Supporting Information

Bipartite Tetracysteine Display Reveals Allosteric Control of Ligand-Specific EGFR Activation

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Materials and Methods

Materials.

All purchased reagents were used without further purification. Unlabeled EGF was purchased from Fisher. AlexaFluor 647-labeled EGF and ReAsH were purchased from Invitrogen. British Anti-Lewisite (BAL) was purchased from VWR. Additional growth factors (TGFα, HB-EGF, and HRG) and fibronectin were purchased from Sigma. Unless otherwise noted, cell culture media and buffers were purchased from Gibco. All other reagents and chemicals were purchased from Sigma-Aldrich or Fisher.

Site-directed mutagenesis.

The parent plasmid (pcDNA3.1) containing the sequence encoding full-length EGFR with an N-terminal FLAG tag was generously provided by the Kuriyan lab. Site-directed mutagenesis was accomplished using Quikchange or Quikchange Lightning (Agilent) according to the manufacturer's instructions. Please see Table S1 for details regarding the mutagenesis primers.

Cell Culture.

CHO-K1 cells were maintained in Kaighn's modified F-12 media (F12-K, Cell-gro) supplemented with 10% fetal bovine serum, pen-strep, and 10 mM HEPES. COS7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, pen-strep, and 10 mM HEPES.

EGFR activation studies.

CHO-K1 cells (roughly 1.2 x 10^6) were seeded into 100 mm dishes (BD Falcon) and incubated at 37 °C in 5% CO_2 for 24 h. Transfection of full-length EGFR variants was accomplished using TransIT-CHO (Mirus) according to the manufacturer's instructions (briefly, 36 µL TransIT-CHO, 18 µg DNA, and 18 µL CHO Mojo reagent per 100 mm dish). After 8 h, the cells were serum-

starved in serum-free F-12K for 16 h. At this time, cells were harvested using 1X cell dissociation media (Sigma) and washed, first with DPBS and then with warm media. Cells from each dish were divided into two wells of a 96 well plate and pelleted at 400 × g for 2 min. To each well was added either 0.2 mL of unlabeled EGF (100 ng/mL, 16.7 nM) in serum-free media or 0.5 mL of serum-free media. [Please note: when other growth factors were used in place of EGF, the concentration was 16.7 nM] The plates were incubated at 37 °C for 5 min, and then the cells were pelleted at 400 × g for 5 min. The supernatant was removed and the cells were washed once with serum-free media. The cells were pelleted again at 400 × g for 2 min, and then resuspended in 200 µL of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% Triton X-100, pH 7.5) containing 1 mM sodium orthovanadate and a protease inhibitor cocktail (Roche) and were incubated on ice for 1.5 h. The lysates were clarified by centrifugation at 14,000 × g for 25 min and the supernatant was stored in aliquots and frozen. Total protein concentrations were determined using the Bradford protein assay (Bio-Rad) in order to normalize the total amount of protein loaded onto the gels (10 µg per lane). SDS-PAGE analysis was accomplished using 10% polyacrylamide gels (BioRad) and was followed by transfer to PVDF membranes (iBlot apparatus, Invitrogen). The membranes were blocked with 5% milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.1 % Tween, pH 7.4) for 2-3 h, followed by incubation with primary (mouse α-FLAG or rabbit α-pY1173) antibodies for 16-18 h at 4 °C. The membranes were washed three times with 5% milk in TBST (2X 5 min; 1X 15 min) and exposed to secondary HRP-conjugated α-mouse (FLAG) or α-rabbit (pY) antibodies for 1.5 h. The membranes were washed again using TBS-T, as above, and then were developed using ImmunStar WesternC chemiluminescent reagents (BioRad). Chemiluminescent detection was performed using a ChemiDoc XRS+ (BioRad)

Surface biotinylation studies.

CHO-K1 cells (roughly 1.2 x 10^6) were seeded into 100 mm dishes (BD Falcon) and incubated at 37 °C in 5% CO_2 for 24 h. Transfection of full-length EGFR variants was accomplished using TransIT-CHO (Mirus) according to the manufacturer's instructions (briefly, 36 µL TransIT-CHO, 18 µg DNA, and 18 µL CHO Mojo reagent per 100 mm dish). After 8 h, the cells were serum-starved in serum-free F-12K for 16 h. At this time, the cells were rinsed twice with DPBS and then each dish was incubated with 2 mL of 1 mg/mL sulfo-NHS-LC-biotin (Pierce) at 4 °C for 45 min. After the biotinylation protocol, the remaining unreacted biotin was quenched by washing three times with cold DPBS containing 100 mM glycine. The cells were then washed twice with DPBS, harvested from the dishes using 1X cell dissociation media (Sigma), and then washed first with DPBS and then with warm media. Cells from each dish were divided into two wells of a 96 well plate and pelleted at 400 × g for 2 min. To each well was added either 0.2 mL of unlabeled EGF (100 ng/mL, 16.7 nM) in serum-free media or 0.5 mL of serum-free media. The plates were incubated at 37 °C for 5 min, and then the cells were pelleted at 400 × g for 5 min. The supernatant was removed and the cells were washed once with serum-free media. The cells were pelleted again at 400 × g for 2 min, and then resuspended in 200 µL of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% Triton X-100, pH 7.5) containing 1 mM sodium orthovanadate and a protease inhibitor cocktail (Roche) and were incubated on ice for 1.5 h. The lysates were clarified by centrifugation at 14,000 × g for 25 min and the supernatant was stored in aliquots and frozen. The FLAG-containing protein (EGFR) was immunoprecipitated using the FLAG immunoprecipitation kit (Sigma). Briefly, to 40 µL of packed ANTI-FLAG M2-Agarose Affinity Gel in a spin column (Pierce), was added 35 µL of cell lysate that was diluted to 750 µL with lysis buffer. The beads were incubated on a rotisserie shaker at 4 °C overnight. At this time, the unbound protein was removed *via* centrifugation. The resin-bound protein was washed three times with wash buffer (Sigma). Bound FLAG-EGFR was eluted by boiling for 5 min with 2X

