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Supplementary Information for

Mechanical Disruption of E-cadherin Complexes with Epidermal Growth Factor Receptor Actuates Growth Factor-Dependent Signaling

.

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Supplementary Information Text

Extended technical description of results and full details of mathematical models.

Materials

Cell Culture. The A-431D^{Ecad} cells were cultured in Dulbecco's modified Eagle's medium (DMEM 4.5g/l glucose) supplemented with 10% (v/v) fetal bovine serum (FBS), 1mM sodium pyruvate, 1% (v/v) penicillin–streptomycin (Corning Cell Grow, Manassas, VA) and 200µg/ml geneticin (G418), unless otherwise stated. MCF-10A cells (from B. Gumbiner) were grown in DMEM/F12 medium (Invitrogen) containing 5% horse serum (Invitrogen), 20ng/ml of EGF (Peprotech), 0.5mg/ml Hydrocortisone (Sigma), 100ng/ml cholera toxin (Sigma), 10µg/ml insulin (Sigma), and 1% pen/strep.

FRET measurements were done with HEK293T cells that were transfected with EGFR-mTurq and/or E-cadherin-eYFP using Lipofectamine 3000 (Invitrogen, CA). For FRET experiments, 3-5µg of total DNA was co-transfected in a donor to acceptor (EGFR-mTurq to E-cadherin-eYFP) ratio of 1:3. Control experiments used for calibration were performed with cells that were transfected with 3µg of either EGFR-mTurq or E-cadherin-eYFP DNA as described (1). Twelve hours post-transfection, the cells were rinsed twice with starvation medium (phenol red-free, serum-free DMEM) to remove traces of phenol red, and then serum-starved for 12 hours overnight to ensure that no soluble ligands were present.

Lyophilized hEGF was resuspended in a solution of 1mg/mL BSA in 1XPBS to a final concentration of 5µM. This stock solution was aliquoted and stored at -20 °C until use. All materials used here were pre-treated with BSA solution to minimize ligand surface adsorption. Ten minutes prior to imaging, the starvation medium was removed and hypo-osmotic media (10% starvation media, 90% water, 25mM HEPES) was added to unwrinkle the cell membrane under reversible conditions as described (2). In the cases where EGF ligand was used, aliquots of EGF were diluted to the desired final concentration with swelling medium and mixed thoroughly before adding to the cells. Cells under reversible osmotic stress were imaged under these conditions for 1-1.5 hours. Previous work demonstrated the reversibility of swelling cells using this method (2). Swelling does not alter the measured FRET efficiencies, nor compromise cell integrity. A spectrally-resolved two-photon microscope with line-scanning capabilities (OptiMis True Line Spectral Imaging system, Aurora Spectral Technologies, WI) was used to collect images of the cells under reversible osmotic stress, following published protocols (1, 3-7).

Antibodies and Reagents. Monoclonal, rat anti-EGFR antibody (Cell Signaling Technology, 4267) was used for Western blots of EGFR and for immunoprecipitation. Polyclonal, anti-phospho-EGFR (Tyr845) antibody (Fisher Scientific, 44-784G) and monoclonal phospho-EGFR (Tyr1173) antibody (Cell Signaling Technology, 4407) produced in rabbit was used to detect EGFR phosphorylation. Immunoblots and immunoprecipitation measurements of E-cadherin used mouse, monoclonal anti-E-cadherin (Fisher Scientific, BDB610181). Phospho-Erk1/2 and total Erk1/2 were detected with polyclonal phospho-p44/42 MAPK antibody (Cell Signaling Technology, 9101S) and monoclonal p44/42 MAPK antibody (Cell Signaling Technology, 4695) produced in rabbit. Secondary antibodies anti-rabbit IgG horse radish peroxidase (HRP) (Sigma, A0545) and anti-mouse IgG HRP (Promega, W402B) were used to detect protein levels by chemiluminescence (Thermo Scientific). Anti-human EGF-neutralizing antibody (R&D Systems,

10825), anti-fibronectin (16G3) (from Kenneth Yamada; NIH, Bethesda, MD), and DECMA-1 (Millipore Sigma, MABT26) was used to block EGF, fibronectin, and E-cadherin, respectively. Recombinant E-cadherin ectodomains C-terminally tagged with the Fc domain of human IgG (Ecad-Fc) was produced from HEK293T cells engineered to stably express and secrete the protein (8). Fibronectin (Sigma) was used to coat PDMS membranes. Carrier-free human epidermal growth factor (hEGF) was from Cell Signaling Technology (Danvers, MA). Gefitinib (Selleckchem, Houston, TX)—a non-competitive EGFR inhibitor—was used to block EGFR function.

Soluble, recombinant E-cadherin extracellular domains were expressed in HEK293T cells that were stably transfected with canine E-cadherin extracellular domains with a C-terminal, human Fc-tag (E-cad-Fc), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10v/v% fetal bovine serum (FBS), 3.5g/L D-glucose, and 1.5g/L sodium bicarbonate at 37 °C and 5% CO_2. Cells were maintained for up to 25 passages, after which they were discarded.

Methods

Cell stretching. Polydimethylsiloxane (PDMS) membranes (Sylgard 184, Dow Corning) were cast in a cut out area of a silicon membrane and prepared with an elastomer base to crosslinker ratio of 26:1. The membranes were coated with 20□g/ml of fibronectin or 20µg/ml of DECMA-1 in 1X Dulbecco's phosphate-buffered saline (DPBS). Substrates were also coated with 100□g/ml of E-cad-Fc in 20mM HEPES buffer containing 2mM $CaCl_2$. Proteins were incubated with the substrates for 3hr at 37°C. The membranes were washed once with buffer prior to use, to remove unbound protein.

A-431D^{Ecad} and MCF10A cells were serum starved overnight and seeded onto the protein-coated membranes in serum-free medium, at either sub-confluent (3 x 10^5 cells/membrane) or confluent (3 x 10^6 cells/membrane) density. In studies with cells on either E-cadherin or DECMA-1 coated membranes, measurements were done within 5hr to minimize interference by secreted fibronectin. Including fibronectin-blocking antibody, 16G3 (1:100 dilution) also minimized integrin interference. As a control for 16G3, a non-binding isotype 13G12 was used at 1:100 dilution (see Fig. S4). To block EGFR activation, cells were treated with 15□□□ of Gefitinib 2hr prior to EGF treatment and/or cell stretch.

In studies of confluent monolayers on fibronectin, cells were plated in serum-containing medium and then serum starved overnight at 37°C (5%CO_2), to maintain conditions consistent with the co-IP assays (see below). In order to prevent EGFR activation by EGF, cells were treated for 30min with 250ng/ml (final concentration) of anti-human EGF neutralizing antibody. Cells were then subjected to 10% cyclic (15 cycles/min) or static stretch, with or without EGF. In controls for E-cadherin contributions to EGFR activation, 30min prior to mechanically perturbing monolayers on fibronectin, intercellular junctions were disrupted with the anti-E-cadherin antibody, DECMA-1 (1:100 dilution).

After mechanically stretching cells and prior to Western blots, samples were washed once with ice cold DPBS and lysed on ice for 30min with 1% Triton X-100 lysis buffer (20mM Tris HCl pH7.4, 137mM NaCl, 1.2mM $CaCl_2$) supplemented with protease/phosphatase inhibitor cocktails (Roche Applied Science) and Orthovanadate (Na_3VO_4).

