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Spatial Control of Membrane Receptor Function using Ligand Nano–Calipers

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Abstract

The spatial organization of membrane-bound ligands is thought to regulate receptor mediated signaling. However, direct regulation of receptor function by nanoscale distribution of ligands has not been demonstrated. We developed rationally designed DNA origami nanostructures modified with ligands at well-defined positions. Using these types of *nano-calipers* to present ephrin ligands, here we show that the nanoscale spacing of ephrin-A5 directs the levels of EphA2 receptor activation in human breast cancer cells. Furthermore, we found that the nanoscale distribution of ephrin-A5 regulates the invasive properties of breast cancer cells. The ligand nano-caliper method we introduce here has the potential to provide new insights into the roles of the spatial distribution of ligands at the nanoscale in signaling through membrane receptors.

Introduction

Cells that are nearest neighbors coordinate their activities in tissues through interactions between membrane-bound receptors that specifically bind ligands presented by adjacent cells. This coordination is often dysregulated in cancer, allowing cells to acquire abnormal functions such as aberrant proliferation and invasion of neighboring tissues. The nanoscale distribution of ligands is hypothesized to regulate signaling mediated by membrane receptors, translating subtle differences in ligand nanoarchitecture into a diversity of cellular responses¹. However, the underlying mechanisms are poorly understood due to difficulties in controlling the spatial distribution of protein assemblies at the nanoscale. Previous approaches for studies of ligand and receptor clustering have either used patterning of surface-anchored ligands^{2,3} or antibody-mediated clustering in solution⁴. In contrast to these methods, we present an approach to control the spatial distribution of ligands that offers accurate nanoscale distance manipulation, which can be tuned independently of ligand concentration. This method, which we call ligand nano-calipers,

produces precise patterns of ligands, enabling us to map the relationships between the nanoscale spacing of ligands and receptor activation, intracellular signaling and cellular outcomes. Further, nano-calipers display the ligands in solution-phase, similarly to the antibody clustering method, allowing nano-calipers to be added to the media of cells cultured in standard conditions.

Importantly, because of the solution-phase display, the method can be used to manipulate cells in 3D tissue models and, as such, has the potential to provide new knowledge for development of therapeutic strategies.

Here we apply our ligand nano-caliper method to the investigation of the roles of the spatial distribution of ephrin ligands in Eph receptor function. Eph receptors bind ephrins in adjacent cells, inducing receptor phosphorylation and activation of intracellular signaling pathways that primarily regulate cell migration and proliferation. Eph receptor signaling is critical in many developmental processes and is frequently disrupted in cancer, showing both tumor promoting and suppressing effects, depending on the cellular microenvironment⁵⁻⁸. Mounting evidence suggests that the nanoscale spatial distribution of ephrin ligands is a relevant physical signal in Eph receptor activation^{1,2,9}, potentially contributing to the diversity of cell outcomes. For example, soluble recombinant ephrin dimers induce Eph receptor phosphorylation to a larger extent than monomers^{10,11}, reflecting the requirement for receptor dimerization for the release of auto-inhibition of the kinase domain. Moreover, higher order clusters of recombinant ephrins in solution, produced by combining ephrin-Fc fusion proteins with anti-Fc antibodies, were found to produce a subset of cellular responses *in vitro*, such as the formation of endothelial tubes, which are not elicited by ephrin dimers¹². Interestingly, constraining the lateral mobility of ephrin-A1 ligands in supported lipid bilayers caused spatial reorganization of EphA2 receptors and modulated cytoskeletal morphology in overlying cells². *In vivo*, EphA4 has been shown to have higher-order cluster dependent functions in neurodevelopment such as hindlimb locomotion,

as well as cluster independent activities¹³. Using ligand nano-calipers, here we show that the nanoscale spatial distribution of ephrin-A5 ligands tunes EphA2 receptor activation levels and the invasive properties of breast cancer cells.

Results

Design and synthesis of ephrin-A5 nano-calipers

To investigate the roles of ligand nanoscale spacing in EphA2 receptor activation, we developed a collection of nanostructures, where each set of structures displayed ephrin-A5 ligands at different distances. This was achieved using DNA nanotechnology^{14,15}, which recently showed increasing promise for nanoscale positioning of proteins¹⁶⁻²². Here we produce solution-phase, ligand nanopatterns that bind EphA2 receptors and are able to move laterally on the cell membrane. By using DNA origami^{15,23,24}, we designed a base nano-caliper structure and then modified it to display short single-stranded DNA (ssDNA) sequences at the surface at regular intervals. Ephrin-A5-Fc chimeric protein, which spontaneously forms ephrin-A5 dimers, was conjugated to the 3' end of a target ssDNA oligonucleotide (**Supplementary Fig. 1**) that was designed to hybridize to the sequences displayed at the surface of the nano-calipers (**Fig. 1a,b**). These ephrin-A5-Fc oligonucleotide conjugates are henceforth referred to as ephrin-A5 conjugates.

The base design for our nano-calipers is a DNA origami structure comprising 18 parallel double helices in a hollow tube-like arrangement (**Supplementary Fig. 2** and **Supplementary Table 1**). We modified this base design (NC-empty, **Fig. 1c**) to display protruding 5' ssDNA handles to bind ephrin-A5 conjugates through hybridization (**Supplementary Fig. 3**). Each binding site was implemented as a pair of protruding ends separated by three double-helices in a honey-comb arrangement²³, to ensure high yield of bound ephrin-A5 conjugates at each site. The binding sites were then separated along the helical direction by 100.1 nm or 42.9 nm, hereafter referred to as

NC100 (**Fig. 1e**) and NC40 (**Fig. 1f**), respectively. Further, we included one design with a single binding site, NC0 (**Fig. 1d**). Membrane microdomains with dimensions ranging from 10 nm to 200 nm are thought to mediate spatial regulation of receptor signaling.^{3,25,26} Therefore, we designed our tool to control spatial distributions of ligands within this range. The 100 nm spacing is on the limit of what can be achieved with a rigid DNA–origami with the currently available scaffolds and the 43 nm spacing was selected as an intermediate distance (**Supplementary Figs. 2 and 3**). The DNA origami structures were folded as described elsewhere^{15,23,27}.