SDS-PAGE sample buffer containing 50 mM DTT. SDS-PAGE analysis was accomplished using 10% polyacrylamide gels (BioRad) and was followed by transfer to PVDF membranes (iBlot apparatus, Invitrogen). The membranes were blocked with 5% milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.1 % Tween, pH 7.4) for 2-3 h, followed by incubation for 16-18 h at 4 °C with primary (mouse α-FLAG or rabbit α-pY1173) antibodies. For incubation with primary mouse α-FLAG antibodies, 8% bovine serum was used as a blocking agent. After overnight incubation, the membranes were washed three times with 5% milk (or 8% BSA) in TBST (2X 5 min; 1X 15 min) and exposed to secondary HRP-conjugated α-mouse (FLAG; biotin) or α-rabbit (pY) antibodies for 1.5 h. The membranes were washed again using TBS-T, as above, and then were developed using ImmunStar WesternC chemiluminescent reagents (BioRad). Chemiluminescent detection was performed using a ChemiDoc XRS+ (BioRad).

Surface ReAsH labeling.

CHO-K1 cells (75,000) were seeded into glass-bottomed MatTek 35 mm dishes coated with fibronectin and cultured for 24 h. Transfection with the EGFR construct of interest was accomplished using TransIT-CHO (Mirus) according to the manufacturer's instructions (briefly, 4 μL TransIT-CHO, 2 μg DNA, 2 μL CHO Mojo reagent per 35 mm dish). After 8 h, the cells were serum-starved using 1% serum in F-12K for 16 h. Receptor endocytosis was inhibited by incubation with an ATP synthesis inhibition cocktail (10 mM NaN_3, 2 mM NaF and 5 mM 2-deoxy-*D*-glucose) for 1 h. At this time, the cells were stimulated with 1 mL of unlabeled EGF (100 ng/mL in serum-free ATP inhibition media) or media alone for 30 min at 8 °C. The EGF solution was removed, and the cells were washed once with serum-free ATP inhibition media before incubation with 150 μL ReAsH labeling solution (2 μM ReAsH + 20 μM BAL + 2 μM disperse blue) for 60 min at 37 °C. The ReAsH labeling solution was removed and replaced with 2

mL of serum-free ATP inhibition media containing 100 μM BAL. This media was removed and the cells were incubated with 2 mL of serum-free ATP inhibition media containing 100 μM BAL for 10 min at 37 °C. The media was removed and the cells were fixed using 4% paraformaldehyde (PFA) for 30 min at RT. The PFA was removed and cells were rinsed once with DPBS and then blocked with 10% BSA in DPBS (PBSB) for 30 min at 37 °C. Cells were labeled with primary antibodies (α-FLAG, 1:1000 dilution in PBSB, 1h, 37 °C) and then washed three times with PBSB (1X immediate, 2X 5 min). The cells were then incubated with FITC-labeled secondary antibodies (α-mouse, 1:200 dilution in PBSB, 1 h, 37 °C) and washed as above using DPBS. Total internal reflection fluorescence microscope (TIRFM) imaging was performed using an Olympus IX81 inverted microscope fitted with TIRF optics, a temperature controlled stage, and a 63X/1.45 NA oil immersion TIRF objective. Images were collected on an EMCCD camera (Andor, Belfast). Signal from FITC-labeled antibodies (green) was monitored using the 488 nm line of an Ar/Kr laser for excitation, and emission was collected using a LP500 filter. ReAsH labeling was monitored using the 568 nm line of a He/Ne laser for excitation and a LP585 emission filter. Acquired images were analyzed using ImageJ. The mean red fluorescence was measured for 1) a peripheral region (R1) of a transfected cell 2) a comparable region (R2) of a neighboring untransfected cell and 3) a nearby region (B) of background from the glass. The mean green fluorescence (G1) was also measured for the identical region R1 to account for varying levels of receptor expression. The fold increase (normalized for receptor expression) was assessed as: fold= ((R1-B)/(R2-B))/G1. Error bars represent the 95% confidence interval. Statistical analysis was performed using GraphPad Prism Software.

Endosomal ReAsH labeling.