Co-immunoprecipitation Assays. Cells were washed once in ice cold DPBS and lysed on ice with 1% Triton X-100 lysis buffer (20mM Tris HCl pH7.4, 137mM NaCl, 1.2mM $CaCl_2$) supplemented with protease/phosphatase inhibitor cocktails (Roche Applied Science) and 2mM orthovanadate for 30min at 4°C under gentle rocking. To remove cellular debris, lysates were centrifuged at 14,000xg for 5min at 4°C with an Eppendorf 5415C benchtop centrifuge, followed by the collection of the supernatant. Protein-G conjugated magnetic beads (BioRad 1614023) were incubated with 1:100 dilution of monoclonal anti-E-cadherin or monoclonal anti-EGFR antibodies for 30min at 4°C, before gently washing three times with 1X PBST (0.1% Tween 20) to remove excess antibody. The lysate supernatant and magnetic beads were then mixed and incubated at 4°C for 1hr. The beads were then washed three times with PBST, before eluting the protein with sample running buffer (50mM Tris-HCL pH 6.8, 5% 2-□-mercaptoethanol, 2%SDS,

0.01% bromophenol blue and 10% glycerol) at 80°C for 10min. Western blot assessed the protein content in the resulting samples.

SDS-PAGE and Western blot analysis. Proteins in lysates were separated on an 7.5% SDS-PAGE gel and then transferred onto a PVDF membrane (Biorad), followed by blocking with 5% (w/v) bovine serum albumin (BSA) in TBST (10mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Tween-20) for 1hr at room temperature. The membrane was incubated with primary antibody overnight at 4°C using 1:1000 dilutions for all antibodies. The blot was then washed thrice with TBST for 10min a wash, followed by the addition of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000, Sigma) or anti-mouse IgG (1:10,000, Promega) secondary antibodies containing 5% (w/v) BSA in TBST for 1hr at room temperature. The blot was then washed thrice in TBST for 10min a wash prior to quantification using a chemiluminescence based ECL western blotting substrate (Thermo Scientific) and the iBright imaging system (Invitrogen). Following imaging, the blot was stripped with stripping buffer (62.5mM Tris-HCL pH 6.8, 2% SDS, and 0.7% 2-mercaptoethanol) at 50°C for 20min followed by 5X, 10min washes with TBST before blocking and re-probing the membrane.

EGFR Phosphorylation Array. A human EGFR Phosphorylation Antibody Array (Raybiotech, Georgia, AAH-PER) was used to identify phosphorylation sites affected by cyclic stretch and EGF. Cell treatments were conducted as described for the co-immunoprecipitation studies. After treatment, the cells were washed 1X with ice cold DPBS before lysing with 500ul of 1X lysis buffer (Raybiotech, Georgia) for 30min at 4°C, under gentle rocking. Lysates were clarified by centrifugation at 14,000xg for 5min with an Eppendorf 5415C benchtop centrifuge at 4°C, followed by the collection of the supernatant. The array membranes were blocked with the provided blocking buffer for 1hr at room temperature under gentle shaking. The blocking buffer was removed, after which the lysate was incubated with the arrays for 2hr at room temperature. The membranes were then washed 5X with wash buffer I (Raybiotech, Georgia) for 5min per wash. Next, the blots were rinsed 3X with wash buffer II at 5min per wash. A 1ml cocktail of biotin conjugated anti-EGFR (Raybiotech, Georgia) in blocking buffer was then added to the membranes, and incubated overnight at 4°C. The antibody was then washed off by using the described wash steps. Then 1ml of HRP-conjugated streptavidin in blocking buffer was added and incubated with the array for 2hr at room temperature. After rinsing, detection buffer (Raybiotech, Georgia) was added and blots were imaged with the iBright imaging system (Invitrogen).

Modeling E-cadherin/EGFR hetero-interactions. The FSI-FRET method can predict the stability and stoichiometry of protein hetero-complexes (1, 3). This is made possible by the development of physical-chemical models for various hetero-complex stoichiometries, which are each fit to the FSI-FRET data to identify which model best describes the data. A detailed explanation of the data fitting process is described by Del Piccolo *et al.* (3). Here, models are generated based upon our current knowledge of E-cadherin and EGFR homodimerization. Full-length E-cadherin is a constitutive dimer at all concentrations, as reported by Singh *et al*. (4). Full-length EGFR homodimerizes in a monomer-dimer equilibrium with an association constant of (8.8 ± 0.7) x 10^{-3} μm^2/receptor (dissociation constant, K_{diss}=114 ± 9 receptors/μm^2), as reported by Macdonald and Pike (9). When the intracellular domains are replaced with a fluorescent protein, as done in the FRET experiments, E-cadherin and EGFR each exist in a monomer-homodimer equilibrium. The reported association constant for E-cadherin is (1.6 ± 0.3) x 10^{-3} μm^2/receptor (K_{diss} is 624 ± 103 receptors/μm^2) and for EGFR is (3.6 ± 0.2) x 10^{-4} μm^2/receptor (K_{diss} is 2,812 ± 197 receptors/μm^2) (4, 5). Because both proteins can exist as monomers or dimers, there are several possible stoichiometries that the E-cadherin/EGFR complex could form at the plasma membrane: (i) hetero-dimers of an E-cadherin monomer and an EGFR monomer, (ii) hetero-trimers of E-cadherin dimers and EGFR monomers, (iii) hetero-trimers of E-cadherin monomers and EGFR dimers, and (iv) hetero-tetramers of E-cadherin dimers with EGFR dimers. Here, we describe the physical-chemical models of the binding equilibria for each of these hetero-complex stoichiometries, where E-cadherin and EGFR are denoted by C and R, respectively.

Hetero-dimers. It is possible that the E-cadherin/EGFR interactions are mediated by monomers where an E-cadherin monomer interacts with an EGFR monomer. These equations consider the fact that both C and R form homodimers, CC and RR, in addition to the hetero-dimer, CR:

$$[C] + [C] \overset{K_C}{\leftrightarrow} [CC]$$
$$[R] + [R] \overset{K_R}{\leftrightarrow} [RR]$$
$$[C] + [R] \overset{K_{CR}}{\leftrightarrow} [CR] \quad (1)$$

The homodimer association constants, K_C and K_R, and the hetero-dimer association constant, K_{CR} can be written in terms of the monomer and dimer concentrations:

$$K_C = \frac{[CC]}{[C]^2}$$
$$K_R = \frac{[RR]}{[R]^2}$$
$$K_{CR} = \frac{[CR]}{[C][R]} \quad (2)$$

Equations for mass conservation can be written for the total protein concentrations under the assumption that the total concentration of each protein is constant—an assumption which is valid for the conditions under which the FSI-FRET experiments were performed (10).

$$[C_{total}] = [C] + 2[CC] + [CR]$$
$$[R_{total}] = [R] + 2[RR] + [CR] \quad (3)$$

The total concentrations can be written in terms of the monomer and hetero-dimer concentrations by the use of equation *(2)*:

$$[C_{total}] = [C] + 2K_C[C]^2 + [CR]$$
$$[R_{total}] = [R] + 2K_R[R]^2 + [CR] \quad (4)$$

