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Supplemental Information

Phosphorylated EGFR Dimers

Are Not Sufficient to Activate Ras

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SUPPLEMENTAL FIGURES AND LEGENDS

Fig. S1: SNAP-EGFR expressed at low levels has low background phosphorylation and is responsive to EGF in multiple cell lines, related to Figure 1 (A) Uncropped western blot from figure 1D shown at one scan intensity with labeled MW ladder. (B) Representative FACS plots of wild-type HEK293 cells and cells after stable transduction with SNAP-EGFR and selection by FACS sorting. The HEK293 cells used in signaling assays were sorted for low expression to achieve low background phosphorylation of EGFR in the absence of treatment. Cells were lifted with PBS without Ca^{2+} and Mg^{2+}, with 0.04% EDTA, and labeled with 1 µM BG-Alexafluor488 for 30 minutes on ice. (C) Quantum MESF beads were used to quantify surface receptor number counts on SNAP-EGFR cell lines that were labeled with a non-cell permeable BG-Alexafluor dye and analyzed by flow cytometry. Error bars are standard deviation of three separate experiments. (D) Western blot of H1299 cells stably expressing SNAP-EGFR and treated with either 8 nM of EGF or 2 µM $(DNA\text{-}BG)_2$. (E) Western blot of murine suspension Ba/F3 cells stably expressing SNAP-EGFR and treated with either 8 nM of EGF or 2 µM $(DNA\text{-}BG)_2$. (F) Western blot of breast epithelial MCF10A cells stably expressing SNAP-EGFR and treated with either 8 nM of EGF or 2 µM $(DNA\text{-}BG)_2$.