Characterization of ephrin–A5 nano–calipers

We verified the successful fabrication of the ephrin–A5 nano–calipers by transmission electron microscopy (TEM) (**Fig. 1c–f** and **Supplementary Fig. 4**). In addition to TEM, agarose gel shift assays confirmed efficient hybridization of the ephrin–A5 conjugates to the nano–calipers, indicating that the fractions of NC100 and NC40 bound with two dimers were close to 90% (**Fig. 1g, Supplementary Figs. 5–8**).

To analyze the binding ability of the ephrin–A5 conjugates and complete ephrin–A5 nano–calipers to the EphA2 receptor, we performed surface plasmon resonance (SPR) measurements. We compared the binding curves of the ephrin–A5 conjugates to the extracellular domain of human EphA2 receptor to the binding curves of unmodified ephrin–A5–Fc (**Supplementary Fig. 9**). The measurements showed no clear loss of binding activity upon conjugation of the ligand, which indicates that the bioavailability of ephrin–A5 was retained. This is in line with our estimate that more than 95% of the binding domains of ephrin–A5 are available for EphA2 binding after conjugation (**Supplementary Fig. 10**). Further, we performed SPR analysis on ephrin–A5 nano–calipers (**Fig. 2a**, explanation of curves in **Supplementary Fig. 11**, curve fitting in **Supplementary Fig. 9**) and the results showed that NC0 exhibited similar binding affinity as ephrin–A5–Fc and ephrin–A5 conjugates, indicating that conjugation and further hybridization to

the nano-calipers does not interfere with the ephrin-A5 binding activity. Moreover, NC100 and NC40 showed an enhancement in EphA2 binding affinity when compared to NC0, indicating that NC100 and NC40 are able to bind EphA2 with avidity. To complement the SPR binding assay, we performed a magnetic bead based EphA2 pull-down assay with the ephrin-A5 nano-calipers (**Supplementary Fig. 12**) to probe the amount of ephrin-A5 on the nano-calipers available for EphA2 binding. The results showed clear EphA2 binding activity that scaled well with the designed number of binding sites on the nano-calipers and, importantly, NC100 and NC40 showed similar binding. Together, the results from TEM imaging, gel shift assays, SPR binding and EphA2 pull-down assay support the conclusion that the ephrin-A5 nano-calipers are produced with the anticipated stoichiometry of biological active ligands.

To investigate whether ephrin-A5 nano-calipers were capable of binding EphA2 in an MDA-MB-231 human breast cancer cell line, we performed fluorescence-activated cell sorting (FACS) analysis. These results showed extensive binding of ephrin-A5 nano-calipers to cells whereas NC-empty showed minimal binding. Cells treated with IgG-clustered ephrin-A5 showed a similar increase in binding to EphA2 compared to IgG treated cells (**Fig. 2b**). Thus, ephrin-A5 nano-calipers efficiently bind to EphA2 expressing cells.

Ephrin-A5 nano-calipers direct EphA2 phosphorylation

To quantify EphA2 receptor phosphorylation, we used an *in situ* proximity ligation assay (PLA)²⁸. To enable accurate quantification, we cultured MDA-MB-231 cells as single cells on substrates with micropatterned fibronectin islands of defined size, which prevent receptor activation through cell-cell contact, resulting in a PLA that is highly sensitive and reproducible. To validate the method, we analyzed the phosphorylation of the EphA2 receptor in response to commonly used conditions to study Eph receptor signaling using recombinant ligands (using 10 nM of ephrin-A5-Fc). We observed the predicted gradual increase in receptor phosphorylation in cells treated

with IgG, ephrin–A5 monomers, ephrin–A5 dimers (ephrin–A5–Fc) and IgG–clustered ephrin–A5–Fc (**Fig. 3a,b** and **Supplementary Fig. 13**). EphA2 immunoprecipitations followed by anti-phosphotyrosine immunoblotting of cells treated with IgG, ephrin–A5 monomer, ephrin–A5 dimer and IgG–clustered ephrin–A5 showed similar activation trends as the PLA (**Supplementary Fig. 14**). Immunoprecipitation of cells treated with NC–empty, NC0, NC100 and NC40 showed that ephrin–A5 nano–calipers induced receptor phosphorylation at levels comparable to those triggered by ephrin–A5 dimers and IgG–clustered ephrin–A5 (**Supplementary Fig. 14**). To achieve higher resolution and results not confounded by cell–cell contact, in this study we quantified receptor phosphorylation induced by ephrin–A5 nano–calipers using PLA.

All ephrin–A5 nano–calipers (using 10 nM total concentration of ephrin–A5–Fc), NC0, NC100 and NC40, induced receptor phosphorylation compared to NC–empty control. Further, NC0, which contains a single ephrin–A5 dimer, showed similar activation levels of EphA2 as NC100, at equivalent molar amounts of ephrin–A5, suggesting that no proximity effects are observed in EphA2 phosphorylation induced by NC100. Interestingly, NC40 was more efficient at inducing EphA2 receptor phosphorylation than NC100 (**Fig. 3c,d**). The conclusion that this effect is due to proximity and not differences in binding is strengthened by the fact that the NC100 and NC40 show similar binding affinity in SPR (**Fig. 2a**) and similar binding stoichiometry in the EphA2 pull–down assay (**Supplementary Fig. 12**).