These quadratic equations can be solved for the monomer concentrations:

$$[C] = \frac{-1 + \sqrt{1 - 8K_C([CR] - [C_{total}])}}{4K_C}$$
$$[R] = \frac{-1 + \sqrt{1 - 8K_R([CR] - [R_{total}])}}{4K_R} \quad (5)$$

By rearranging equation *(2)*, the hetero-dimer concentration can be written in terms of the monomer concentrations and the hetero-dimer association constant:

$$[CR] = K_{CR}[C][R] \quad (6)$$

By substituting the monomer concentrations from equation *(5)* into equation *(6)*, we arrive at an expression in which the hetero-dimerization terms, K_{CR} and [CR], are defined in terms of $[C_{total}]$, $[R_{total}]$, K_C, and K_R. The association constants, K_C and K_R, are known from previous FRET experiments (4, 5). The $[C_{total}]$ and $[R_{total}]$, along with the FRET efficiency, are measured directly from the plasma membrane of cells using the FSI-FRET method (1, 3-5). Therefore, the remaining unknown parameters in our equations are the hetero-dimerization association constant, K_{CR}, and the concentration of hetero-dimers [CR]. We can determine the concentration of hetero-dimers [CR] in our FRET experiments because [CR] is related to the FRET efficiency by:

$$E = \frac{[\mathrm{CR}]\tilde{E}}{[R_{total}]} = \frac{K_{CR}[\mathrm{C}][R]\tilde{E}}{[R_{total}]} \quad (7)$$

where E is the measured FRET efficiency and Ẽ is the intrinsic FRET—a structural parameter that depends on the distance between the two fluorophores and their relative orientation (11). We perform a two-parameter fit of the FRET data, which optimizes the values for Ẽ and K_{CR}.

Hetero-trimers. There are two possible hetero-trimers that could form when E-cadherin and EGFR interact: either an E-cadherin dimer (CC) associates with an EGFR monomer (R) to form a trimer CCR as shown in Fig. 6B, or an E-cadherin monomer (C) interacts with an EGFR dimer (RR) to form a trimer CRR (Fig. 6C). In either case, three coupled reactions are needed to describe hetero-trimerization.

$$[C] + [C] \overset{K_C}{\leftrightarrow} [CC]$$
$$[R] + [R] \overset{K_R}{\leftrightarrow} [RR]$$
$$[CC] + [R] \overset{K_{CCR}}{\longleftrightarrow} [CCR] \quad or \quad [C] + [RR] \overset{K_{CRR}}{\longleftrightarrow} [CRR]$$

(8)

The homodimer association constants, K_C and K_R, and the hetero-trimer association constant, K_{CCR} or K_{CRR}, can be written in terms of the monomer, dimer, and hetero-trimer concentrations:

$$K_C = \frac{[CC]}{[C]^2}$$
$$K_R = \frac{[RR]}{[R]^2}$$
$$K_{CCR} = \frac{[CCR]}{[CC][R]} \quad or \quad K_{CRR} = \frac{[CRR]}{[C][RR]}$$

(9)

The total concentration of each receptor is given by the equations for mass conservation:

$$[C_{total}] = [C] + 2[CC] + 2[CCR] \quad or \quad [C_{total}] = [C] + 2[CC] + [CRR]$$

$$[R_{total}] = [R] + 2[RR] + [CCR] \quad or \quad [R_{total}] = [R] + 2[RR] + 2[CRR]$$

(10)

These mass conservation equations can be rewritten in terms of the association constants from equation *(9)*:

$$[C_{total}] = [C] + 2K_C[C]^2 + 2[CCR] \quad or \quad [C_{total}] = [C] + 2K_C[C]^2 + [CRR]$$
$$[R_{total}] = [R] + 2K_R[R]^2 + [CCR] \quad or \quad [R_{total}] = [R] + 2K_R[R]^2 + 2[CRR]$$

(11)

These quadratic equations can be rearranged and solved, in terms of each of the monomer concentrations:

$$[C] = \frac{-1 + \sqrt{1 - 8K_C(2[CCR] - [C_{total}])}}{4K_C} \quad or \quad [C] = \frac{-1 + \sqrt{1 - 8K_C([CRR] - [C_{total}])}}{4K_C}$$
$$[R] = \frac{-1 + \sqrt{1 - 8K_R([CCR] - [R_{total}])}}{4K_R} \quad or \quad [R] = \frac{-1 + \sqrt{1 - 8K_R(2[CRR] - [R_{total}])}}{4K_R}$$

(12)

The hetero-trimer concentration can be written in terms of the monomer concentrations and the hetero-trimer association constant, by rearranging equation *(9):*

$$[CCR] = K_{CCR}[CC][R] = K_{CCR}K_C[C]^2[R] \quad or \quad [CRR] = K_{CRR}[C][RR] = K_{CRR}[C]K_R[R]^2$$

(13)

The monomer concentrations from equation *(12)* can be substituted into equation *(13)* to obtain an expression in which the hetero-trimerization terms, K_{CCR} or K_{CRR} and [CCR] or [CRR], are defined in terms of $[C_{total}]$, $[R_{total}]$, K_C, and K_R. The association constants, K_C and K_R, are known from previous FRET experiments (4, 5). The $[C_{total}]$ and $[R_{total}]$, along with the FRET efficiency, are measured directly on the plasma membranes of cells, using the FSI-FRET method (1, 3-5). Therefore, the remaining unknown parameters in our equations are the hetero-trimer association constant, K_{CCR} or K_{CRR}, and the hetero-trimer concentration, [CCR] or [CRR]. We can

determine the concentration of hetero-trimers [CCR] or [CRR] in our FRET experiments because the hetero-trimer concentration is related to the FRET efficiency:

$$E = \frac{[\mathrm{CCR}]\tilde{E}}{[R_{total}]} = \frac{K_{CCR}K_C[C]^2[R]\tilde{E}}{[R_{total}]} \quad or \quad E = \frac{[\mathrm{CRR}]\tilde{E}}{[R_{total}]} = \frac{K_{CRR}[C]K_R[R]^2\tilde{E}}{[R_{total}]} \tag{14}$$

where E is the measured FRET efficiency and Ẽ is the intrinsic FRET (11). We perform a two-parameter fit of the FRET data, to optimize the values for Ẽ and K_{CCR} or K_{CRR}.