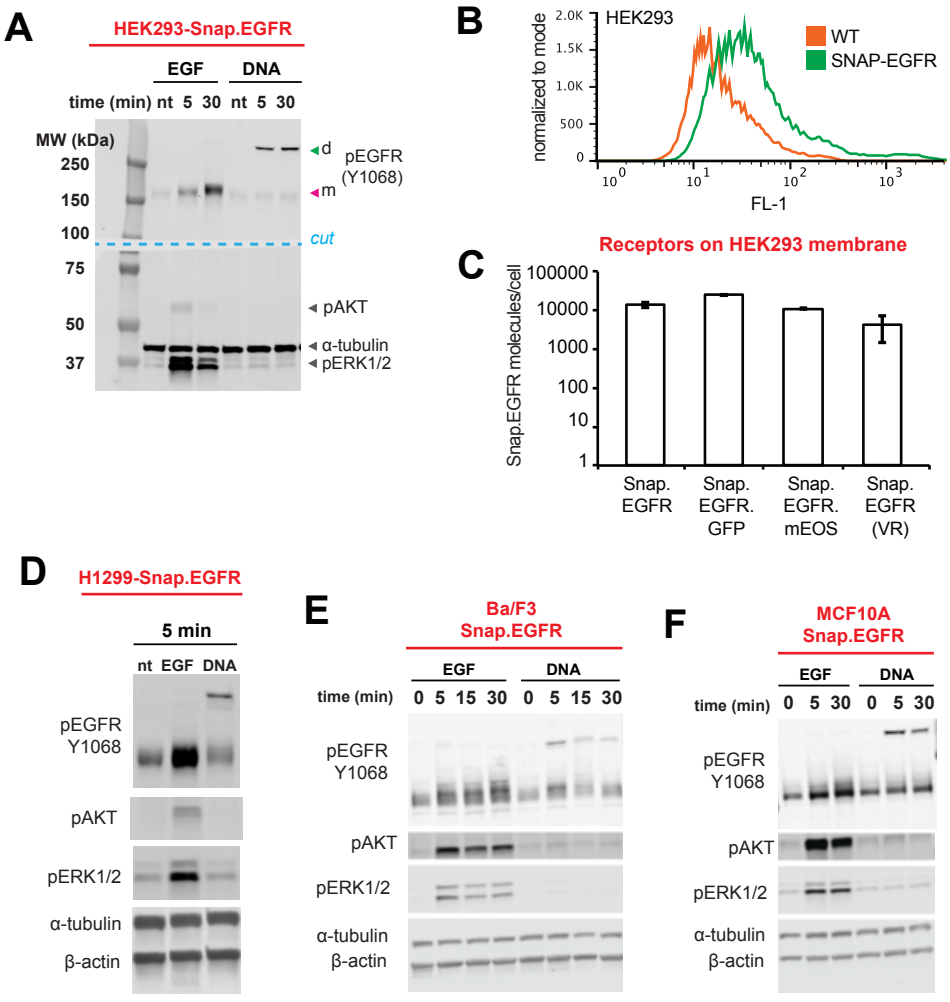


Fig. S2: EGFR phosphorylation occurs without ERK or AKT activation when the structure of chemical cross-linkers is altered, related to Figure 1. (A) Western blot of cells treated with different lots of $(DNA\text{-}BG)_2$ at 2 µM as well as double the $(DNA\text{-}BG)_2$ concentration at 4 µM. No pERK was observed at either concentration of $(DNA\text{-}BG)_2$ compared to stimulation with 8 nM EGF. (B) Western blot of cells stimulated with either 8 nM of EGF, 2 µM $(DNA\text{-}BG)_2$, or sequential addition $(DNA\text{-}BG)_2$ and EGF. For sequential addtion, 2 µM $(DNA\text{-}BG)_2$ was first added to cells for 15 minutes, then 8 nM EGF was added for the indicated times. (C) Quantitation of pERK levels from the conditions described in (B) in relation to EGF-treated cells. pERK levels were normalized to loading controls and baseline levels of pERK, and then plotted as the percent of the value of pERK for cells treated with EGF for 5 minutes. Error bars are standard deviation of three independent experiments. (D) Western blot of cells treated with flexible and uncharged BG-dimerizers, which were synthesized from PEGs of the indicated lengths. Approximate PEG lengths are 2 nm (n = 5), 3.6 nm (n = 9), 10.5 nm (n=26). EGFR phosphorylation was observed without ERK or AKT activation with these PEG-based crosslinkers. (E) Cells expressing SNAP.EGFR and cells expressing mutant SNAP.EGFR(V942R) were both treated with EGF and BG-PEG_{26}-BG for 5 minutes. (F) Western blot of cells treated with 20mer DNA duplexes having 6, 8, 10, and 20 contiguous complementary bases designed to decrease the lifetime of the duplex. T_m of the pairs are included in the SI with the corresponding DNA sequences. No increase in pERK signal was observed for any of the duplexes.

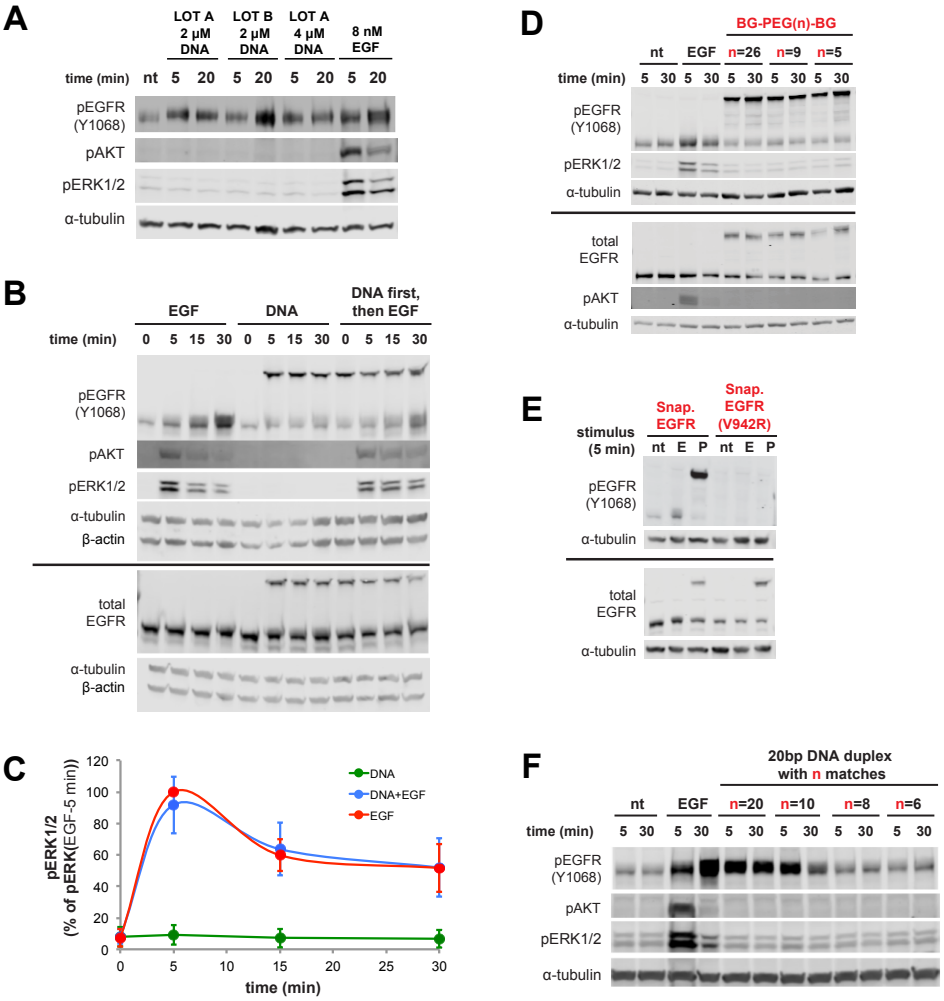


Fig. S3: Panel of representative images of HEK293-SNAP-EGFR-mEos cells (related to Figure 4) were incubated with 8 nM EGF, 2 μM $(DNA\text{-}BG)_2$, or serum-free media (no treatment) for 10 minutes. Cells were imaged by STORM and images in Figure 6a are included here in larger form, along with another set of representative images. Scale bar is 10 μm.