COS7 (60,000) or CHO-K1 (75,000) cells were seeded into glass-bottomed MatTek 35 mm dishes coated with fibronectin and cultured for 24 h. The cells were transfected with the EGFR construct of interest using TransIT-CHO according to the manufacturers instructions. After 8 h, the cells were serum-starved using 1% serum in F-12K for 16 h. Cells were stimulated with 1 mL of AlexaFluor647 EGF (1 μg/mL in serum-free media) for 30 min at 8 °C. At this time, cells were washed once with serum-free media, and then incubated with 150 μL ReAsH (2 μM ReAsH + 20 μM BAL + 2 μM disperse blue) for 60 min at 37 °C. The ReAsH labeling solution was removed and replaced with 2 mL of serum-free media containing 100 μM BAL. This media was removed and the cells were incubated with 2 mL of serum-free ATP inhibition media containing 100 μM BAL for 10 min at 37 °C. The media was removed and the cells were fixed using 4% paraformaldehyde (PFA) for 30 min at rt. The PFA was removed and the cells were rinsed once with DPBS before incubation with Hoescht for 1 h at rt. Confocal microscopy was accomplished on a Yokagawa-type SDCM system mounted onto an inverted microscope (IX71; Olympus) equipped with a 1Kb × 1Kb electron-multiplying charge-coupled device camera (Hamamatsu Photonics) and a temperature-controlled stage set at 37 ° C controlled by Ultraview ERS software (PerkinElmer). Samples were imaged in DPBS using a 60 × 1.4 NA oil phase objective. Excitation was achieved using 405 nm (Hoescht) and 568-nm argon/krypton (ReAsH) lasers and 640 nm (Alexa647) (Perkin Elmer). Acquired images were analyzed using ImageJ.

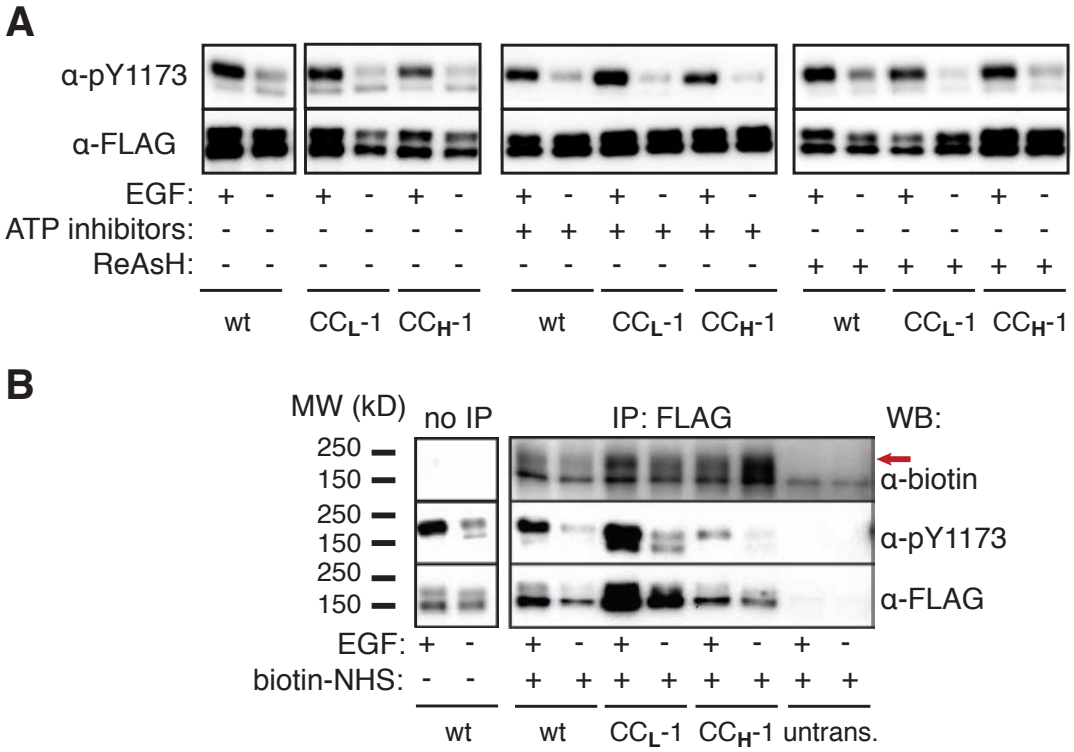
Disulfide exchange assay.

Terminal monocysteine variants of the helical JM monomer segment were prepared using solid-phase peptide synthesis (CEM). Purified monomers (C-helix: KRTLRRLLQERELVE-GGYGC; N-helix: CGYGG-KRTLRRLLQERELVEPLTPS) were combined and/or allowed to oxidize in air in a solution of 30-50mM sodium phosphate buffer, pH 9.3. The resulting homo- and

heterodimer solutions were purified by HPLC (Varian). To evaluate a preference for monomer or dimer (parallel or antiparallel), a solution containing 15 μM C-homodimer and 15 μM N/C-heterodimer was incubated with redox buffer (250 μM oxidized glutathione, 250 μM reduced glutathione, 0.2M KCl, 0.1M Tris, 1mM EDTA, pH 8.7) *(1)* for varying amounts of time at rt. The equilibration was quenched at each timepoint using 10% glacial acetic acid (v/v). Th e resulting solution was analyzed by HPLC (Shimadzu), monitoring tyrosine fluorescence.