To investigate the effects of local stoichiometry on the activation of EphA2 receptor, we compared the phosphorylation levels induced by NC40 and NC–sat, which has a saturated amount of protruding ends, resulting in eight binding sites for ephrin–A5–Fc separated by 14.3 nm. Interestingly, using this approach to increase the local stoichiometry of ephrin–A5 dimers in nano–calipers, we observed no increase in the levels of phosphorylated EphA2 receptors detected

by PLA (**Supplementary Fig. 15**). Together, these results indicate that the nanoscale spatial distribution of ephrin–A5 tunes the levels of activated EphA2 receptors.

Nano–calipers induce dynamin dependent EphA2 endocytosis

Activation of Eph receptors is followed by endocytosis leading to termination of the signal^{29,30}. To investigate the endocytosis of EphA2 in response to ephrin–A5 nano–calipers, we performed immunocytochemistry experiments of EphA2 receptor and Early Endosome Antigen 1 (EEA1), upon treatment with NC–empty, NC0, NC100 and NC40. The results showed EphA2 receptor internalization and translocation to early endosomes after two hours of stimulation with ephrin–A5 nano–calipers, similarly to cells treated with ephrin–A5 dimers or IgG–clustered ephrin–A5 (**Fig. 4a,c**). In contrast, treatment with NC–empty, IgG or ephrin–A5 monomer did not cause detectable receptor endocytosis at this time (**Fig. 4a,c**). These results suggest that there is endocytosis of EphA2 following activation upon treatment with ephrin–A5 nano–calipers. Moreover, to investigate the mechanisms of endocytosis following receptor activation, we inhibited endocytosis using a dynamin inhibitor, Dyngo–4a. Dynamin inhibition hampered EphA2 endocytosis in ephrin–A5 nano–caliper treated cells, similarly to cells treated with ephrin–A5 dimer or IgG–clustered ephrin–A5 (**Fig. 4b,d**). Therefore, EphA2 endocytosis following ephrin–A5 nano–caliper stimulation is, at least partially, mediated by dynamin.

Ephrin–A5 nano–calipers modulate cell invasion

The EphA2 receptor is overexpressed in many cancers and high expression levels generally correlate with poor prognosis⁵. EphA2 is often weakly phosphorylated in tumors and engages in ligand–independent signaling that promotes cell invasion^{6,31}. Receptor activation, using recombinant ligands or activating antibodies, inhibits cell proliferation and migration and shows tumor suppressive effects³². Accordingly, IgG–clustered ephrin–A5 caused a decrease in cell invasion compared to IgG alone (**Supplementary Fig. 16**). EphA2 activation in MDA–MB–231

cells by ephrin–A5 nano–calipers significantly decreased cell invasiveness compared to cells treated with empty nano–calipers (**Fig. 4e**). Notably, NC40 caused a larger decrease in cell migration than NC100. This is in line with the higher activation of EphA2 receptor by NC40 compared to NC100 (**Fig. 3d**). In conclusion, ephrin–A5 nano–calipers appear capable of modulating suppression of migration by the activated EphA2 receptor.

Discussion

We have developed a method to efficiently present ligands to cell membrane–bound receptors with tailor–made spatial nanoscale distributions. Using DNA origami nanofabrication, the positions of ligands on DNA nano–calipers can be defined with nanometer resolution. Notably, these DNA origami/ligand conjugates offer the opportunity to investigate the roles of nanoscale spatial distribution of ligands on receptor activation and cellular responses in a solution phase display. Here, we use this method to demonstrate that the levels of phosphorylation of the EphA2 receptor are controlled by the nanoscale distribution of ephrin–A5 ligands. Analysis of the crystal structure of EphA2 associated with ephrin–A5 gave rise to the hypothesis that ligand–receptor binding is followed by recruitment of non–ligand–bound EphA2, in a seeding mechanism.^{33–35} We hypothesize that NC40 is more efficient at triggering this effect than NC100. Moreover, NC–sat did not further increase the activation levels compared to NC40. This suggests that the decrease in spacing of ephrin–A5 in NC–sat, and increase in local stoichiometry, does not induce more efficient seeding of EphA2 clusters. Interestingly, NC0, which contains a single ephrin–A5 dimer, showed similar activation levels of EphA2 as NC100. This points to a loss of proximity effects in EphA2 activation for NC100. Together, these results show that EphA2 receptor activation is sensitive to the initial ephrin–A5 spatial distribution upon binding. Furthermore, we show that the nanoscale spatial distribution of ephrin–A5 tunes the invasive properties of breast cancer cells.

Ligand nano-calipers can control the spatial organization of signaling at the cell membrane, which in turn tunes receptor activity and cellular outcomes. This may potentially be relevant in targeting Eph mediated signaling, which can simultaneously activate tumor promoting and suppressing signaling pathways⁸. We suggest that this method could be used to address the roles of nanoscale distribution of ligands in other signaling pathways regulating cell-to-cell communication. The knowledge gained has the potential to provide new insights relevant for the development of pharmacological interventions that target the biophysical context of the ligand-receptor interaction.

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Author contributions

BH and AIT conceived and designed the study. VL, AS, and EP performed most of the experimental work. FF and EB contributed to nanostructure and conjugate production and nano-caliper characterization. AA, AH and AB contributed to cell culture and the setting up and validating of the PLA assay. AB performed immunoprecipitation and immunoblotting

experiments. VL, AS, BH and AIT wrote the manuscript. All authors contributed to manuscript proofing and discussion.