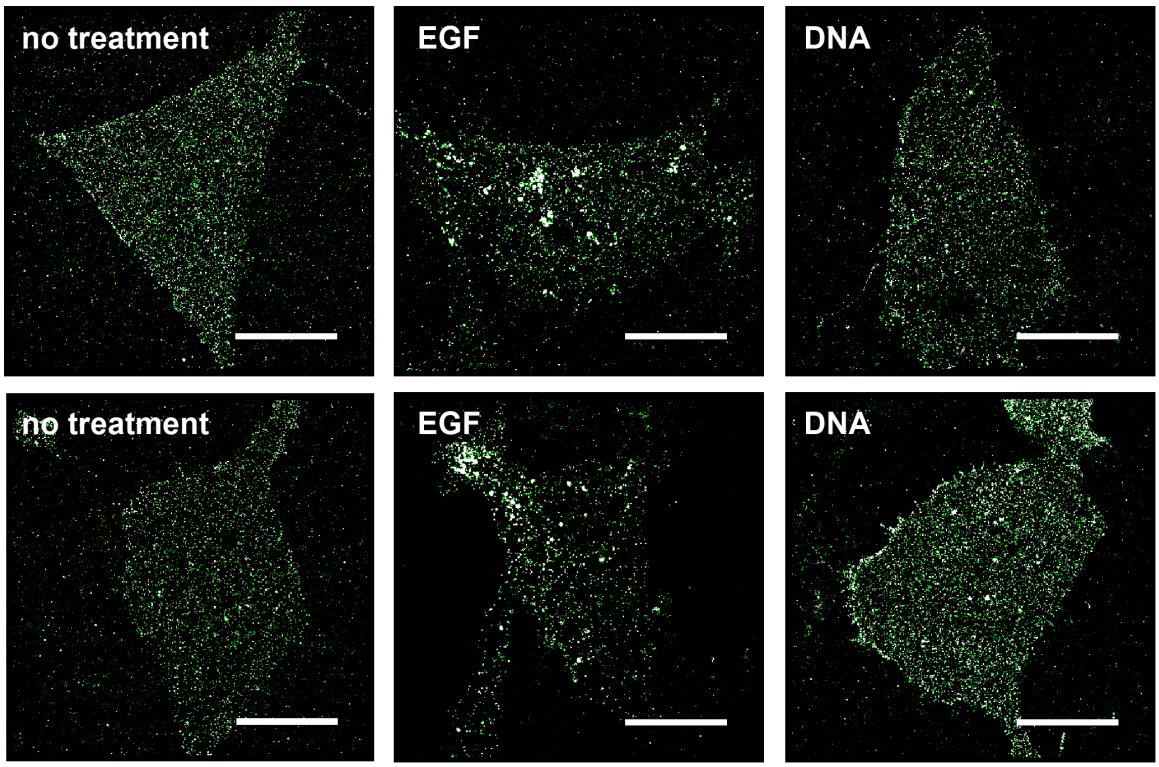
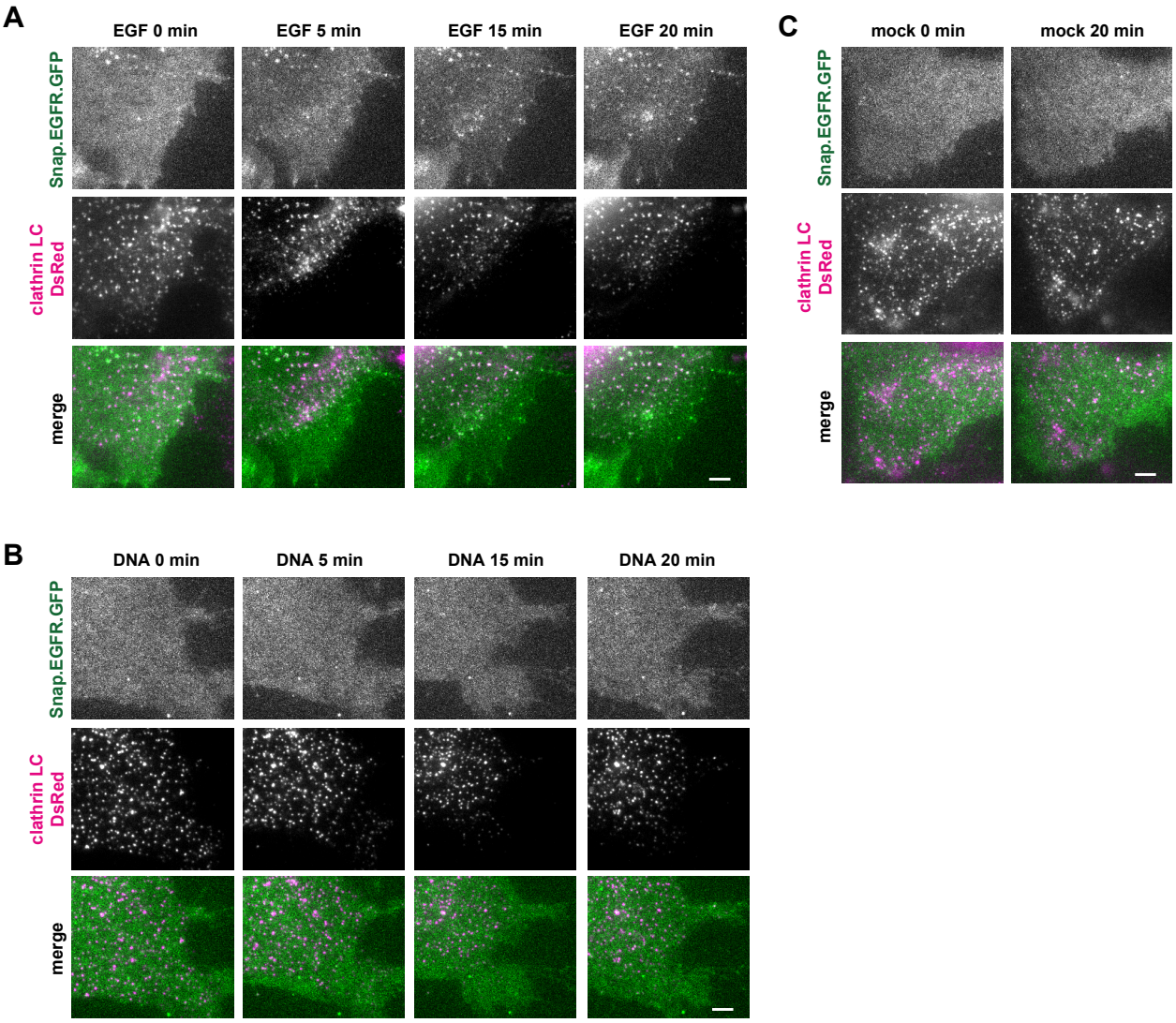