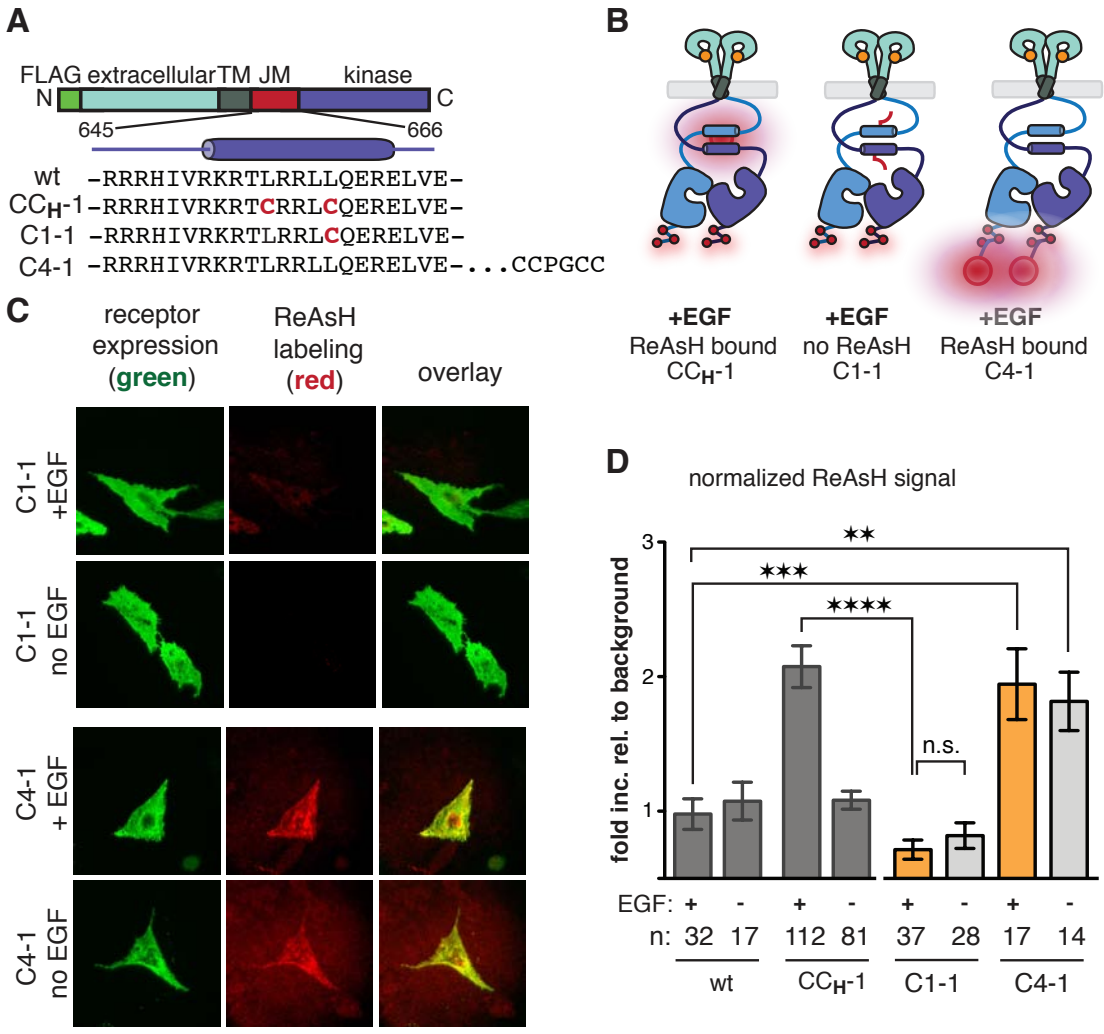
Structural Analysis.

Homology models for the TGFα-bound receptor that included domain IV were based on coordinates of the original structure bound to TGFα (1MOX) *(2)* and three unique EGFR structures that included domain IV: 3NJP *(3),* 1YY9 *(4),* and 1NQL *(5)*. Coordinates for 3NJP, 1YY9 and 1NQL were aligned separately to domain III (residues 311-500) the two peptide chains of 1MOX by least squares refinement of main chain atoms. Domain IV of the aligned structures (residues 501 onwards) were then appended to the 1MOX chains to generate three distinct homology models.