Competing financial interests

The authors declare no competing financial interests.

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Figure 1: The nano-caliper principle and implementation with ephrin ligands. (a,b)

Schematic example showing that by slightly varying the DNA oligonucleotides used for assembly of a DNA origami nano-caliper, protruding 5' sequences can be placed in different positions. Using a protruding version of the black and green sequences or using protruding versions of the black and blue sequences, leaves binding sites at different positions for proteins conjugated to the 3' ends of a DNA-oligo bearing a common complementary binding sequence (black strands with orange circles). **(c-f)** 150 nm x 35 nm TEM micrographs of NC-empty **(c)**, NC0 **(d)**, NC100 **(e)** and NC40 **(f)**. The nano-caliper used here is an 18-helix bundle DNA origami nanotube. The structures were prepared without protein binding sites, with one binding site or with two binding sites, 101.1 nm or 42.9 nm apart, referred to as NC-empty, NC0, NC100 and NC40, respectively. **(g)**, 2% agarose gel run with **i** 1 kb DNA ladder, **ii** p7560 ssDNA, **iii** NC-empty, **iv** NC0, **v** NC100, **vi** NC40 and stained with ethidium bromide.

Figure 2: Ephrin–A5–Fc conjugate and ephrin–A5 nano–caliper binding abilities. (a)

Binding curves of ephrin–A5–Fc conjugates (grey) and ephrin–A5 nano–calipers (green, blue and purple) to the extracellular domain of human EphA2 measured by SPR. The apparent dissociation constants (K_d^{app} (nM) fitted with a 1:1 Langmuir binding model, Supplementary Fig. 9) are presented as an insert. **(b)** FACS analysis of MDA–MB–231 cells stimulated for 15 min with IgG–clustered ephrin–A5 or NC–sat. IgG and NC–empty were used as controls. 488–conjugated anti–human IgG was used to pre–cluster the ephrin–A5 for 15 min. Or, in the case where nano–calipers were used, DyLightTM 488–conjugated anti–human IgG was added to the cell culture medium 15 min after EphA2 stimulation with NC–sat and NC–empty. The percentage of fluorescently labeled cells is indicated in the plots, calculated as average $\pm$ s.e.m. from two independent biological repeats.

Figure 3: The spatial distribution of ephrin–A5 ligands directs the phosphorylation levels of the EphA2 receptor. (a) Phosphorylation of the EphA2 receptor in MDA–MB–231 cells cultured on fibronectin micropatterns after 15 min of treatment with IgG, ephrin–A5 monomer, ephrin–A5 dimer and IgG–clustered ephrin–A5 detected with PLA (magenta dots). (b) Quantification of the PLA of phosphorylated EphA2 receptor (averages). Error bars represent s.e.m., $n = 7$ biological replicates for all conditions except monomer where $n = 5$. (c) Phosphorylated EphA2 receptor (magenta dots) in MDA–MB–231 cells cultured on fibronectin micropatterns detected by PLA. Cells were treated with NC–empty, NC0, NC100 and NC40 for 15 min. Scale bar, 10 μm . (d) Quantification of the PLA of phosphorylated EphA2 receptor in MDA–MB–231 cells grown on fibronectin micropatterns (averages). Error bars represent s.e.m., $n = 6$ biological replicates for all conditions except NC0 where $n = 4$, $P < 0.0001$. 1–Way ANOVA analysis of variance was used followed by Tukey’s Multiple Comparison Test. Cells in (a) and (c) were stained for the actin cytoskeleton using Alexa488–phalloidin (turquoise) and the nuclei using DAPI (blue). In all cases, PLA signal quantification was performed on over 20 cells for each condition, for each biological repeat (* $P < 0.05$, *** $P < 0.001$).

Figure 4: Ephrin–A5 nano–calipers modulate EphA2 mediated responses in human breast cancer cells. (a–d) Immunocytochemistry of MDA–MB–231 cells using an antibody against the EphA2 receptor (turquoise) and the endosomal marker Early Endosome Antigen (EEA1, magenta). Cells were stimulated for 120 min with IgG, ephrin–A5 monomer, ephrin–A5 dimer and IgG–clustered ephrin–A5 (a and b) or with NC–empty, NC0, NC100 or NC40 (c and d). Cells in (b) and (d) were treated with ephrin–A5 in the presence of Dyngo–4a, a dynamin inhibitor. (e) Relative average fold decrease in matrix invasion of the breast cancer cells MDA–MB–231, after treatment with NC–empty, NC100 or NC40. Error bars represent s.e.m., $n = 2$, $P=0.0001$. 1–Way ANOVA analysis of variance was used followed by Tukey’s Multiple Comparison Test (** $P < 0.01$, *** $P < 0.001$).