Fig. S4: Representative images of Snap-EGFR-EGFP co-localization to clathrin coated pits, related to Figure 4. (A) TIRF images of HEK293 cells co-transfected with SNAP-EGFR-EGFP and clathrin-light chain-dsRed after treatment with 8 nM of EGF at time points from 0-20 minutes. (B) 2 μM of DNA, (C) or media as a mock treatment. Scale bars are 1 micron.



SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Synthesis of benzylguanine-modified oligonucleotides

Benzylguanine-conjugated N-Hydroxysuccinimide (BG-GLA-NHS) was purchased from New England Biolabs. All phosphoramidites and DNA synthesis reagents and solvents were purchased from Glen Research. Amine-modified oligonucleotides were synthesized on an Applied Biosystems Expedite 8909 DNA synthesizer using a default coupling protocol, with the addition of a 5'-Amino-Modifier C6 phosphoramidite (Glen Research, cat. # 10-1906) resuspended at 100 mM as the last step. CPG beads from up to five 1 µmol syntheses of amine-modified DNA were aliquoted among several microcentrifuge tubes. 2 mg of the BG-GLA-NHS ester was dissolved in 400 µl of dry DMSO (Sigma Aldrich), mixed with 100 µl of dry N,N-Diisopropylethylamine (DIPEA, Sigma Aldrich) under argon, and then quickly transferred evenly among the microcentrifuge tubes to completely submerge the CPG beads. The microcentrifuge lids were secured with parafilm and the reaction agitated on a vortex mixer overnight. The beads were then washed 3 times with dimethylformamide (DMF, Sigma Aldrich), 3 times with dichloromethane (DCM, Sigma Aldrich), 1 time with acetonitrile, and then dried completely on a speed vac system. In order to maintain the BG, a mild cleavage solution without methylamine was used. The DNA was cleaved off the beads using a mixture of 30% ammonium hydroxide for 2 hours at 65 °C, dried on a speed vac system, resuspended in 100 mM triethylamine acetate (TEAA), and filtered through 0.2-µm spin filters. The BG-conjugated strand with a 3' conjugated biotin was prepared similarly, but using biotin-TEG-conjugated CPG support from Glen Research (cat. # 20-29550).

Oligonucleotides were then purified by reversed-phase high-performance liquid chromatography (Agilent 1260 Infinity Series HPLC System) using a 5 um Eclipse XDB-C18 9.4 x 250 mm column (Agilent). An elution gradient of 8-15% acetonitrile in 100 mM TEAA (pH7) over 20 minutes, followed by a gradient of 15-70% acetonitrile over 6 minutes, was used to purify BG-DNA, which eluted around 22-23 minutes from the start of the program. Fractions containing BG-modified oligonucleotides were washed with Millipore MilliQ-purified water and lyophilized three times. Prior to use, the DNA was resuspended in water and its concentration was determined by its absorbance at 260 nM measured on a NanoDrop (Thermo Scientific).

DNA sequences were selected as previously described (Hsiao et al., 2009) to form dimer constructs with minimal secondary structure and are listed below:

Name	Sequence	calculated Tm*
BG-20A	Benzylguanine-5'-GTAACGATCCAGCTGTCACT-3'	66.28 ˚C
BG-20A'	Benzylguanine -5'-AGTGACAGCTGGATCGTTAC-3'	
BG-20A-biotin	Benzylguanine -5'-AGTGACAGCTGGATCGTTAC-3'-biotin	
BG-20A'-MM10	Benzylguanine -5'-gtaagCAGCTGGATCtcact-3'	51.26 ˚C
BG-20A'-MM12	Benzylguanine -5'-gtaacgAGCTGGATgtcact-3'	37.64 ˚C
BG-20A'-MM14	Benzylguanine -5'-gtaagttGCTGGAcgtcact-3'	26.28 ˚C

*calculated at 2 µM concentration, 154 mM Na^+, 0.81 mM Mg^{2+} and considering only the directly complementary sequence shown in caps.