Hetero-tetramers. Finally, it is possible that the interactions between E-cadherin and EGFR are mediated by interacting homodimers as illustrated in Fig. 6D. In this model, the interaction of two dimers, CC and RR, creates a hetero-tetramer, CCRR. The formation of hetero-tetramers can be described by three coupled equations:

$$\begin{gathered}[C] + [C] \overset{K_C}{\leftrightarrow} [CC] \\ [R] + [R] \overset{K_R}{\leftrightarrow} [RR] \\ [CC] + [RR] \overset{K_{CCRR}}{\longleftrightarrow} [CCRR]\end{gathered} \tag{15}$$

The homodimer association constants, K_C and K_R, and the hetero-tetramer association constant, K_{CCRR}, can be written in terms of the monomer, dimer, and hetero-tetramer concentrations:

$$\begin{gathered}K_C = \frac{[CC]}{[C]^2} \\ K_R = \frac{[RR]}{[R]^2} \\ K_{CCRR} = \frac{[CCRR]}{[CC][RR]}\end{gathered} \tag{16}$$

The total concentration is given by the equations for mass conservation:

$$\begin{aligned}[C_{total}] &= [C] + 2[CC] + 2[CCRR] \\ [R_{total}] &= [R] + 2[RR] + 2[CCRR]\end{aligned} \tag{17}$$

and these equations can be rewritten in terms of the association constants from equation *(16):*

$$\begin{aligned}[C_{total}] &= [C] + 2K_C[C]^2 + 2[CCRR] \\ [R_{total}] &= [R] + 2K_R[R]^2 + 2[CCRR]\end{aligned} \tag{18}$$

Upon rearranging equation *(18)*, these quadratic equations can be solved for the monomer concentrations:

$$\begin{aligned}[C] &= \frac{-1 + \sqrt{1 - 8K_C(2[CCRR] - [C_{total}])}}{4K_C} \\ [R] &= \frac{-1 + \sqrt{1 - 8K_R(2[CCRR] - [R_{total}])}}{4K_R}\end{aligned} \tag{19}$$

The hetero-tetramer concentration can be written in terms of the monomer concentrations and the hetero-tetramer association constant by rearranging equation *(16)*:

$$[CCRR] = K_{CCRR}[CC][RR] = K_{CCRR}K_C[C]^2K_R[R]^2 \tag{20}$$

The monomer concentrations from equation *(19)* can be substituted into equation *(20)* to arrive at an equation where the hetero-tetramerization terms, K_{CCRR} and [CCRR], are defined in terms of $[C_{total}]$, $[R_{total}]$, K_C, and K_R. The association constants, K_C and K_R, are known from

previous FRET experiments (4, 5). The [C_{total}] and [R_{total}], along with the FRET efficiency, are measured directly from the plasma membrane of cells, using the FSI-FRET method (1, 3-5). Therefore, the remaining unknown parameters in our equations are the hetero-tetramer association constant, K_{CCRR}, and the concentration of hetero-tetramers [CCRR]. We can determine the concentration of hetero-tetramers [CCRR] in our FRET experiments since [CCRR] is related to the FRET efficiency:

$$E = \frac{[\mathrm{CCRR}]\tilde{E}}{[R_{total}]} = \frac{K_{CCRR}K_C[C]^2K_R[R]^2\tilde{E}}{[R_{total}]} \quad (21)$$

where E is the measured FRET efficiency and $\tilde{E}$ is the intrinsic FRET [7]. We perform a two-parameter fit of the FRET data, which optimizes the values for $\tilde{E}$ and K_{CCRR}.

We determine the best model for the data by fitting each of the binding models to the measured FRET data and by determining the mean square error (MSE) for each of the fits. The model with the lowest MSE is considered to be the best descriptor of the data. We fit each of these models to the FSI-FRET data for the E-cadherin/EGFR complex in the absence of EGF ligand. Our findings suggest that the hetero-trimer model consisting of two E-cadherin and one EGFR best fits the FRET data. The association constant, K_{CCR}, for the $(\text{E-cadherin})_2$-EGFR complex, whereby the intracellular domains are replaced by a fluorescent protein is (1.03 ± 0.05) x 10^{-3} μm^2/receptor (dissociation constant, K_{diss}, is 969 ± 52 receptors/μm^2) in the absence of ligand. The hetero-receptor association constant is weaker than E-cadherin homodimerization, but stronger than EGFR homodimerization, in the absence of EGF.

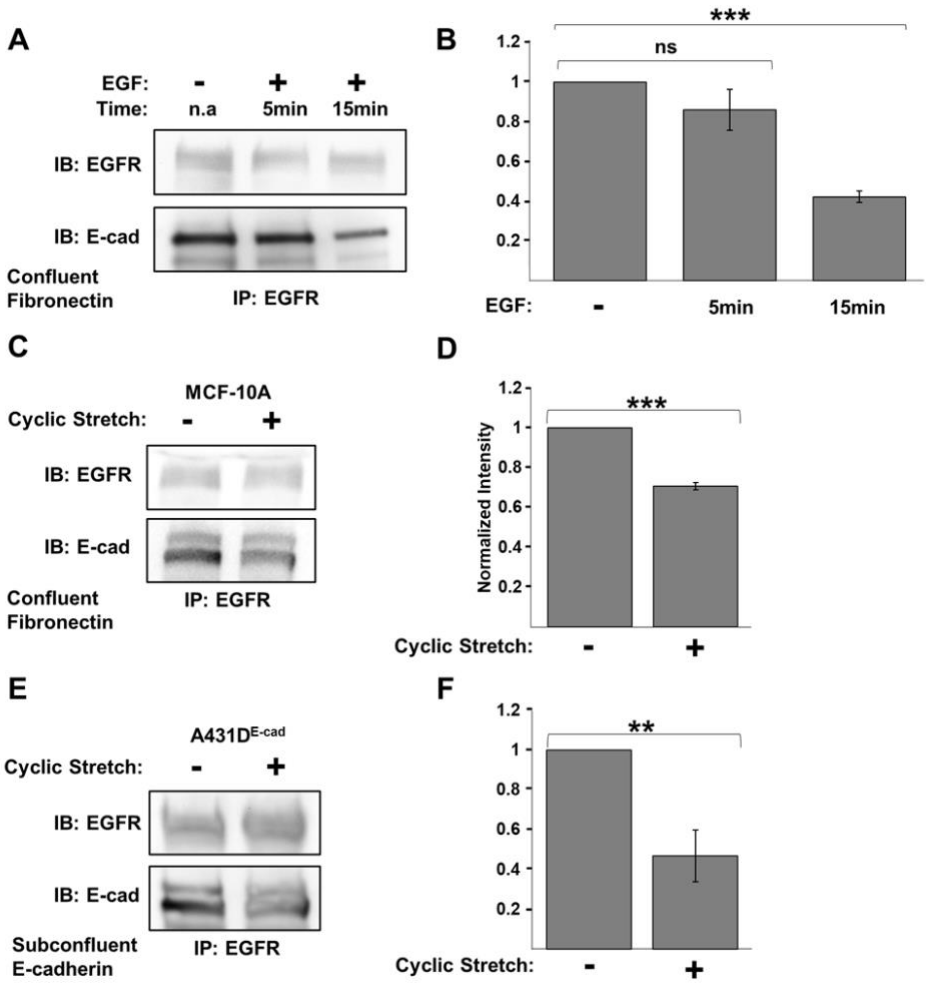

Fig. S1. EGF and cyclic stretch disrupt E-cadherin/EGFR complexes. Panels A, C, and E, show co-IP results obtained with cells (A) treated with 3nM EGF or (C,E) subjected to cyclic stretch. With cells that were not treated with EGF, EGF-neutralizing antibody was included in the medium. (A) Confluent A-431D$^{E\text{-}cad}$ cells on fibronectin-coated PDMS membranes were treated with 3nM EGF for either 5 or 15min. (B) Normalized E-cadherin band intensities from measurements under the conditions in panel A (n = 3). The intensities were normalized to the untreated condition. (C) Co-IP results obtained with serum starved, confluent MCF-10A cells on fibronectin-coated PDMS membranes were subjected to 10% cyclic stretch for 30min. (D) Normalized E-cadherin band intensities determined under conditions in panel C (n = 3). Intensities are normalized relative to the unstretched condition. (E) Co-IP results obtained with stretched subconfluent A-431D$^{E\text{-}cad}$ monolayers on E-cad-Fc coated PDMS membranes. Integrin blocking antibody 16G3 was included to prevent integrin interference. (F) Normalized E-cadherin band intensities obtained under conditions in panel E (n = 3). Band intensities are normalized to the unstretched condition. The error bars are the s.e.m. (* p = 0.05, ** p = 0.005, *** p = 0.0005).