Methods

p7560 Scaffold ssDNA Preparation From a single colony of *E. coli* JM109 that was cultured overnight in 25 ml lysogeny broth (LB), 3 ml were diluted in 250 ml of 2xYT medium with 5 mM MgCl₂ and placed in a 37 °C shaker. When the Optical Density at 600 nm (OD₆₀₀) reached 0.5, p7560 phages were added at a Multiple of Infection (MOI) of one and incubation with shaking continued for an additional 5 h. The culture was transferred to a 250 ml centrifuge bottle and was centrifuged at 4,000 g for 30 min and the supernatant was centrifuged again at 4,000 g for 20 min. 10 g PEG and 7.5 g NaCl (VWR international) were added to the supernatant containing p7560 phages, which was then incubated on ice for 30 min and centrifuged at 10,000 g for 40 min. Next, the supernatant was removed, the pellet was resuspended in 10 ml 10 mM Tris (pH 8.5, VWR international) and transferred to a 70 ml centrifuge bottle. 10 ml of a solution with 0.2 M NaOH (VWR international), 1 % SDS, was added, mixed gently by inversion and incubated at room temperature for 3 min. Then 7.5 ml of 3 M KOAc pH 5.5, was added, gently mixed by swirling and incubated on ice for 10 min. The mixture was centrifuged at 16,500 g for 30 min. The supernatant containing ssDNA was poured into fresh centrifuge bottles and 50 ml 99.5% EtOH was added, mixed gently by inversion and incubated on ice for 30 min. The solution was centrifuged at 16,500 g for 30 min. After decanting the supernatant, the pellet was washed with 75% EtOH and air dried at room temperature for 15 min. Finally, the pellet was resuspended in 10 mM Tris pH 8.5 and the concentration and quality were characterized by UV–Vis (Nanodrop, Thermo Scientific) and a 1.5% agarose gel, respectively.

Staple Oligonucleotides Preparation Oligonucleotides were purchased from Bioneer in 96 well plates. The staples in each well were diluted to a final concentration of 100 µM. Staples were mixed according to the method described in Supplementary Fig. 3. The final concentration of the staples was adjusted to 200 nM each.

Ligand Conjugation *1. 4FB modification of 3' amino modified oligonucleotide.* A pellet of 46 nmoles of 3' amino modified oligonucleotide (Bioneer) was dissolved in 460 μ l reaction buffer and washed with the same buffer three times in a Vivaspin 5k MWCO spin filter (centrifuged at 15,000 g, 12 min, room temperature, Sartorius) and concentrated to 23 μ l (2 mM of the oligonucleotide concentration). To this solution, 12.5 μ l of 0.172 M Sulfo-S-4FB (Solulink) in DMF were added and incubated for 1 h at room temperature with occasional mixing. This procedure was repeated once. The reaction mixture was diluted with conjugation buffer (PBS pH 6.0) and transferred to a Vivaspin 5k MWCO spin filter (pre-wetted with conjugation buffer) and washed with the same buffer seven times (centrifugation at 15,000 g, 12 min, room temperature) and concentrated to 20 μ l. The 4FB modified oligonucleotide was then stored at 4 °C until further usage.

2. HyNic modification of ligand and conjugation of modified ligand with modified oligonucleotide. 200 μ g of lyophilized ephrin-A5-Fc (Recombinant Human Ephrin-A5-Fc Chimera, R&D Systems) was dissolved in 200 μ l PBS pH 7.4 and split to two 100 μ l fractions, which were processed identically. 100 μ l of ligand solution was buffer exchanged to PBS pH 7.4 with Zebaspin desalting columns 7k MWCO (Thermo Fisher Scientific). 2 μ l of 7.3 μ M Sulfo-S-HyNic (Solulink) in DMF was added and incubated at room temperature for 2 h with occasional mixing. The ligand solution was buffer exchanged to PBS pH 6.0 with Zebaspin desalting columns 7k MWCO and 10 μ l of the 4FB modified oligonucleotide (ca. 10 eq.) was added and incubated at room temperature with occasional mixing. Upon completion, the ligand conjugate solution was diluted with PBS pH 7.4 to 450 μ l, transferred to an Amicon Ultrafiltration Unit 50k MWCO (Millipore) and washed four times with PBS pH 7.4. After the final wash, the volume was adjusted to 100 μ l. To evaluate the efficiency of conjugation of ephrin-A5 to DNA, we measured absorption of light at 350 nm, giving an estimate of the ligand-to-oligonucleotide ratio.

By carefully controlling of the conjugation chemistry, we were able to limit the average number of oligonucleotides conjugated to each ligand between 0.9 and 1.3.

3. Rhodamine conjugation to oligonucleotide modified ligand.

Rhodamine–NHS (Invitrogen) dissolved in DMF was added to the oligonucleotide modified ligand in 100 fold molar excess and incubated at room temperature overnight. The excess rhodamine was removed with Zebaspin desalting columns 7K pre–equilibrated with PBS pH 7.4. The rhodamine conjugates were hybridized to nano–calipers and characterized as described in Supplementary Fig. 7.

Ephrin–A5 Nano–caliper Production The standard folding conditions used in this study were as follows: 20 nM ssDNA scaffold, 100 nM per staple, 13 mM MgCl₂, 5 mM Tris pH 8.5 and 1 mM EDTA. Folding was carried out by rapid heat denaturation followed by slow cooling from 80 °C to 60 °C over 20 min, then 60 °C to 24 °C for 14 h. Removal of excess staples or ligand conjugate was done by washing (repetitive concentration and dilution) the nano–calipers with PBS pH 7.4 10 mM MgCl₂ in 100 kDa MWCO 0.5 ml Amicon Centrifugal Filters (Millipore). Samples were diluted to 450 µl and transferred to a pre–wetted centrifugal filter and centrifuged at 14,000 g, 15 °C for 2 min, then diluted again to 450 µl, mixed well and centrifuged again under same conditions. The volume was adjusted with PBS pH 7.4 10 mM MgCl₂ to a nano–caliper concentration of 20 nM and the sample was collected via centrifugation at 1,000 g for 2 min. To remove excess staples, the nano–calipers were washed five times.