Synthesis of benzylguanine-modified PEG linkers

All reagents and solvents were purchased from commercial sources and used as received without further purification. Reactions were carried out under an inert atmosphere of argon in flame-dried glassware. Reactions were purified by HPLC using an Agilent 1200 series liquid chromatography system with Agilent Eclipse XDB C-18 5 µm, 4.6 x 250 mm column. An elution gradient of 5-90% acetonitrile in 0.1% aqueous trifluoroacetic acid over 25 minutes was used for purification. Mass spectrometry was performed on a LC-MS (Waters Acquity UPLC/ESI-TQD) with the Acquity BEH C18 1.7 um, 2.1x50 mm column.

Synthesis of bis(BG)-PEG26 crosslinker: O,O'-Bis(2-aminoethyl)hexacosaethylene glycol (1.5 mg, 1.2 µmol, Santa Cruz Biotechnology) was dissolved in anhydrous dimethylsulfoxide (0.5 mL). A drop of N,N-diisopropylethylamine was added to the solution followed by addition of BG-GLA-NHS (2.0 mg, 4.15 µmol). The solution was allowed to stir at room temperature overnight. The reaction mixture was concentrated under reduced pressure, redissolved in 3:1 water:acetonitrile, filtered and purified via HPLC. The purified sample was lyophilized to yield the desired product (0.65 mg, 0.33 µmol, 28% yield): MS (LC-MS) *m/z* calculated for $C_{92}H_{152}N_{14}O_{33}$ [M+H]+ 1982.07, found 1982.54.

Synthesis of bis(BG)-PEGn crosslinker: $BS(PEG)_n$ (9 μmol, Thermo Fisher Scientific) was dissolved in anhydrous dimethylsulfoxide (37.5 μL). N,N-diisopropylethylamine (70 μL, 0.4 mmol) was added to the solution followed by addition of 6-((4-(aminomethyl)benzyl)oxy)-7H-purin-2-amine (54 mg, 0.2 mmol). The solution was allowed to stir at room tempearture overnight. The reaction mixture was concentrated under reduced pressure, redissolved in 3:1 water:acetonitrile, filtered and purified via HPLC. The purified sample was lyophilized to yield the desired product.

Bis(BG)-PEG_5. (1.7 mg, 2.0 μmol, 22% yield) MS (LC-MS) *m/z* calculated for $C_{40}H_{50}N_{12}O_9$ [M+H]+ 843.39, found 843.83.
Bis(BG)-PEG_9. (2.1 mg, 2.1 μmol, 23% yield) MS (LC-MS) *m/z* calculated for $C_{48}H_{68}N_{12}O_{13}$ [M+H]+ 1020.50, found 1020.03.

Plasmids and generation of stable cell lines
Lentiviral plasmids for SNAP-EGFR, SNAP-EGFR-EGFP, and SNAP-EGFR(VR) were cloned into the DT39 vector by Gibson assembly using a homemade mixture. In each case, the signal peptide was fused in front of the SNAP protein to guide membrane localization. The backbone DT39 was a generous gift from the Weiner lab at UCSF, and the Snap construct was a generous gift of the Huang lab. The open reading frames of the signal peptide, the SNAP protein, and the remainder of EGFR, and GFP, were amplified by PCR and purified by agarose gel. Each piece contained 25 bp homology to its neighboring piece or the vector, and the pieces were assembled by Gibson assembly. The VR mutant was made by quick change of the wild type construct. The plasmid for SNAP-EGFR-mEos was made by replacing the DNA sequence encoding GFP from the SNAP-EGFR-EGFP plasmid with a DNA sequence encoding mEOS amplified using an enzymatic inverse PCR strategy. Lentivirus or retrovirus plasmids were prepared in larger scale using the Qiagen Midi-prep kit and delivered to UC San Francisco's Viracore for lentivirus production. Cells were transduced by plating at 25% confluency on 10 cm diameter tissue culture plates and incubating in 5 ml of media with 250 ul of virus at 37 ˚C. After 6-24 hours, 5 ml of media with 10% serum were added to the cells. The virus-containing media was replaced with fresh media after 48 hr, and the cultures split after 72 hours. HEK293 and H1299 cells were cultured with DMEM-H21 (UCSF Cell Culture Facility) with 10% FBS (UCSF Cell Culture Facility). MCF10A cells were cultured with DMEM/F12 (UCSF Cell Culture Facility), 5% horse serum (UCSF Cell Culture Facility), 20 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), and 10 μg/ml insulin (Invitrogen). Ba/F3 cells were cultured in RPMI (UCSF Cell Culture Facility) with 10% FBS and 1 ng/ml mouse IL-3 (Invitrogen).