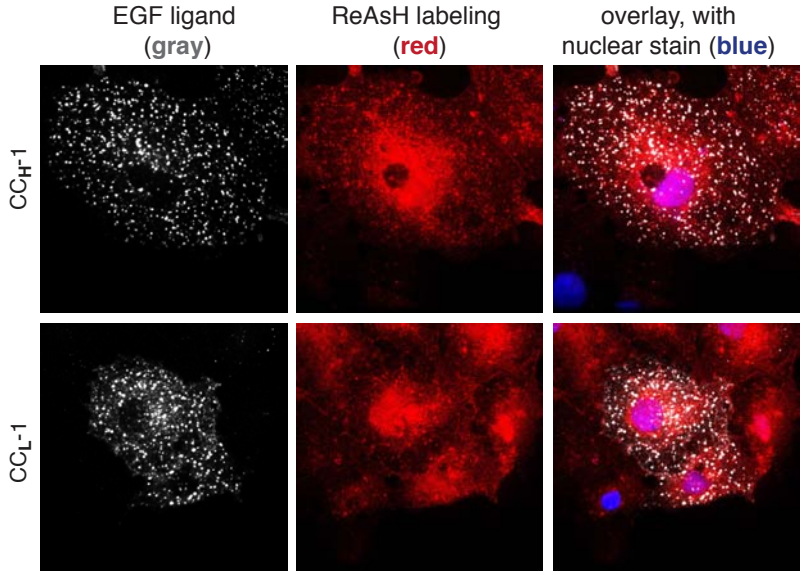
Supporting Figure S1. Bipartite EGFR variants are activated by EGF and expressed on the cell surface.

(a) Western blot analysis of wild-type, CC_L-1 and CC_H-1 EGFR stimulated with EGF (or not) in the presence of ReAsH or in the presence of ATP synthesis inhibitors to prevent endocytosis. An N-terminal FLAG epitope is used to monitor receptor expression levels. In all cases, treatment with EGF leads to receptor activation and the phosphorylation of Y1173. Neither wild-type, CC_L-1 or CC_H-1 EGFR were activated by the addition of ReAsH, and the use of an ATP inhibition cocktail did not prevent receptor activation and autophosphorylation. (b) Surface biotinylation assay. An N-terminal FLAG epitope is used to monitor the expression of wild-type, CC_L-1, and CC_H-1 EGFR and as an affinity tag for immunoprecipitation. Th is assay confirms that the top band (see red arrow) is the mature cell surface form (positive biotin signal) and that this form becomes activated in response to EGF (pY signal). Controls that were not treated with biotin do not yield any signal when probed with an anti-biotin antibody. A control where the surface biotinylation assay/IP protocol was performed on non-transfected cells (or with no lysate, not shown) reveal that a non-specific band (bottom) results from the beads used for immunoprecipitation. Together, these results confirm that wild-type, CC_L-1 and CC_H-1 EGFR variants are expressed on the cell surface, and are able to be activated by EGF.



Supporting Figure S2. Monitoring EGFR dimerization using bipartite tetracysteine display. (a) Domain and partial sequence information for the helical bipartite variant CC_H-1, the single cysteine variant C1-1, and the linear tetracysteine variant C4-1. (b) Cartoon depicting the helical bipartite variant CC_H-1, the single cysteine variant C1-1, and the linear tetracysteine variant C4-1. (c) Representative TIRFM images showing ReAsH labeling for CHO cells expressing the single cysteine variant C1-1 or the C-terminal linear tetracysteine variant C4-1 in the presence or absence of EGF. (d) Quantification of TIRFM results expressed as a fold increase in ReAsH fluorescence relative to background and normalized for receptor expression. Error bars represent the standard error. ****p<0.0001, *** p<0.001; **p<0.01 based on ANOVA with Bonferroni post-test. These results indicate that bipartite display detects the engineered ReAsH binding site only when four Cys are present, and that bipartite tetracysteine display performs at a level that compares well with linear tetracysteine display.