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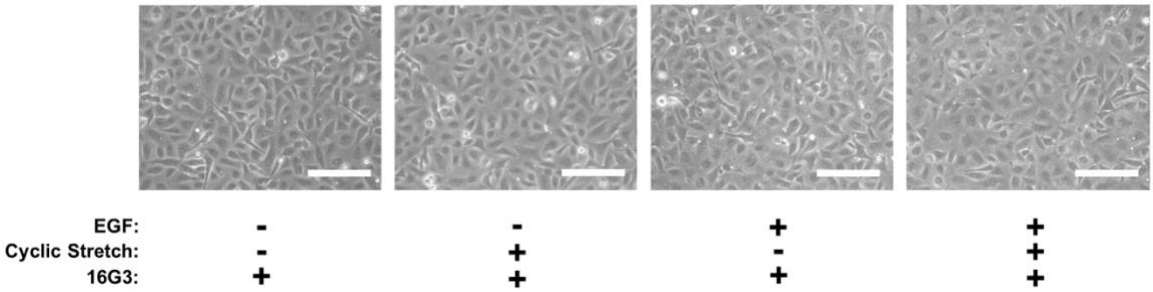

Fig. S2. Confluent A-431D$^{E\text{-}cad}$ cells on E-cad-Fc-coated PDMS membranes remain intact during cyclic stretching, in the presence and absence of EGF. DIC images are of A-431D$^{E\text{-}cad}$ cells that were serum starved overnight and then seeded on E-cad-Fc coated PDMS membranes at confluent density for 5hr. 16G3 antibody was added to prevent integrin interference, and control samples without EGF were treated with EGF-neutralizing antibody. Before acquiring DIC images to assess monolayer integrity, cells were subjected to either of 4 conditions: ± 10% cyclic stretch for 30min and ± 3nM EGF. Scale bar is 200μm.

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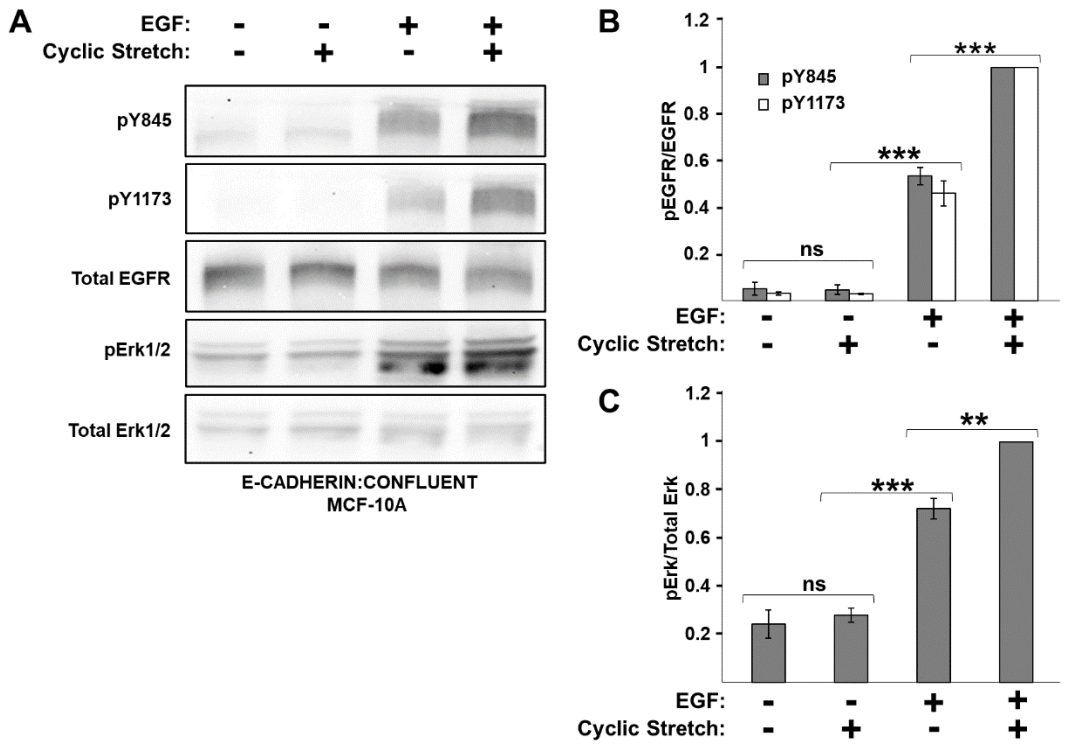


Fig. S3: **Western blot analysis of EGFR and Erk1/2 activation in confluent MCF10A monolayers on E-cad-Fc substrates.** MCF10A cells were serum starved overnight and plated at monolayer density on E-cad-Fc coated PDMS membranes. Cells were allowed to attach for 5hr in the presence of 16G3 antibody, which blocks integrin binding to fibronectin. Cells were subjected to either of 4 conditions: ± 10% cyclic stretch for 30min, in the presence or absence of 3nM EGF. EGF neutralizing antibody was included in samples that were not treated with EGF. (A) Western blot analyses assessed pEGFR (Y845 and Y1173) and pErk1/2 levels following cyclic stretch, in the absence and presence of 3nM EGF. Control cells were not stretched or treated with EGF. (B) Normalized band densities of pY845 (n = 4) and pY1173 (n = 4) determined under the four conditions in panel A. Band intensities are normalized to the total EGFR. (C) Normalized pErk1/2 (n = 4) levels determined under the conditions in panel A. Band intensities are normalized to total Erk1/2. Error bars are s.e.m. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