In order to generate cell lines with low levels of SNAP-EGFR, SNAP-EGFR(VR), SNAP-EGFR-EGFP, and SNAP-EGFR-mEOS expression, transduced cells were subjected to a round of fluorescence activated cell sorting (FACS). Cells were lifted with Ca^{2+} and Mg^{2+} free PBS containing 0.04% EDTA, then incubated in PBS with 1 μM of BG-Alexafluor488 dye (New England Biolabs) for 10 minutes at 37 ˚C. Then the cells were washed 3 times with PBS with 1% BSA and resuspended in 1 ml of PBS with 1% BSA. Cells were then analyzed on the Facs Aria III (BD Biosciences) and sorted into 4 populations based on their level of fluorescence above non-transduced control cells. All cell signaling assays and STORM assays were performed using the 25% of cells incorporating the lowest amount of dye, as these cells were to have the lowest levels of background EGFR signaling. The number of SNAP-EGFR and SNAP-EGFR(VR) receptors were quantified on HEK293 cells using Quantum MESF 488 microspheres (Bangs Laboratories, Inc.) after cell labeling with 1 μM BG-Alexafluor 488 (New England Biolabs) for 30 minutes on ice. SNAP-EGFR-EGFP and SNAP-EGFR-mEOS receptors were quantified on HEK293 cells using Quantum MESF 647 microspheres (Bangs Laboratories, Inc.) after cell labeling with 1 μM BG-Alexafluor 647 (New England Biolabs) for 30 minutes on ice. Cells were then washed with PBS with 1% BSA three times and analyzed on a Facs Calibur (BD Biosciences), using the Quantum MESF microspheres to generate a standard curve for the total number of AF488 or AF647 molecules based on fluorescence intensity.

Cell signaling assays
6X stocks of the stimuli (EGF, $(BG\text{-}DNA)_2$, BG-PEG-BG) were prepared prior to all experiments in serum free media: 6X BG-DNA stock (12 uM); 6X BG-PEG-BG stock (12 uM); 6X EGF stock (300 ng/ml, 48 nM). 1X lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin) was prepared by diluting a 10X stock (Cell Signaling Technologies) and adding 1 tablet of phosphatase inhibitor cocktail (PhosSTOP, Roche) and 1 tablet of protease inhibitor cocktail (cOmplete Mini, Roche). Cells were typically cultured in 12-well plates until 70-80% confluency. They were then serum starved with 1 ml of serum-free DMEM-H21 for 6-8 hours so as to achieve the lowest possible level of background phosphorylation of EGFR/ERK/AKT. Before stimulating the cells, they were

washed and placed in 250 µl of serum-free media. 50 ul of pre-warmed 6X stocks were then added to each well to initiate the experiment. Cells were moved onto ice, quickly washed 2 times with ice cold PBS, and lysed in 60 ul of lysis buffer on ice for 15 minutes. The cells were scraped off the plates with the back of a pipette tip, and the lysates were transferred to microcentrifuge tubes and centrifuged at 13000xg 10 minutes at 4 ^{o}C in order to pellet out insoluble cell debris. The supernatant were then transferred to a new tube and 4X Laemmli sample buffer (Bio-Rad, 277.8 mM Tris-HCL (pH6.8), 44.4% (v/v) glycerol, 4.4% lithium dodecyl sulfate, 0.02% bromophenol blue) with 250 µM DTT or 10% BME as a reducing agent was added to the lysates. Lysates were stored at -20 ^{o}C and efforts were taken to minimize freeze thaw cycles. A similar protocol was used for co-immunoprecipitation experiments, but using a 10 cm culture plate.

Western blotting and analysis

For western blots, samples were loaded onto BioRad 4-15% gradient TGX gels in SDS-PAGE buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). For denatured $(BG\text{-}DNA)_2$-treated samples where BG-DNA-modified EGFR runs as a monomer, the lysates were boiled immediately before loading on the gel. The gels are run at 250V for 35 minutes resulting in warm buffer throughout electrophoresis. For non-DNA denatured samples, the lysates run at 200V for 45-60 minutes in pre-chilled running buffer that maintained a cool temperature throughout electrophoresis. All western blots are transferred using a BioRad Criterion wet transfer system onto nitrocellulose membranes (BioRad) in a Tris-Glycine buffer (25 mM Tris-HCL, 192 mM glycine) with 20% methanol. Blots were transferred at 150V in ice cold buffer for 25 or 27 minutes for a monomer or dimer transfer, respectively. The blots are then washed briefly with 1X TBST (150 mM NaCl, 20 mM Tris Base, pH 7.4, 0.1% Tween 20) and the blocked in 5% non-fat milk solids in TBST for at least 1 hour at room temperature with gentle rocking. The blots are then washed 3 times with 1X TBST and incubated in the primary antibody overnight on a rocker.