A representative confocal images



B quantification of colocalization

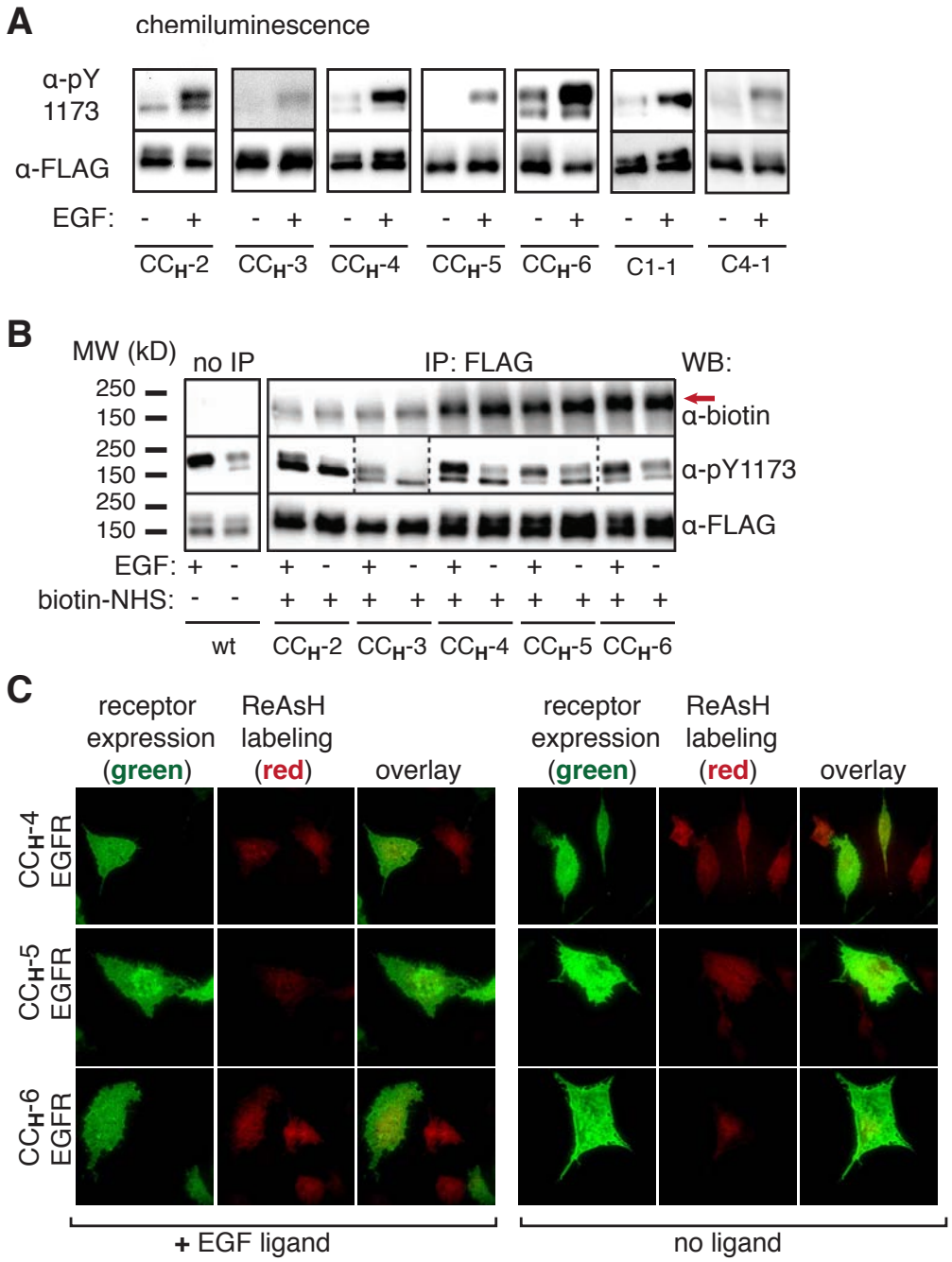
CC_H-1 EGFR

cell type	image #	Pearson's R
COS7	1*	0.447
COS7	2	0.357
COS7	3	0.311
CHO	4	0.587
CHO	5	0.627

CC_L-1 EGFR

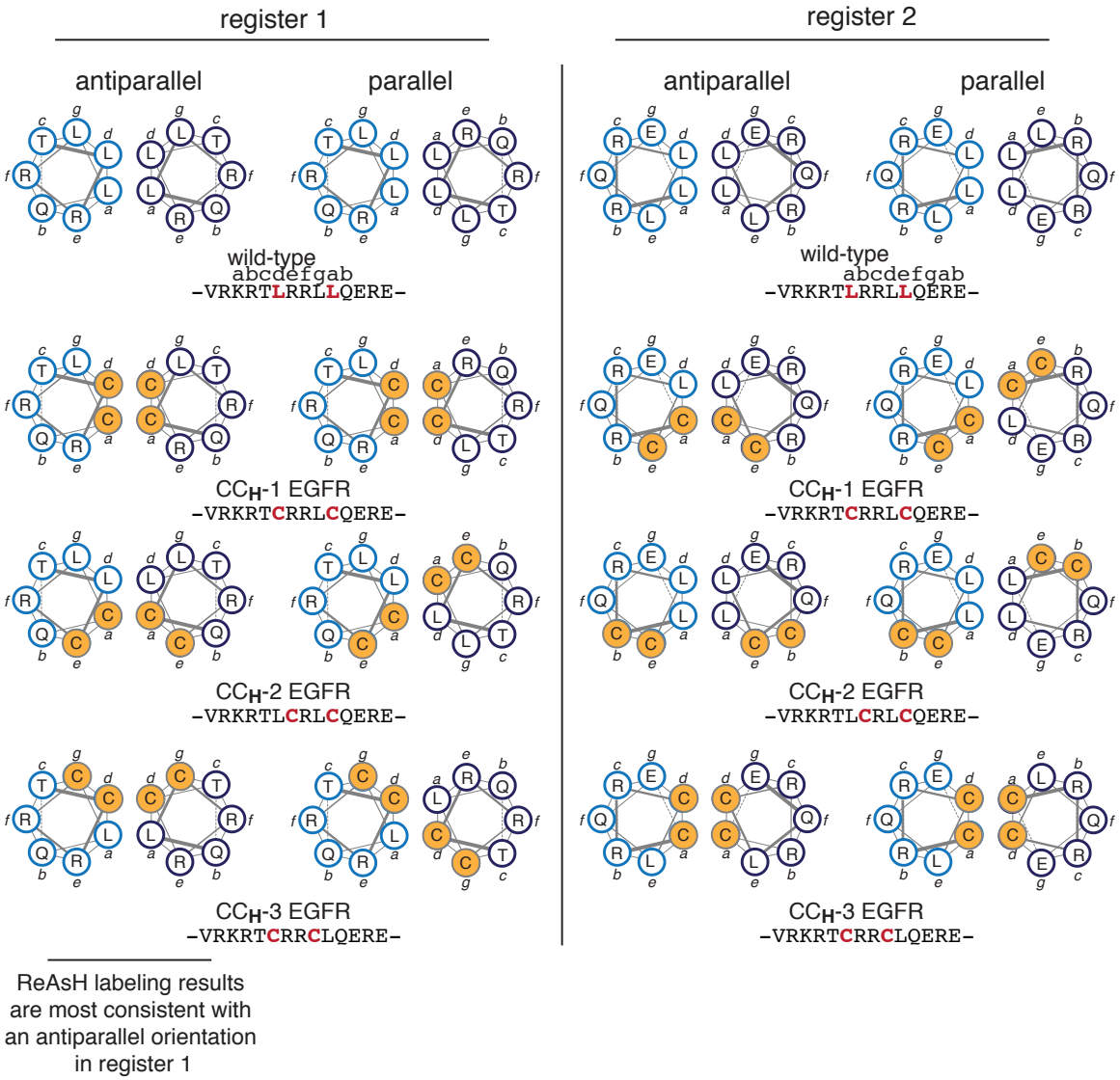
cell type	image #	Pearson's R
COS7	1*	0.237
COS7	2	0.310
COS7	3	0.187
CHO	4	0.493
CHO	5	0.211

