB3-Fc (2 µg/ml), or AP20187 (250 nM). AP20187-induced clustering was sufficient to activate EphA4 receptors. (F) Western blot of exogenous substrate phosphorylation by 2 and 3FKBP isoforms of EphA4. The exogenous EphA4 substrate (JMA4-GST) consists of GST and the juxtamembrane segment of EphA4 (Egea et al., 2005). HeLa cells were transfected with JMA4-GST together with EphA4-2FKBP or EphA4-3FKBP. Cells were stimulated as indicated with control stimuli (Fc/ethanol), preclustered ephrinB3-Fc (2 µg/ml), or AP20187 (250 nM) for 40 min. JMA4-GST was immunoprecipitated from cell lysates and probed with anti-phosphotyrosine antibodies or anti-GST antibodies. Overall levels of EphA4 and tubulin are shown. Numbers in blots and graph indicate relative substrate phosphorylation levels quantified from blots with unsaturated exposure normalized to ephrinB3-Fc stimulation. Bottom band in JMA4-GST blot is likely to be endogenous GST (indicated with an asterisk) because it is present in untransfected cells (unpublished data). The experiment was repeated three times with the same outcome.

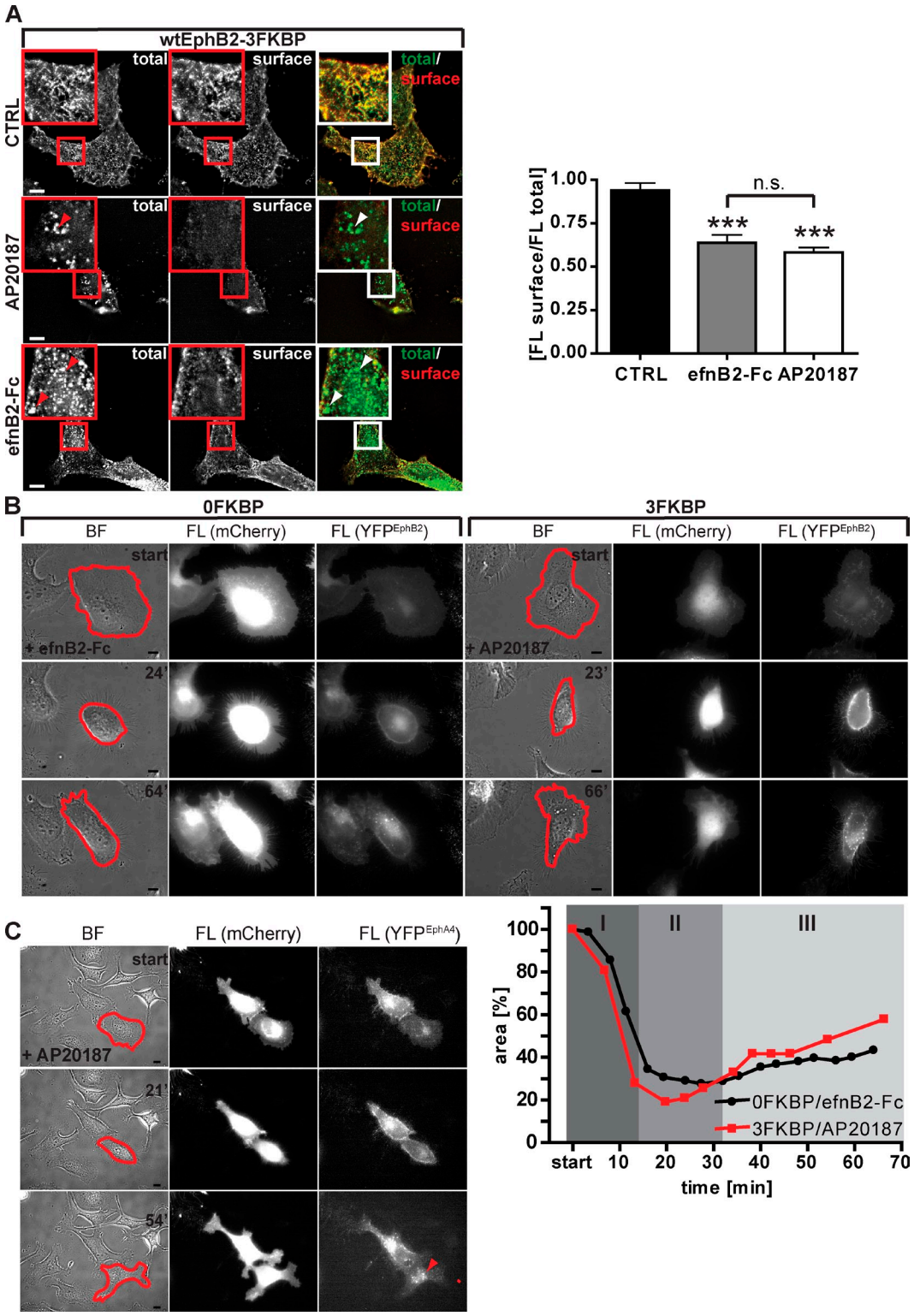


Figure S4. **Dimerizer-induced EphB2 clustering is sufficient to trigger cellular responses.** (A) Dimerizer-induced EphB2 internalization. HeLa cells transiently expressing wtEphB2-3FKBP receptors (carrying YFP) were left untreated (CTRL) or stimulated with preclustered ephrinB2-Fc (2 µg/ml) or AP20187 (250 mM) for 90 min, and then fixed without permeabilization and stained for surface EphB2 with anti-Flag antibodies. YFP clusters that do not colocalize with anti-Flag staining (green in the merge) represent intracellular