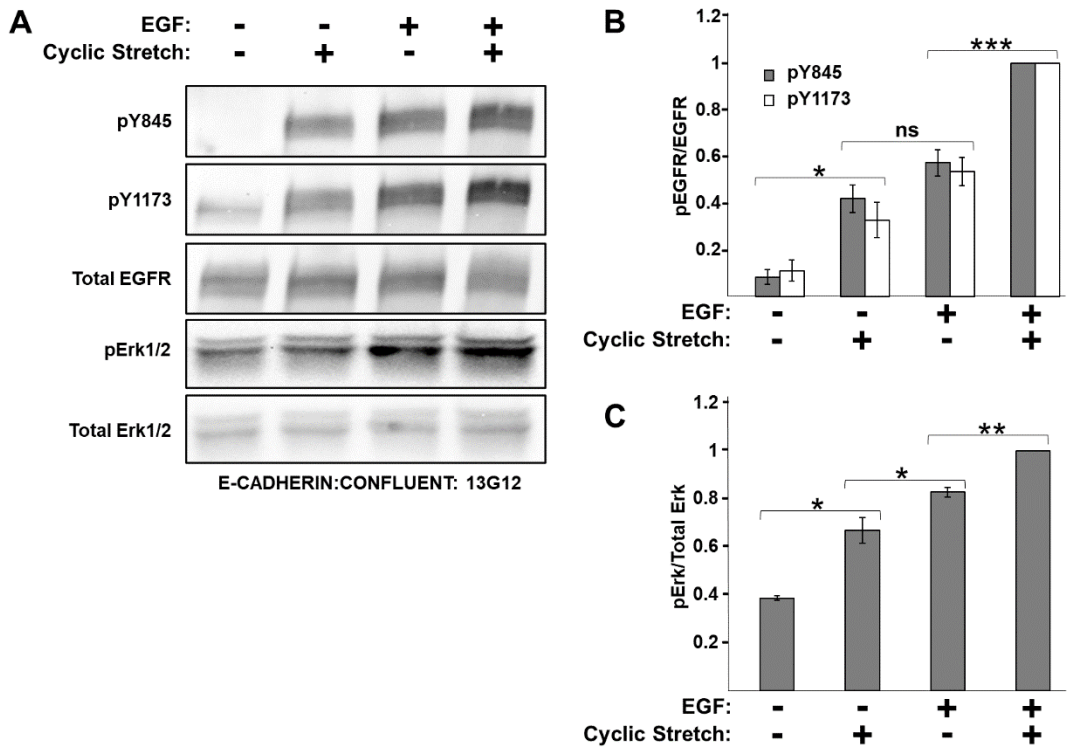
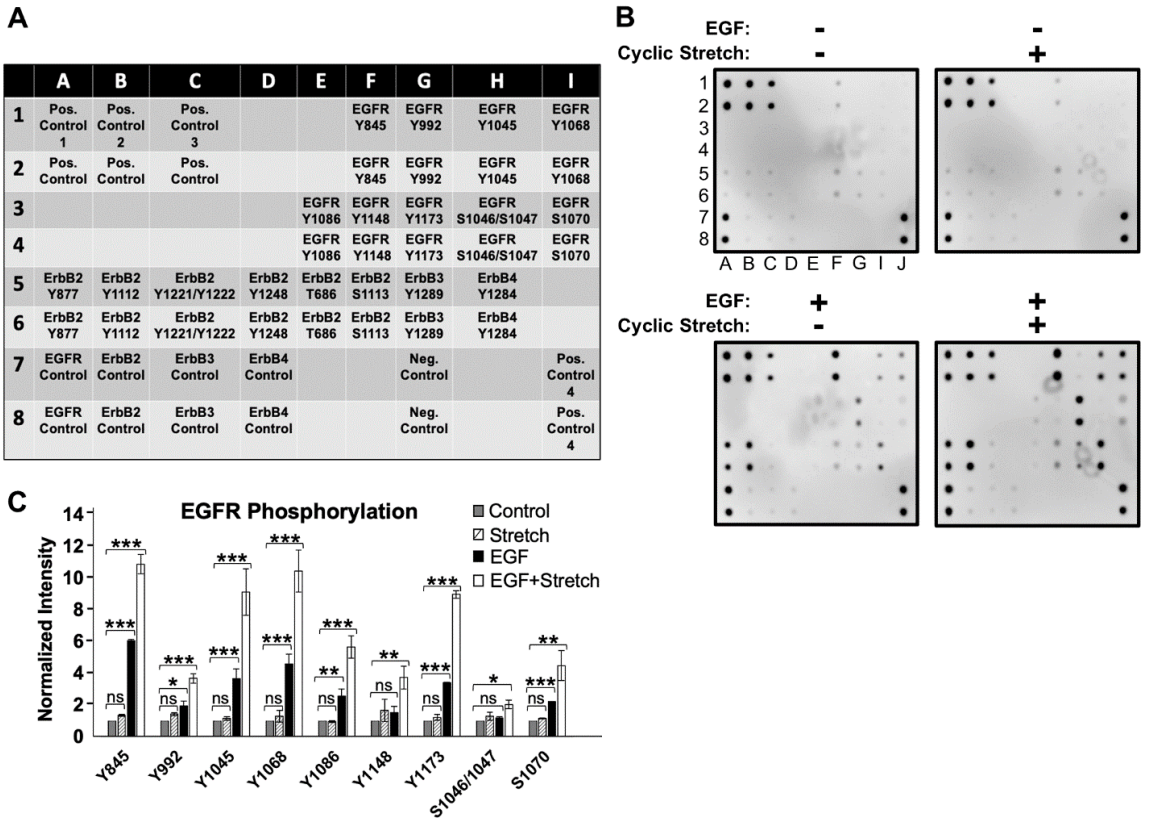


Fig. S4: **Control 13G12 antibody does not affect EGFR and Erk1/2 activation in confluent A431D$^{E\text{-}cad}$ monolayers on E-cad-Fc coated membranes.** Cells were serum starved overnight and plated at confluent density on E-cad-Fc coated membranes, in the presence of the non-blocking control antibody 13G12. Cells were subjected to 4 conditions: ± 10% cyclic stretch for 30min, in the presence or absence of 3nM EGF. EGF neutralizing antibody was included in samples that were not treated with EGF. (A) Cells were analyzed by Western blot analysis to assess EGFR phosphorylation at p845 and pY1173 and total EGFR. Cells were also analyzed for pErk1/2 and total Erk1/2. (B) Normalized band densities of pY845 (n = 4) and pY1173 (n = 4) determined under the four conditions in panel A. Band intensities are normalized to total EGFR. (C) Normalized pErk1/2 (n = 3) levels determined under the four conditions in panel A. Band intensities are normalized to total Erk1/2. Error bars are s.e.m. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.



	A	B	C	D	E	F	G	H	I
1	Pos. Control 1	Pos. Control 2	Pos. Control 3			EGFR Y845	EGFR Y992	EGFR Y1045	EGFR Y1068
2	Pos. Control	Pos. Control	Pos. Control			EGFR Y845	EGFR Y992	EGFR Y1045	EGFR Y1068
3					EGFR Y1086	EGFR Y1148	EGFR Y1173	EGFR S1046/S1047	EGFR S1070
4					EGFR Y1086	EGFR Y1148	EGFR Y1173	EGFR S1046/S1047	EGFR S1070
5	ErbB2 Y877	ErbB2 Y1112	ErbB2 Y1221/Y1222	ErbB2 Y1248	ErbB2 T686	ErbB2 S1113	ErbB3 Y1289	ErbB4 Y1284	
6	ErbB2 Y877	ErbB2 Y1112	ErbB2 Y1221/Y1222	ErbB2 Y1248	ErbB2 T686	ErbB2 S1113	ErbB3 Y1289	ErbB4 Y1284	
7	EGFR Control	ErbB2 Control	ErbB3 Control	ErbB4 Control			Neg. Control		Pos. Control 4
8	EGFR Control	ErbB2 Control	ErbB3 Control	ErbB4 Control			Neg. Control		Pos. Control 4

Fig. S5. Cyclic stretch increases EGFR phosphorylation at EGF-dependent sites. An EGFR phosphorylation array was used to detect different phosphorylated sites on EGFR. Confluent A431D$^{E\text{-}cad}$ cells on E-Cad-Fc coated membranes were subjected to 10% cyclic stretch, in the presence or absence of 3nM EGF. Monolayers were serum starved for 24hr prior to 5hr seeding on E-cad-Fc coated PDMS membranes. 16G3 antibody was applied to prevent integrin interference and cells that were not treated with EGF were cultured with EGF neutralizing antibody. (A) The phospho-array map depicting the global pEGFR sites targeted by the assay. (B) Array images of all the phosphorylation sites shown in panel A for the 4 different cell conditions mentioned above. (C) Bar plots representing the normalized intensities associated with EGFR phosphorylation sites in panel B (n = 4). Sites were normalized to the positive controls and to samples that were not subjected to cyclic stretch or EGF treatment, as recommended by the manufacturer. Error bars are s.e.m. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