Supporting Figure S3. Monitoring endocytosis of ReAsH-labeled EGFR
(a) Representative confocal images for ReAsH labeled cells expressing CC_H-1 and CC_L-1 EGFR in the presence of Alexa-fluor647 labeled-EGF. (b) Quantification of colocalization using Pearson's correlation coefficient. * represents the image pictured. The correlation between the fluorescent signal of ReAsH-labeled endosomes (ReAsH, red) that also contained fluorescently labeled EGF (AlexaFluor647, gray) was stronger for CC_H-1 EGFR than for CC_L-1 EGFR, as assessed using Pearson's correlation coefficient. We conclude that ReAsH labeling of CC_H-1 EGFR is contingent on EGF binding, whereas ReAsH labeling of CC_L-1 EGFR can occur in the ligand-bound or ligand-free homodimer.

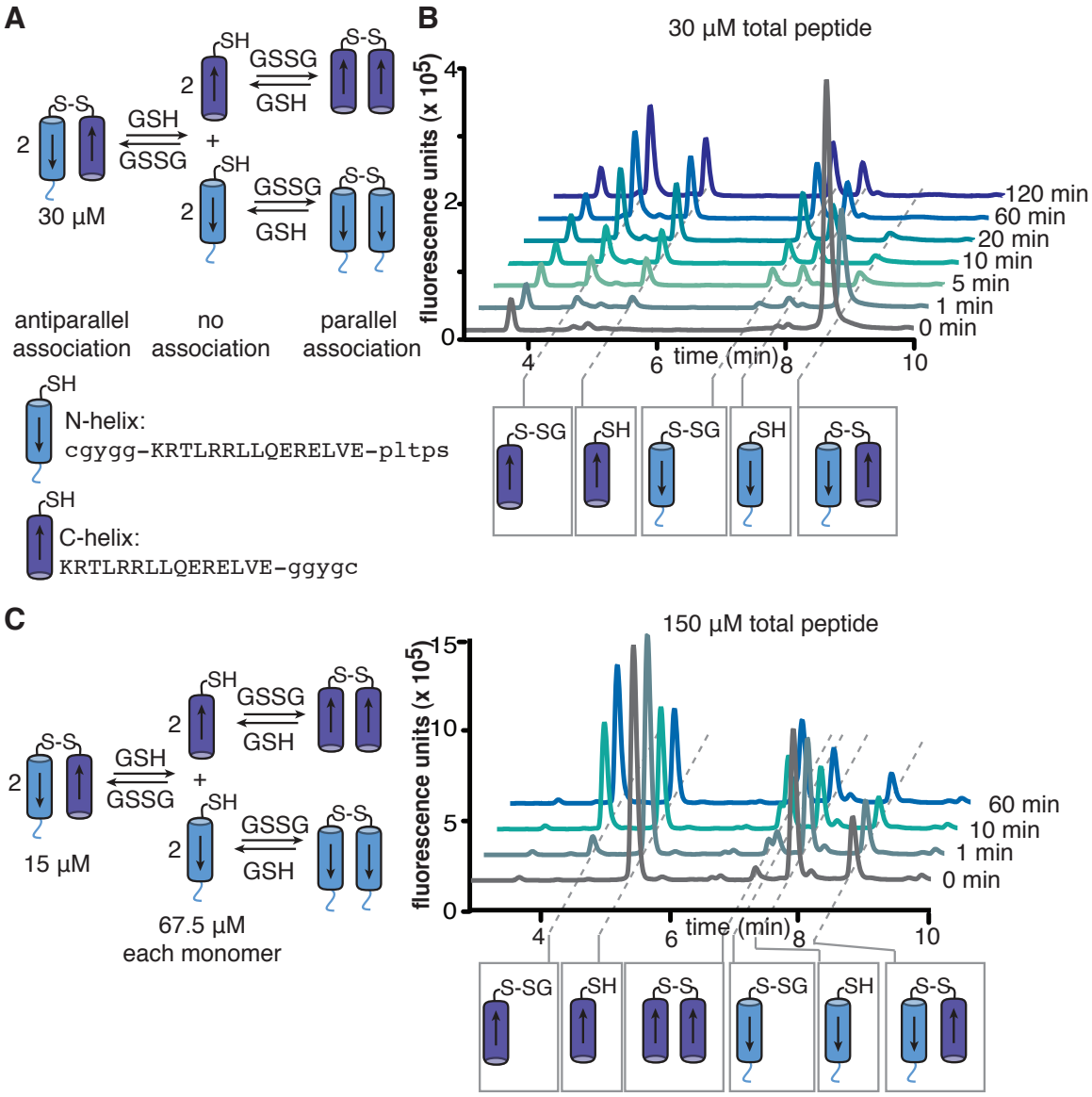


Supporting Figure S4. An antiparallel coiled coil is present when EGFR is activated by EGF. (a) Western blot analysis of lyates from cells expressing CC_H-2, CC_H-3, CC_H-4, CC_H-5, CC_H-6, C1-1, or C4-1 EGFR stimulated with EGF (or not). CC_H-2 and CC_H-6 display higher basal levels of pY1173 in the absence of EGF, and CC_H-3 and C4-1 do not achieve as robust levels of autophosphorylation overall. Despite these differences, the fact remains that all variants show a ligand-dependent increase in autophosphorylation (activation). (b) Surface biotinylation assay for CC_H-2, CC_H-3, CC_H-4, CC_H-5, and CC_H-6 EGFR. An N-terminal FLAG epitope is used to monitor receptor expression levels and as an affinity tag for immunoprecipitation. See also

Supporting Figure S1 for additional controls. (c) Representative TIRFM images of cells expressing CC_H-4, CC_H-5, and CC_H-6 EGFR after ReAsH treatment in the presence and absence of EGF. These results provide additional evidence that the proposed JM antiparallel helical interaction is assembled within the activated EGFR homodimer (see also Figure 2 and Supporting Figure S5)