The ligand conjugates were added with a twofold excess to each protruding site on the nano–calipers and incubated in the PCR machine with a temperature ramp starting from 1 h at 37 °C followed by 14 h at 22 °C and immediately after incubation the nano–calipers were stored at 4 °C. Removal of the excess conjugates was carried out by passing the samples consecutively through two sepharose 6B (Sigma–Aldrich) loaded spin columns (Thermo Scientific, the first

column loaded with 400 μ l and the second column loaded with 260 μ l resin) and spun at 15 °C, 800 g for 3 min. The final concentration of the nano-calipers was adjusted to 20 nM by UV-Vis A260 measurement (default extinction coefficient for dsDNA in Nanodrop) of NC-empty as standard and the ephrin-A5 nano-caliper concentration was calculated according to the gel band intensity ratio when compared to the standard (Supplementary Fig. 8).

Agarose gels for characterization of the ephrin-A5 nano-calipers. We prepared 2% agarose gels with 0.5x TBE supplemented with 11 mM $MgCl_2$ (Sigma-Aldrich) and 0.5 mg/ml ethidium bromide (Sigma -Aldrich). We typically loaded 4 μ l of 20 nM suspensions of nanostructures in each lane and ran the gels in 0.5x TBE with 11 mM $MgCl_2$ at 70 V for 4 h cooled in an ice-water bath. We imaged the gels using a UV gel imaging system fitted with a digital camera (Uvipro Silver, Uvitec ltd) and analyzed the images with ImageJ (NIH), MultiGauge (Fujifilm), and GelBandFitter³⁶.

Surface Plasmon Resonance (SPR) The extracellular domain of the human EphA2 receptor (Recombinant Human EphA2 CF, R&D Systems) was dissolved in 10 mM sodium acetate buffer pH 4.5 and immobilized on a CM3 chip (GE Healthcare) according to manufacturer instructions. Ephrin-A5-Fc and Ephrin-A5-Fc oligonucleotide conjugate samples were diluted to concentrations ranging from 12 nM to 120nM in PBS pH 7.4 supplemented with 10 mM $MgCl_2$. Ephrin-A5 nano-caliper samples were diluted in PBS supplemented with 10 mM $MgCl_2$ to a final concentration ranging from 6 nM 24 nM and the binding was measured by BIAcore 2000 (GE Healthcare). The flow rate of the samples was adjusted to 5 μ l/min and a total amount of 35 μ l was injected. Sensorgram data was processed with BIAevaluation 3.2 software.

EphA2 pull-down experiment DNA nano-calipers were designed for pull-down experiments containing anchoring sites for the polyA-linked magnetic Dynabeads® (Life Technologies) and sites with extra toehold sequences for elution of ephrin-A5/EphA2 complexes from the nano-

calipers. The control nano-calipers with zero, one, two and three pairs of sites as well as NC100 and NC40 were folded in 13 mM Mg^{2+} Folding Buffer (5 mM TRIS, 1 mM EDTA, pH8.5). The excess of staples were washed away with Millipore Amicon 100K spin columns. Ephrin-A5 oligonucleotide conjugates were bound to structures by incubating them in a thermal-cycler on a 15 h long thermal-program from 37 °C to 4 °C. Ephrin-A5 nano-calipers were purified with spin column based size-exclusion purification with Sepharose 6B beads. After the purification, the concentration of nano-calipers was measured with NanoDrop and was adjusted to 15 nM. 90 µl of 15 nM ephrin-A5 nano-caliper solution was incubated with magnetic Dynabeads® (Life Technologies) overnight at 4 °C. Unbound nano-calipers were removed by washing with 10 mM Mg^{2+} Folding Buffer. After washing, the bound ephrin-A5 nano-calipers were incubated with 50µL of 1 µM of EphA2 for 3 h at room temperature. The unbound fraction of EphA2 was removed by washing with 10 mM Mg^{2+} Folding Buffer. After washing, the ephrin-A5/EphA2 complexes were eluted from the bound nano-calipers by incubation of the nano-calipers with 40µL of 1 µM of invading complementary oligonucleotide solution (10 mM Mg^{2+} Folding Buffer) overnight at 37 °C. Eluted samples were run under denaturing conditions (0.1% SDS) in a 10% PAGE gel (Bio-Rad, mini protean TGX). The gel was stained with the PlusOne Silver Staining Kit (GE Healthcare) and imaged with ChemiDoc XRS gel-imager (Bio-Rad).

Western Blot. Eluted samples were run under non-denaturing conditions in a 10% PAGE gel (Bio-rad, mini protean TGX) and transferred to a PVDF membrane (GE, Amersham Hybond). The membrane was incubated with goat anti-EphA2 antibodies (R&D systems, AF3035) (1:2000 diluted in TBST with 1% milk-powder) overnight at 4 °C. After washing, the membrane was incubated with HRP-conjugated rabbit anti goat IgG (Dako, P0449) (1:2000 diluted in TBST with 1% milk-powder) for 1 h at room temperature. Chemoluminescence reagent (GE, Amersham ECL prime) was applied and the membrane imaged with ChemiDoc MP gel-imager (Bio-Rad).

Transmission Electron Microscopy (TEM) We spotted 2 μ l of the purified nano-caliper suspensions on glow-discharged, carbon-coated Formvar grids (Electron Microscopy Sciences), incubated for 20 s, blotted off with filter paper, and then stained with 2% (W/V) aqueous uranyl-formate solution followed by a final blot with filter paper. We performed TEM analysis using a FEI Morgagni 268(D) transmission electron microscope at 100 kV with nominal magnifications between 28000x and 44000x. Images were recorded digitally using the Advanced Microscopy Techniques Image Capture Engine 5.42.