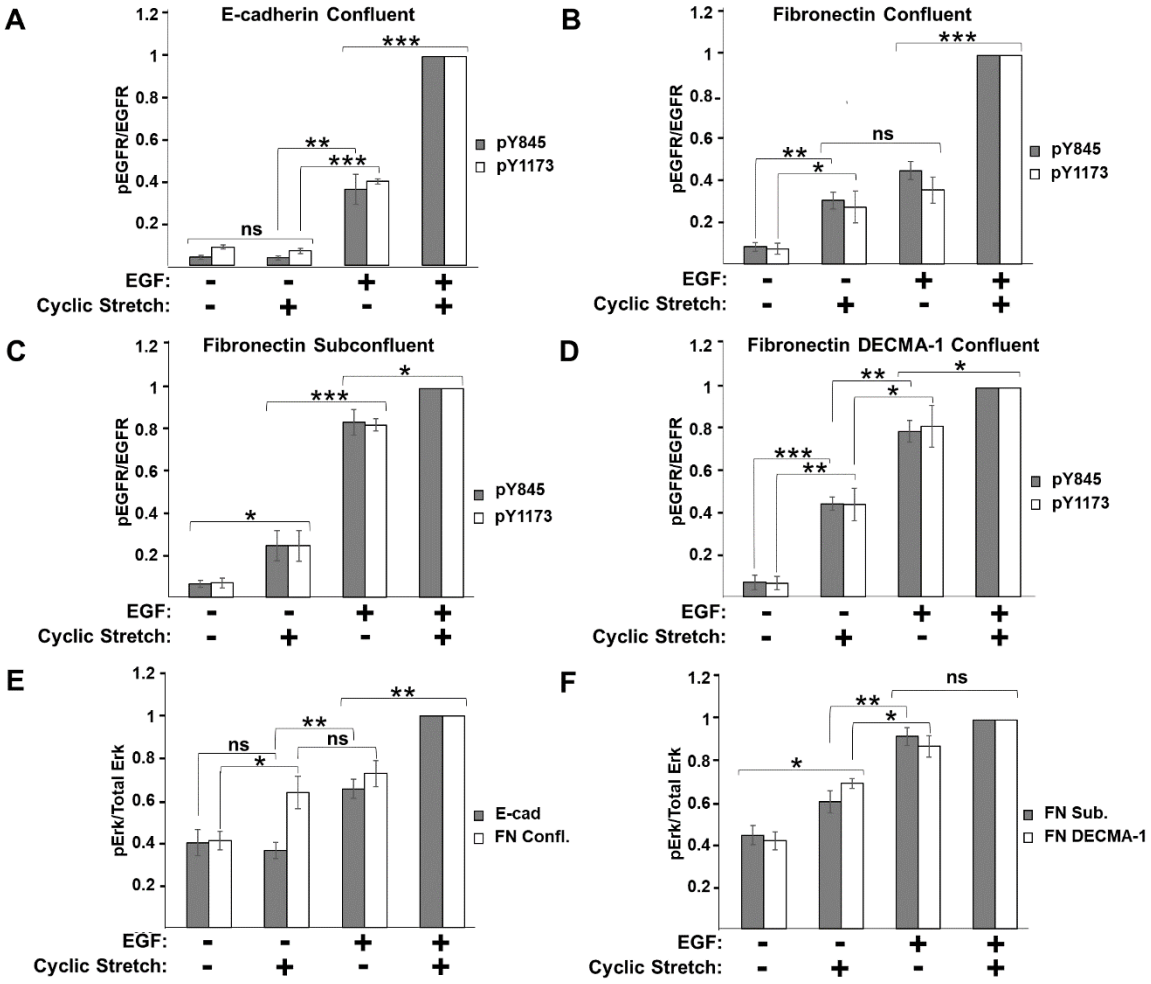


Fig. S6: Normalized band intensities from Western blots of pY845, pY1173, and pErk1/2 in A-431D$^{E\text{-}cad}$ cells on PDMS membranes. Cells were subject to 10% cyclic-stretch for 30 min, in the presence and absence of 3nM EGF. (A,B) EGFR phosphorylation levels (pY845 and pY1173) were measured with confluent A-431D$^{E\text{-}cad}$ cells on membranes coated with (A) E-cad-Fc (n_{845} = 7, n_{1173} = 6, $n_{pErk1/2}$= 5) or (B) fibronectin (n_{845} = 4, n_{1173} = 4, $n_{pErk1/2}$ = 4). (C,D) Normalized pEGFR band intensities determined with subconfluent (n_{845} = 6, n_{1173} = 6, $n_{pErk1/2}$ = 6) and (D) DECMA-1 treated confluent A-431D$^{E\text{-}cad}$ cells (n_{845} = 4, n_{1173} = 4, $n_{pErk1/2}$ = 3) on fibronectin. Phosphorylation levels were normalized to the EGFR intensities determined with stretched cells in the presence of 3nM EGF. (E) Normalized pErk1/2 levels in confluent monolayers on E-cad and FN substrates. Signals were normalized by the pErk1/2 levels in cyclically stretched cells treated with 3nM EGF. (F) Normalized pErk1/2 levels in A431D monolayers on E-cad and FN substrates. Signals were normalized by the pErk1/2 levels in cyclically stretched cells on FN, in the presence of 3nM EGF. Samples did not have intact cell-cell junctions. They were either subconfluent monolayers or monolayers treated with DECMA-1. Error bars are s.e.m. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