clusters. Yellow or red clusters in the merge represent surface clusters. (right) Graph showing receptor internalization was quantified by the mean ratio of fluorescence intensity (surface/total) ± SEM from clustered subcellular regions of $n \geq 5$ cells (***, $P < 0.001$; n.s., not significant; one-way ANOVA with post hoc Bonferroni test). Quantification over many cells from one representative experiment. In total the experiment was repeated three times with the same outcome. Bars: (main images) 10 µm; (insets) 3 µm. (B) Dimerizer-induced EphB2 clustering triggers cell collapse. Fluorescence time-lapse imaging of HeLa cells transiently expressing wtEphB2-3FKBP receptors (carrying YFP) in the presence of 2 µg/ml of preclustered ephrinB2-Fc or 250 mM AP20187. The cells' surface area was scored over time and plotted in the graph (in percentage relative to the start of the experiment). Maximum cell collapse is reached at around 20 min after stimulation (phase II) followed by respreading of the cell starting at around 30 min after stimulation (phase III). Myristoylated mCherry was coexpressed to better visualize the cells. Bars, 10 µm. (C) Fluorescence time-lapse imaging of HeLa cells transiently expressing wtEphA4-3FKBP receptors (carrying YFP) in the presence of AP20187. EphA4$^+$ Cells display the same response pattern as EphB2$^+$ cells shown in B. Images before and 21 and 54 min after stimulation are displayed in BF and fluorescence channels. Myristoylated mCherry was coexpressed to better visualize the cells. Bars, 10 µm.

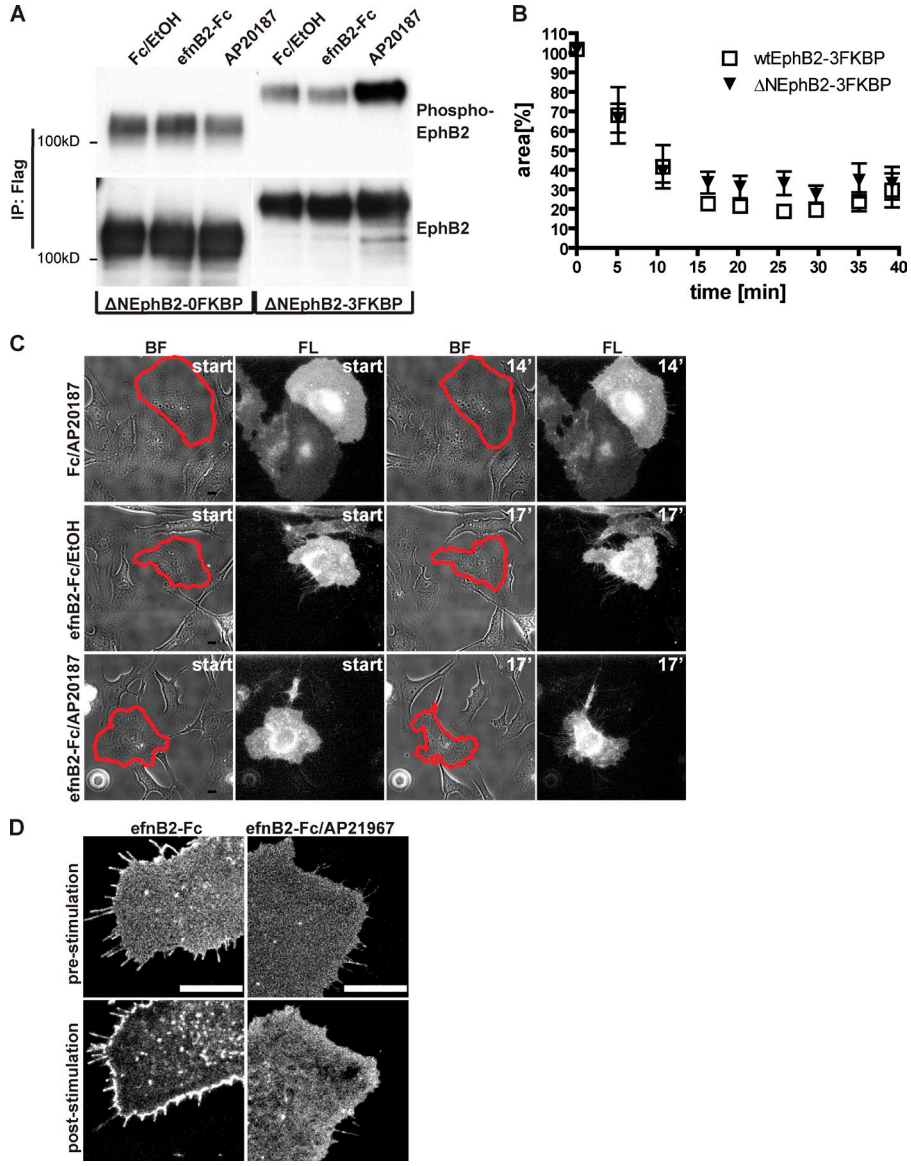
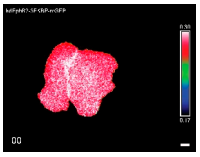
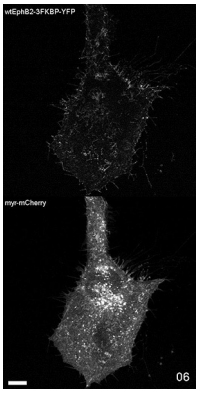