Cell Culture MDA-MB-231 cells (generously provided by the lab of Urban Lendahl, Karolinska Institutet, purchased from ATCC, free of mycoplasma) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin. For immunocytochemistry and PLA experiments, cells were cultured on 16 well glass slides (Thermo Fisher Scientific) or on micropatterned glass slides (CYTOO) to obtain single cells of uniform size and shape, which are unable to form cell-cell contacts. The micropatterns consisted of disc-shaped fibronectin patterns with approximately 30 μ m in diameter spaced 80 μ m apart. Cells were detached using warm TrypLE Express (Gibco) and seeded onto the patterns at 60,000 cells/ml. The cells were allowed to attach for 1 h before the unattached cells were washed off.

Eph Receptor Stimulation Prior to EphA2 receptor stimulation, the cells were serum starved for 1 h. Ephrin-A5-Fc homodimers (Recombinant Human Ephrin-A5-Fc Chimera, R&D Systems, cat. no. 374-EA-200) were pre-clustered with IgG antibodies (polyclonal AffiniPure Goat Anti-Human IgG, Jackson ImmunoResearch Laboratories Inc.) at a mass ratio of 1:10 for 15 min at room temperature, which is a commonly used method to cluster ephrin ligands, hereon referred to as "IgG-clustered ephrin-A5". Cells were treated for 15 min at 37 °C with either 5 μ g/ml IgG or 0.5 μ g/ml of ephrin-A5 monomer (Creative Biomart), ephrin-A5 dimer or IgG-clustered ephrin-

A5. For experiments with nano-calipers, cells were treated with 10 nM of ephrin-A5-Fc, which corresponds to 0.5 µg/ml, conjugated to DNA nano-calipers (NC0, NC100, NC40 and NC-sat) or empty nano-calipers (NC-empty) as controls.

Immunocytochemistry Cells were serum starved for 1 h prior to stimulation with IgG, ephrin-A5 monomer, ephrin-A5 dimer, IgG-clustered ephrin-A5, NC-empty, NC0, NC100 and NC40 for 120 min at 37 °C. For dynamin inhibition, Dyngo-4a (Abcam) was added to the serum free media at 30 µM, and DMSO was used as control. After EphA2 stimulation, cells were rinsed once with PBS and fixed for 20 min in 10% formalin at room temperature. The cells were washed three times with PBS/0.1% Triton X-100 and stained overnight at 4 °C in PBS/0.1% Triton X-100/1% BSA with the following antibodies: anti-EphA2 (1:500, Santa Cruz Biotechnology Inc., cat. no. sc-924) and anti-EEA1 (1:1000, BD Biosciences, cat. no. 610457). The samples were washed six times with PBS/0.1% Triton X-100, then treated with the species specific secondary antibodies Alexa Fluor® 488 (1:500, Invitrogen) and Cy3™ conjugated AffiniPure IgG (1:450, Jackson ImmunoResearch Laboratories Inc.) diluted in PBS/0.1% Triton X-100/1% BSA for 1 h in the dark. Finally, samples were washed with PBS and mounted in Vectashield with DAPI. Images were acquired using a Zeiss LSM700 CLSM confocal microscope.

***In situ* Proximity Ligation Assay (PLA)** Unless stated otherwise, all reagents for the PLA were from Sigma-Aldrich. Upon EphA2 stimulation, cells were washed once with warm PBS and fixed in 10% formalin for 20 min at room temperature. The cells were washed three times for five min each in PBS with gentle agitation, followed by three washes with PBS/0.1% Triton X-100 for 10 min each and three washes with TBS-0.05% Tween 20 for 5 min. The samples were incubated with Duolink II Blocking Solution in a humidity chamber for 1 h at 37 °C. For detection of EphA2 receptor phosphorylation, the following antibodies and dilutions were used: anti-Phosphotyrosine (1:1200, Abcam, cat. no. ab9319) and anti-EphA2 (clone D7, specific for

intra-cellular domain³⁷, 1:1500, Millipore, cat. no. 05–480). Antibodies were diluted in the Duolink II Antibody Diluent (1x) and incubated overnight at 4 °C in a humidity chamber. The next day, the cells were washed three times for 10 min with 1x Duolink II Wash Buffer A. The two PLA probes, Duolink II anti–Mouse PLUS and Duolink II anti–Rabbit MINUS, were diluted 1:80 in Antibody Diluent, and samples were incubated for 60 min in a humidity chamber at 37 °C. All samples were washed three times for 10 min in 1x Duolink II Wash Buffer A. The Duolink II Ligation stock (5x) was diluted five times in water and the Ligase was diluted 1:80 in the ligation mix. Samples were incubated in the ligase–ligation solution for 30 min at 37 °C in a humidity chamber. The slides were washed twice for two min in 1x Duolink II Wash Buffer A. The Duolink II Amplification stock (5x) was diluted five times in water and used to dilute the Polymerase 1:160. The samples were incubated in amplification–polymerase solution in a humidity chamber for 60 min at 37 °C. Finally, the samples were washed twice for 10 min with 1x Duolink II Wash Buffer B and once for five min with PBS. The cells were stained with Alexa Fluor® 488 Phalloidin (1:100, Invitrogen) and mounted with Vectashield with DAPI. Images for PLA signal analysis were acquired using Zeiss Cell Observer fluorescent microscope. Z–stack images with 40x magnification were acquired in “Start/stop mode” (at least 20 stacks with 3 µm step size) for at least 20 cells per condition. The number of biological repeats was: $n = 7$ for IgG, ephrin–A5 dimer and IgG–clustered ephrin–A5; $n = 5$ for ephrin–A5 monomer (two biological replicates were excluded for the monomer because the results were anomalous and we confirmed by native gel electrophoresis the presence of clustered monomers in these repeats); $n = 6$ for NC–empty, NC100 and NC40; and $n = 4$ for NC0 (all performed experiments were included). Maximal intensity projection of the z–stacks was performed using CellProfiler cell image analysis software. Fluorescence images for Fig. 3a and 3c were acquired with a Zeiss LSM700 confocal microscope. We quantified the PLA signals in Fig. 3b and 3d by selecting all single

cells for every experimental group, based on the DAPI images (without seeing the PLA signal). The PLA signals were quantified with BlobFinder using batch processing and all measurements were included in the analysis.