The following rabbit primary antibodies were used at 1:1000 in 5% BSA in TBST: EGFR (D38B1 XP, Cell Signaling Technologies), pEGFR Y1068 (clone D7A5 XP, Cell Signaling Technologies), pEGFR Y1045 (#2237, Cell Signaling Technologies), pEGFR Y1086 (#2220, Cell Signaling Technologies), pEGFR Y1173 (clone 53A5, Cell Signaling Technologies), pERK1/2 Thr202/Tyr204 (clone D13.14.4E XP, Cell Signaling Technologies), pAKT Ser473 (clone D9E XP, Cell Signaling Technologies), and clathrin heavy chain (clone TD.1, Santa Cruz Biotechnology). The loading control mouse antibodies alpha-tubulin (clone DM1A, Sigma-Aldrich) and beta-actin (clone AC-15, Sigma-Aldrich, or similar) were used at 1:10000 in 5% BSA. Following incubation with primary antibody, the blots were then washed 4 times for 5 minutes each in 1X TBST. Then, they were incubated in secondary antibodies conjugated to either AlexaFluor680 (Thermo Fisher Scientific) or DyLight800 (Thermo Fisher Scientific and Rockland) diluted 1:10000 into 5% milk in TBST for 1-2 hours at room temperature, protected from light. After 3 more washes in 1X TBST, the blots were imaged on a Licor imaging system. Scans were then quantified and analyzed on ImageStudioLite (Licor) by densitometry. Measurements were normalized to the geometric mean of the intensity of both loading controls alpha-tubulin and beta-actin.

Ras-activation assay

Ras-GTP was precipitated from lysates using an Active Ras Pull-Down and Detection Kit (Thermo Fisher Scientific). Cells were plated at 80% confluency in a 10 cm dish and serum starved for 6.5 hours. The media was removed and replaced with 5 ml of the indicated ligand as a 1X solution in serum free media, and incubated for five minutes at 37 ^{o}C. The plates were then placed on ice, washed twice with ice cold PBS, and lysed using 500 µl of the 1X lysis buffer included in the kit with protease and phosphatase inhibitor cocktail tablets added. The cells incubated on ice for 15 minutes, then scraped off the plates with the back of a pipette tip, and the lystates were transferred to microcentrifuge tubes and centrifuged at 13000xg 10 minutes at 4 ^{o}C in order to pellet out insoluble cell debris. 30 µl of the fresh lysate was reserved and added to 12 µl of 4X SDS-PAGE buffer containing BME to run as a lysate reference. Ras-GTP was precipitated from crude lysates using an Active Ras Pull-Down and Detection Kit (Thermo Fisher Scientific). Briefly, 100 µl of a 50% slurry of Glutathione Resin was put into a spin cup with a collection tube, washed with 400 µl of lysis buffer, and centrifuged. 400 µl of lysate was then added to the spin cups, which were then capped on both ends and incubated at 4 ˚C while rotating for 1 hour. The spin cup was then unsealed, centrifuged again and the resin was washed 3 more times with lysis buffer. For elution, 50 µl of 2X SDS sample buffer with BME was added to the resin, vortexed, and incubated at room temperature for 2 minutes. The spin tube was then transferred to a fresh collection tube and the flow through was collected. The eluted samples were then heated for 5 minutes at 95 ˚C. For the western blot analysis, 25 µl of each sample was loaded on a gel and the anti-Ras antibody provided in the kit was used to detect Ras.

Co-immunoprecipitation with EGFR

Lysates were prepared as described above for the Ras-GTP Pull Down assay. 250 µl of lysis buffer was added to 150 µl of lysate, 5.5 µl of a mouse anti-EGFR antibody (#2256, Cell Signaling Technology), and incubated at 4 ˚C overnight while on a rotary mixer. The next day, 40 µl of a Protein G-conjugated magnetic bead slurry (#8740, Cell Signaling Technology) was added to the tubes and incubated at 4 ˚C for 2 hours while on a rotary mixer. The beads were then washed 4 times with 500 µl of 1X lysis buffer using a magnetic rack. Proteins were eluted by incubating the beads with 60 µl of 2X sample buffer, quickly vortexed, and boiled for 5 minutes. Finally, the tubes were centrifuged at 13000 g for 1 minute and the supernatant was transferred to a new tube. For the western blot analysis, 20 µl of the samples were run in each lane of the gel and the protocol described above was used with the following primary antibodies at a 1:250 dilution in 5% BSA in TBST: Grb2 (#3972, Cell Signaling Technologies), SHC (ab24787, Abcam), and SOS (clone D3T7T, Cell Signaling Technologies). The same samples were also probed with total EGFR and phosphorylated-EGFR antibodies as controls to ensure that similar amounts of total EGFR were pulled down in each sample.

Live cell total internal reflection fluorescence microscopy (TIR-FM) imaging and analysis

TIR-FM was performed at 37 °C using a Nikon Ti-E inverted microscope equipped for through-the-objective TIR-FM and outfitted with a temperature-, humidity-, and CO_2-controlled chamber (Okolab). Images were obtained with an Apo TIRF 100X, 1.49 numerical aperture objective (Nikon) with solid-state lasers of 488 and 561 nm (Keysight Technologies). An Andor iXon DU897 EMCCD camera controlled by NIS-Elements 4.1 software was used to acquire image sequences every 4 seconds for 20 minutes. Cells were transfected with SNAP-EGFR-EGFP and clathrin light chain-dsRed (CLC-dsRed) using Lipofectamine 2000 (Life Technologies) according to manufacture protocol 48 hours before imaging and then plated on poly-L-lysine (0.0001%, Sigma) coated 35-mm glass-bottomed culture dishes (MatTek Corporation) 24 hours before imaging. Prior to imaging, cells were washed once and imaged live in DMEM without phenol red (UCSF Cell Culture Facility) supplemented with 30 mM HEPES, pH 7.4. Cells were treated as indicated at frame 5 of 301 image sequences. Acquired image sequences were saved as stacks of 16 bit TIFF files. Receptor fluorescence enrichment into clathrin-coated structures (CCSs) was calculated using a mask of CCSs generated using a thresholded average image of the clathrin channel as previously described (Eichel et al., 2016). Enrichment at CCSs was measured as the difference between the average fluorescence in the mask and average fluorescence outside of the thresholded structures. Each condition represents 10 cells pooled across 7 independent experiments.