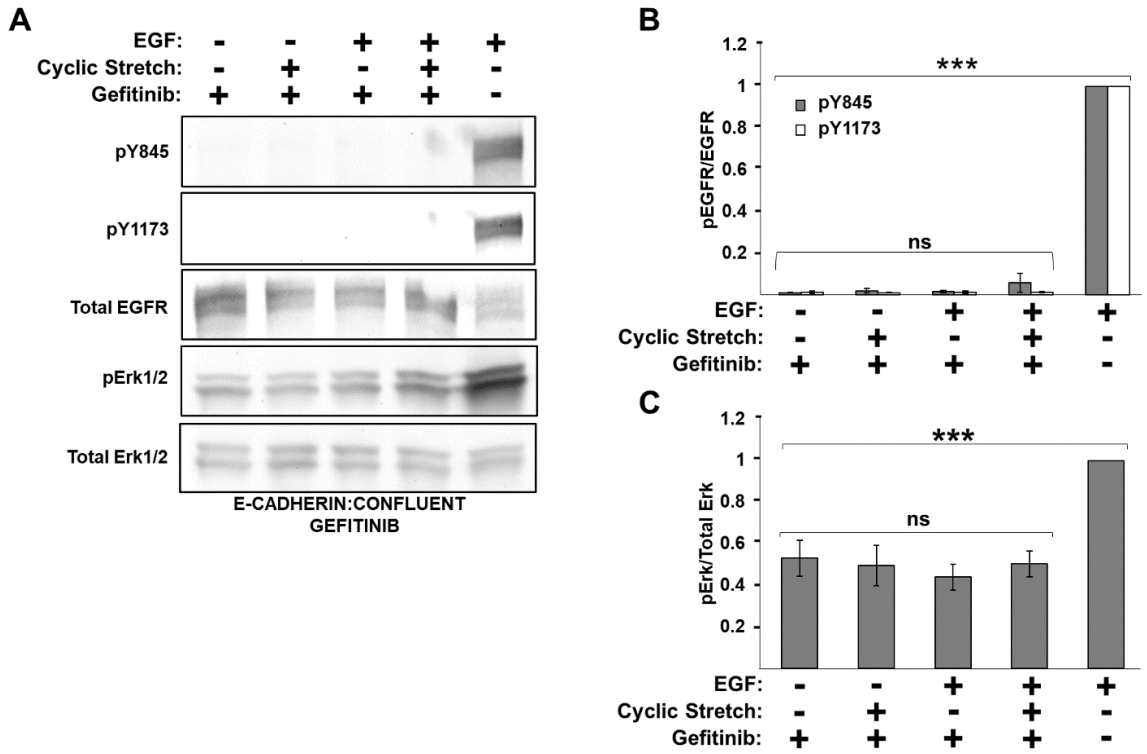


Fig. S7: Gefitinib blocks EGFR and Erk1/2 phosphorylation in confluent monolayers on E-cad-Fc substrates. (A) Western blot analysis of EGFR (Y845 and Y1173) and Erk1/2 phosphorylation levels in serum starved, confluent A-431D$^{E\text{-}cad}$ monolayers on E-cad-Fc coated PDMS membranes. Cells were allowed to attach for 5hr in the presence of anti fibronectin antibody, 16G3. 2hr prior to 10% cyclic stretch and 3nM EGF treatment, cells were treated with 15μM Gefitinib. EGF neutralizing antibody was included in samples that were not treated with EGF. (B) Normalized EGFR phosphorylation levels (pY845 (n = 4) and pY1173 (n = 5)). Band intensities from Western blots as in panel A were normalized by intensities of unstretched, EGF treated cells. (C) Normalized pErk1/2 (n = 5) levels obtained from Western blots as in panel A. Intensities were normalized by the band intensities of unstretched, EGF treated cells. Error bars are s.e.m. *** $p < 0.0005$.

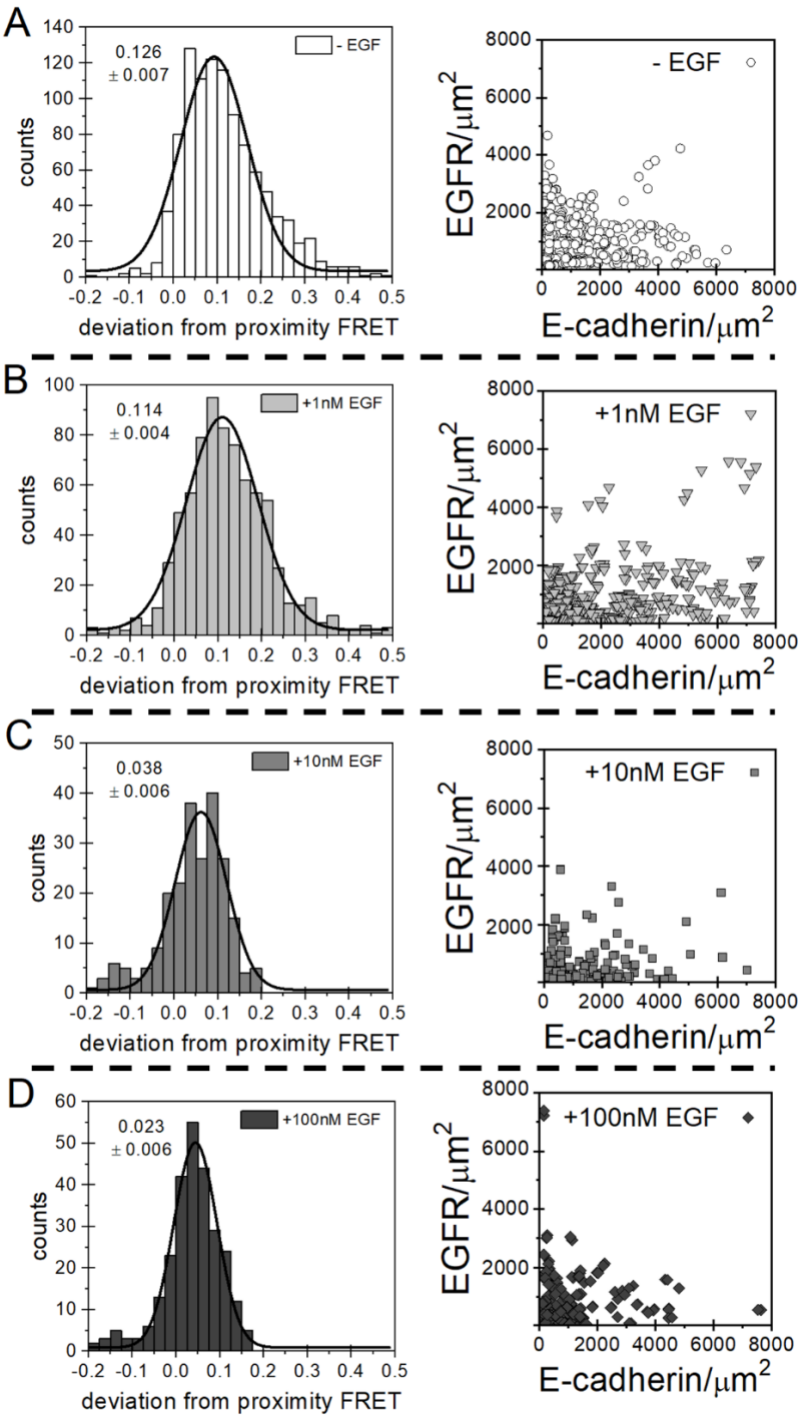


Fig. S8. Corrected FRET histograms and EGFR/E-cadherin expression levels. Histograms of FRET deviations from proximity FRET alongside EGFR-mTurq and E-cadherin-eYFP membrane concentrations plotted for each condition: (A) no EGF (516 cells), (B) 1nM EGF (393 cells), (C) 10nM EGF (176 cells), and (D) 100nM EGF (209 cells). Each histogram was fit to a Gaussian distribution, and the mean value and standard error (written in the top left corner) was

determined. For EGFR and E-cadherin concentrations, the surface expression for both proteins was calculated per cell and the expression patterns were similar for each condition.

comparison	significance	*p*-value
no EGF/1nM EGF	n.s.	0.3589
no EGF/10nM EGF	****	<0.0001
no EGF/100nM EGF	****	<0.0001
1nM EGF/10nM EGF	****	<0.0001
1nM EGF/100nM EGF	****	<0.0001
10nM EGF/100nM EGF	n.s.	0.7261

Table S1. Statistical significance of compared deviations from proximity FRET, measured at different EGF concentrations. Significance was calculated by ANOVA. (**** indicates $p < 0.0001$ and n.s. indicates $p \geq 0.05$).

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