Flow Cytometry To verify that the ephrin-A5 nano-calipers bind MDA-MB-231 cells, we performed flow cytometry on cells stimulated with IgG-clustered ephrin-A5 and NC-sat. Empty nanotubes (NC-empty) and IgG alone were used as controls. Specifically, the MDA-MB-231 cells were collected with a cell scraper and kept on ice until treated. DyLightTM 488-conjugated AffiniPure Donkey Anti-Human IgG (Jackson ImmunoResearch Laboratories Inc.) was used to pre-cluster ephrin-A5-Fc for 15 min (20 µg/ml IgG and 2 µg/ml ephrin-A5 dimer).

Alternatively, AffiniPure Donkey Anti-Human IgG was added to the cell culture medium 15 min after the start of stimulation with NC-sat and NC-empty. Flow cytometry was performed two times per condition on FACSCanto II and the percentage of FITC-positive cells was measured and analyzed using FACSDiva software.

Immunoprecipitation MDA-MB-231 cells were seeded at 12,000 cells/cm² in 6-well plates and stimulated with ephrin-A5 for 15 min as previously described. Cells were lysed in 500 µl of modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM sodium vanadate, 1 mM NaF, phosphatase inhibitor cocktail (Sigma-Aldrich), and protease inhibitor cocktail (Roche)). The lysed material was pre-cleared by centrifugation at 14000 g for 10 min in 4 °C. For the immunoprecipitation experiments, 250 µl of pre-cleared lysate was incubated with 1.25 µg of mouse monoclonal α-EphA2 antibody (Millipore) for 18 h in end-over end rotation at 4 °C. Next, 25 µl of an approximately 50% slurry of Sepharose-G beads (GE healthcare) were added and incubated for an additional 4 h. The beads were collected by centrifugation at 9000 rpm on a bench-top

centrifuge for 30 sec and washed three times in 1 ml of ice-cold lysis buffer. After the final wash, the beads were boiled at 95 °C for 5 min in Laemmli/SDS loading buffer with 5% β -mercaptoethanol and separated in a SDS-page gel (Biorad). Proteins on the gel were transferred to a PVDF membrane (Immobilon-P, Millipore) by wet transfer and blocked by 5% BSA in PBS overnight. The membrane was incubated with an anti-Phosphotyrosine antibody (1:500, Abcam, cat. no. ab9319) overnight, washed extensively in TBS-T buffer, and detected with anti-rabbit-HRP (1:20000 dilution, GE Healthcare) and ECL detection reagents (Millipore). The membranes were stripped at 55 °C in a buffer containing 62.5 mM Tris-HCL pH 6.8, 2% SDS and 7 μ l/ml β -mercaptoethanol, blocked with BSA (5%) in TBS and re-probed with anti-EphA2 antibody (1:1000, Santa Cruz, cat. no. sc-924) followed by anti-rabbit-HRP (1:20000, GE Healthcare) and detected with ECL detection reagents (Millipore).

Cell Invasion Assay We performed a cell invasion assay to study the effects of EphA2 receptor stimulation on MDA-MB-231 cell migration through an extracellular matrix (ECM). The assay was performed in two biological repeats using ECMatrixTM precoated cell culture inserts with 8 μ m pores (Merck Millipore). To rehydrate the ECM layer, the inserts were kept in serum free medium for 2 h at room temperature prior to cell seeding. Cells were collected with a cell scraper and treated for 15 min at 37 °C with either 20 μ g/ml IgG or 2 μ g/ml of ephrin-A5 monomer, ephrin-A5 dimer, IgG-clustered ephrin-A5 (pre-clustered with IgG at a mass ratio of 1:10 for 15 min at room temperature) or with NC-empty, NC100 and NC40. 40,000 cells were seeded in each insert in 200 μ l serum free media and the lower chamber was filled with 500 μ l media containing serum. After 24 h of culture, cells were washed with PBS, fixed for 2 min in 10% formalin at room temperature and permeabilized for 10 min with 100% methanol. Cells were stained using the cell stain supplied by the manufacturer as well as DAPI and non-invading cells

were gently removed from the interior of the inserts using a cotton-tipped swab. Images were acquired using a fluorescent Zeiss Axioscope2 microscope and the total number of cells in seven 5x images was counted for each condition.

Statistical Analysis For multiple comparison analysis in Figures 3b and 3d, we performed one-way ANOVA followed by Tukey post-tests. Using Prism software (GraphPad), we performed D'Agostino and Pearson omnibus normality test, which showed that each group of data follows a normal distribution. Further, Bartlett's test for equal variances showed that the variances are similar between the groups. The number of biological repeats was chosen based on preliminary data of PLA for EphA2 phosphorylation of cells on micropatterns, we did not perform power calculations before the experiment. In Figure 4e, we performed Student's t-test, the sample size, $n = 3$, was too small to determine normality and F test showed that the variances were similar in the different groups. The data in Figure 4e is the average of two biological replicates and was analyzed using one-way ANOVA, and the sample size was too small to determine normality or equal variances.

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EphA2 receptor activation in situ PLA

EphA2/p-Tyr PLA signal
Actin Nuclei