Stochastic Optical Reconstruction Microscopy (STORM) and analysis

8-well chambered coverglass slides (Lab-tek) were cleaned with 1 M KOH for 10 minutes, then washed and coated with poly-lysine (0.01%) for 30 minutes. Stably infected HEK293 cell lines expressing SNAP-EGFR- mEos3.2 were deposited on the washed glass slide, and allowed to adhere for 36 hours. Cells were serum starved for six hours, then incubated for with serum-free media (control), 8 nM EGF, or 2 uM DNA at 37°C for 10 minutes. Cells were fixed with 4% formaldehyde for 10 minutes at 20 °C, washed, and stored in PBS at 4 °C. Fixed cells were imaged using an inverted microscope at 100x magnification and TIRF illumination. Cells were located in the 488 nm (green) channel and then imaged with STORM in the 561 nm (red) channel at 60 Hz, and mEos photoconversion from the green to red state was achieved with 405 nm illumination (once every ten frames). Images were processed using custom scripts, and were corrected for blinking as previously described (Puchner et al., 2013). Blinking-corrected molecular positions were then used to calculate a corrected pair-correlation histogram, which calculates all the pair-wise distances as previously described (van Lengerich et al., 2017). This histogram is further corrected for average density by subtracting the average baseline value (calculated as the average from 500 nm to 1000 nm) from the function. This allowed the peak height and width among different conditions to be compared directly, since different samples may contain slightly different density of molecules. To display cluster size histograms, the blink-corrected molecular positions were subjected to an algorithm that counted the number of neighboring molecules within a certain cut-off radius (here, 50 nm was used, which is the FWHM of the measurement).

Single Molecule Immunoprecipitation and Imaging

HEK293 cells stably transduced with SNAP-EGFR-EGFP were plated at 80% confluency and serum starved for 6.5 hours. Cells were then treated with either 8 nm of EGF-biotin or 2 uM of $(BG\text{-}DNA)_2$ for 5 minutes at 37 ˚C and then lysed. Lysates of HEK293 cells with SNAP-EGFR-EGFP were prepared by dissolving the cells with Triton X-100 lysate buffer (1% Triton X-100, 50 mM Tris-Hcl, 150 mM Nacl, 1 mM EDTA, 10% Glycerol). The supernatant of the lysis buffer was then collected after centrifugation at 15000xg. We then precipitated Triton-X100 disrupted cells onto neutravidin coated slides as previously described (Jain et al., 2011; Lee et al., 2013). In more detail, we immobilized the SNAP-EGFR-EGFP receptors bound with bt-EGF or bt-DNA), we prepared Neutravidin-coated

(0.5 mg/ml) PEG slides. SNAP-EGFR-EGFP in the lysate was pulled down on Neutravidin-coated PEG slides and imaged with by TIRF microscopy. Concentration of each cell lysate was increased on the slides to approximately 2 mg/ml, which was the concentration where there were approximately 600 molecules of well-separated single molecules observable on the 256x512 pixel channel on an Andor EMCCD camera.

After capturing images, each molecule's intensity was drawn into a histogram and then fitted with two Gaussians with a fixed center, where single and double EGFP intensity should be. To verify that each Gaussian actually represented monomer and dimer populations, we also analyzed the proposed monomer and dimer populations by bleaching the molecules and counting the number of bleaching steps. Bleaching steps were counted automatically using a program that finds the number of steps within the bleaching traces of EGFP. Briefly, the program uses the following algorithm:

1. Intensity traces longer than 1 min (enough time for EGFP to be bleached) of selected regions of interest (ROI) with EGFP molecules are drawn.
2. Intensity traces are then smoothed into a step-wise function using the Kalafut-Visscher algorithm.
3. Minimum step heights are determined by control EGFP bleaching experiments are manually set as a user defined variable and used as a threshold.
4. Finally, any step heights of the intensity traces above the threshold are counted.

SNAP-EGFR-EGFP clusters (oligomers larger than a monomer or dimer), which were only observed in the EGF-biotin treated sample, were too bright to completely bleach and quantify for exact receptor number using the bleaching step algorithm. To estimate the number of SNAP-EGFR-EGFP molecules in the clusters, we measured and graphed the pixel intensities of all the regions of interest on the image, which included monomers, dimers, and clusters. The intensities were graphed as a histogram and the intensity of single EGFP was calculated as the population of pixels with the lowest peak intensity level. The SNAP-EGFR-EGFP dimer population was identified as being twice the intensity level as the monomer. And finally, the cluster population's mean intensity was divided by mean value of single EGFP to estimate the number of SNAP-EGFR-EGFP molecules in the cluster.

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