Figure S5. **N-terminally truncated EphB2-FKBP isoforms are functional.** (A) Western blot of anti-Flag–immunoprecipitated, N-terminally truncated ΔNEphB2 either lacking or carrying 3FKBP domains using anti-phospho-EphB2 antibodies. Cells were stimulated with control stimuli (Fc protein plus ethanol), 2 μg/ml of preclustered ephrinB2-Fc, or 250 nM AP20187. Only the 3FKBP isoform shows an increase in EphB2 autophosphorylation after AP20187 stimulation. EphrinB2-Fc is inactive because of the deletion of the ephrin ligand binding domain in EphB2. Blot was stripped and reblotted with anti-EphB2 antibodies. (B) Cell collapse assay comparing cells expressing 3FKBP isoforms of ΔNEphB2 or full-length EphB2 and stimulated with 250 nM AP20187. Cell response patterns do not significantly differ from each other (mean cell area ± SEM of $n = 5$ and $n = 9$ cells of full-length EphB2 and ΔN-EphB2 cells, respectively; $P = 0.27$; Mann-Whitney Wilcoxon nonparametric test). (C) EphB2 dimerization sensitizes cells toward extracellular ephrinB2. Representative images of HeLa cells in BF and fluorescence expressing similar low levels of wtEphB2 carrying 1FKBP domain, before and after stimulation with the indicated combinations of stimuli (used for quantification in Fig. 6 C). Cell collapse was scored as outlined previously from cell surface area measurements over time (red outline around cells). (D) Inhibition of EphB2 clustering reduces sensitivity of cells toward extracellular ephrinB2. Representative images of HeLa cells transiently expressing kdEphB2-3FKBP-YFP and myr-FRB-mCherry, stimulated with ephrinB2-Fc alone or ephrinB2-Fc together with AP21967 ($t = 10$ min). Stimulation with ephrinB2-Fc alone leads to accumulation of EphB2 clusters in the plasma membrane at cell edges (bottom left); the presence of AP21967 prevents this effect (bottom right). Bars, 10 μm. Image processing: 9× 0.2-μm z-stacked epifluorescence images; polynomial optical density correction; adaptive-blind psf deconvolution; sum projection; equal scaling over all images.



Video 1. **Fluorescence anisotropy time-lapse video visualizing Eph receptor clustering kinetics.** COS-7 cells were transfected with kdEphB2-3FKBP-mGFP and stimulated with AP20187 (250 nM) 1 min after start of epifluorescence time-lapse acquisition (frame 2). Images were acquired using an inverted microscope (IX81; Olympus) equipped with a MT20 illumination system. Acquisition at 1 frame/min. Fluorescence anisotropy is displayed according to the color code on the right. Bar, 10 µm.



Video 2. **Confocal time-lapse video showing HeLa cell collapse.** HeLa cells were transfected with wtEphB2-3FKBP-YFP and myr-mCherry (for better visualization) and stimulated with AP20187 (250 nM) immediately before the start of confocal time-lapse acquisition. Images were recorded by time-lapse confocal microscopy (Fluoview FV1000; Olympus) equipped with an Argon ion laser. Acquisition at 1 frame/min. Fluorescence intensity is shown. Bar, 10 µm. The HeLa cell shows a characteristic response pattern with contraction and respreading upon clustering with AP20187. Approximately 30 min after stimulation, internalized EphB2-loaded vesicles become visible (see also Fig. S4).

References

Wimmer-Kleikamp, S.H., P.W. Janes, A. Squire, P.I.H. Bastiaens, and M. Lackmann. 2004. Recruitment of Eph receptors into signaling clusters does not require ephrin contact. *J. Cell Biol.* 164:661–666. http://dx.doi.org/10.1083/jcb.200312001

Egea, J., U.V. Nissen, A. Dufour, M. Sahin, P. Greer, K. Kullander, T.D. Mrsic-Flogel, M.E. Greenberg, O. Kiehn, P. Vanderhaeghen, and R. Klein. 2005. Regulation of EphA 4 kinase activity is required for a subset of axon guidance decisions suggesting a key role for receptor clustering in Eph function. *Neuron*. 47:515–528. http://dx.doi.org/10.1016/j.neuron.2005.06.029