Supporting Figure S5. Helical wheel analysis of alternative registers and orientations. CC_H-1, CC_H-2, and CC_H-3 EGFR were all labeled by ReAsH in the presence (but not the absence) of EGF. These results are most consistent with an antiparallel arrangement in register 1.



Supporting Figure S6. Disulfide exchange assay.
(a) Disulfide exchange scheme in which a purified heterodimer equilibrates in redox buffer containing a mixture of oxidized and reduced glutathione. (b) HPLC analysis over 2 h reveals that there is negligible affinity for association in either the parallel or antiparallel sense. In the stacked HPLC chromatograms, loss of the dimer peak occurs alongside the appearance of monomer (and monomeric glutathione adducts). (c) HPLC analysis at higher peptide concentrations is consistent with a negligible affinity for association. Therefore, we conclude that the helical JM segments have a negligible tendency to associate in isolation.

Table S1. Mutagenesis Primers

CC_H-1	F	5'-cgttcggaagcgcacgtgccggaggctgtgccaggagagggagcttg-3'
	R	5'-caagctccctctcctggcacagcctccggcacgtgcgcttccgaacg-3'
CC_L-1	F	5'-tcttcatgcggaggcgctgctgcgttcggaagcgcacgc-3'
	R	5'-gcgtgcgcttccgaacgcagcagcgcctccgcatgaaga-3'
wt R656,657G	F	5'-aagcgcacgctgggggggctgctgcagg-3'
	R	5'-cctgcagcagccccccagcgtgcgctt-3'
CC_H-1 R656,657G	F	5'-aagcgcacgtgcgggggctgtgccagg-3'
	R	5'-cctggcacagcccccgcacgtgcgctt-3'
V924R	F	5'-cgatgtctacatgatcatgcgcaagtgctggatgatagac-3'
	R	5'-gtctatcatccagcacttgcgcatgatcatgtagacatcg-3'
CC_H-2	F	5'-cggaagcgcacgctgtgcaggctgtgccaggagagggagctt-3'
	R	5'-aagctccctctcctggcacagcctgcacagcgtgcgcttccg-3'
CC_H-3	F	5'-cgttcggaagcgcacgtgccggaggtgcctgcaggagagggagc-3
	R	5'-gctccctctcctgcaggcacctccggcacgtgcgcttccgaacg-3'
CC_H-4	F	5'-tcggaagcgcacgctgtgcaggtgcctgcaggagagggagc-3'
	R	5'-gctccctctcctgcaggcacctgcacagcgtgcgcttccga-3'
CC_H-5	F	5'-gcgcacgctgcggtgcctgctgcagtgcagggagcttgtgg-3'
	R	5'-ccacaagctccctgcactgcagcaggcaccgcagcgtgcgc-3'
CC_H-6	F	5'-cacgctgcggaggtgcctgcaggagtgcgagcttgtggagc-3'
	R	5'-gctccacaagctcgcactcctgcaggcacctccgcagcgtg-3'