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Supplemental Information

Growth Factor Identity Is Encoded by Discrete Coiled-Coil Rotamers in the EGFR Juxtamembrane Region

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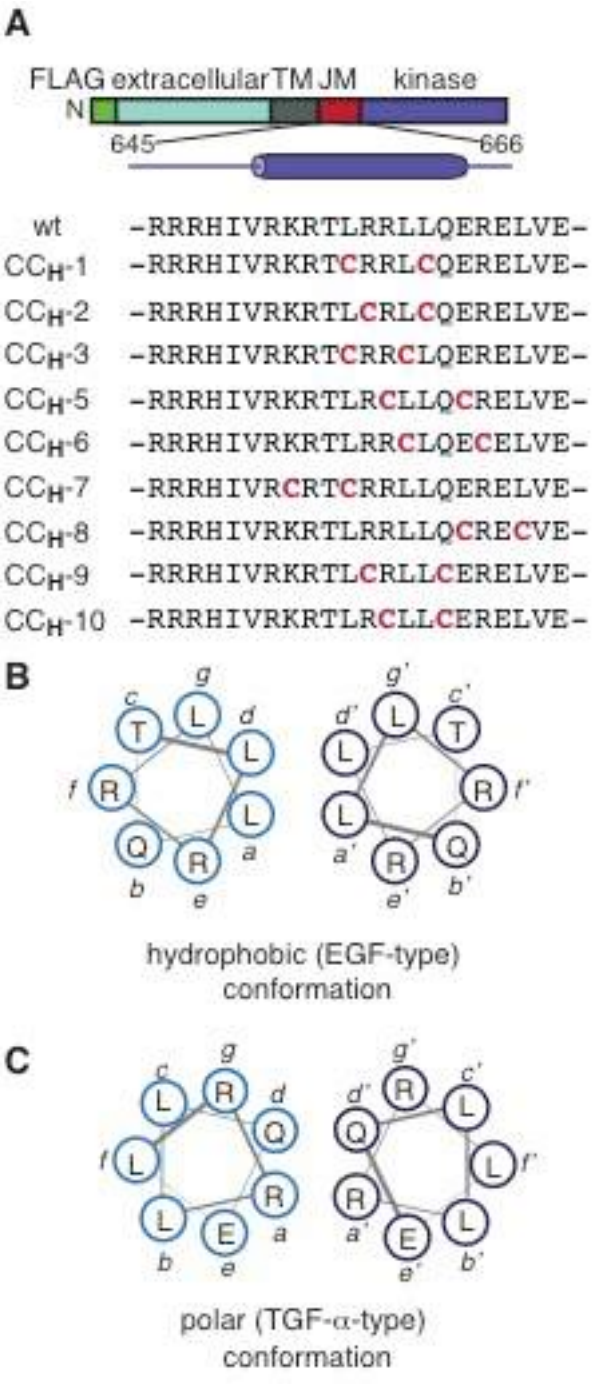
Supplemental Information

Table of Contents



Supplemental Data

Figure S1. Related to Fig 1**.** Probing juxtamembrane segment (JM) structure within full length EGFR on the cell surface using bipartite tetracysteine display and TIRF microscopy. (*A*) Sequences of the JM-A regions of CysCys EGFR variants used in this work. Cys residues that contribute to the ReAsH binding site are shown in red font. (*B*) Helical wheel diagram of the EGF-induced antiparallel JM dimer. (*C*) Helical wheel diagram of the TGF-α-induced antiparallel JM dimer.

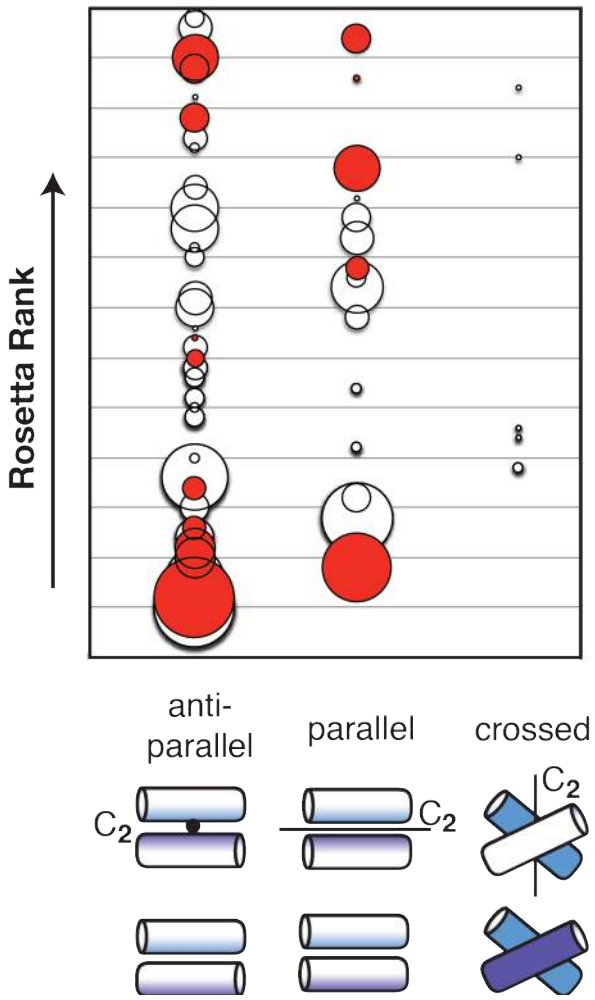


Figure S2. Related to Fig 2**.** Low energy structures identified by RosettaDock ranked in order of increasing Rosetta energy and separated on the basis of strand orientation. Clusters representing structures compatible with the symmetry required for bipartite tetracysteine display are shown in red (these clusters are also depicted on Figure 2B). Incompatible clusters are shown in white.

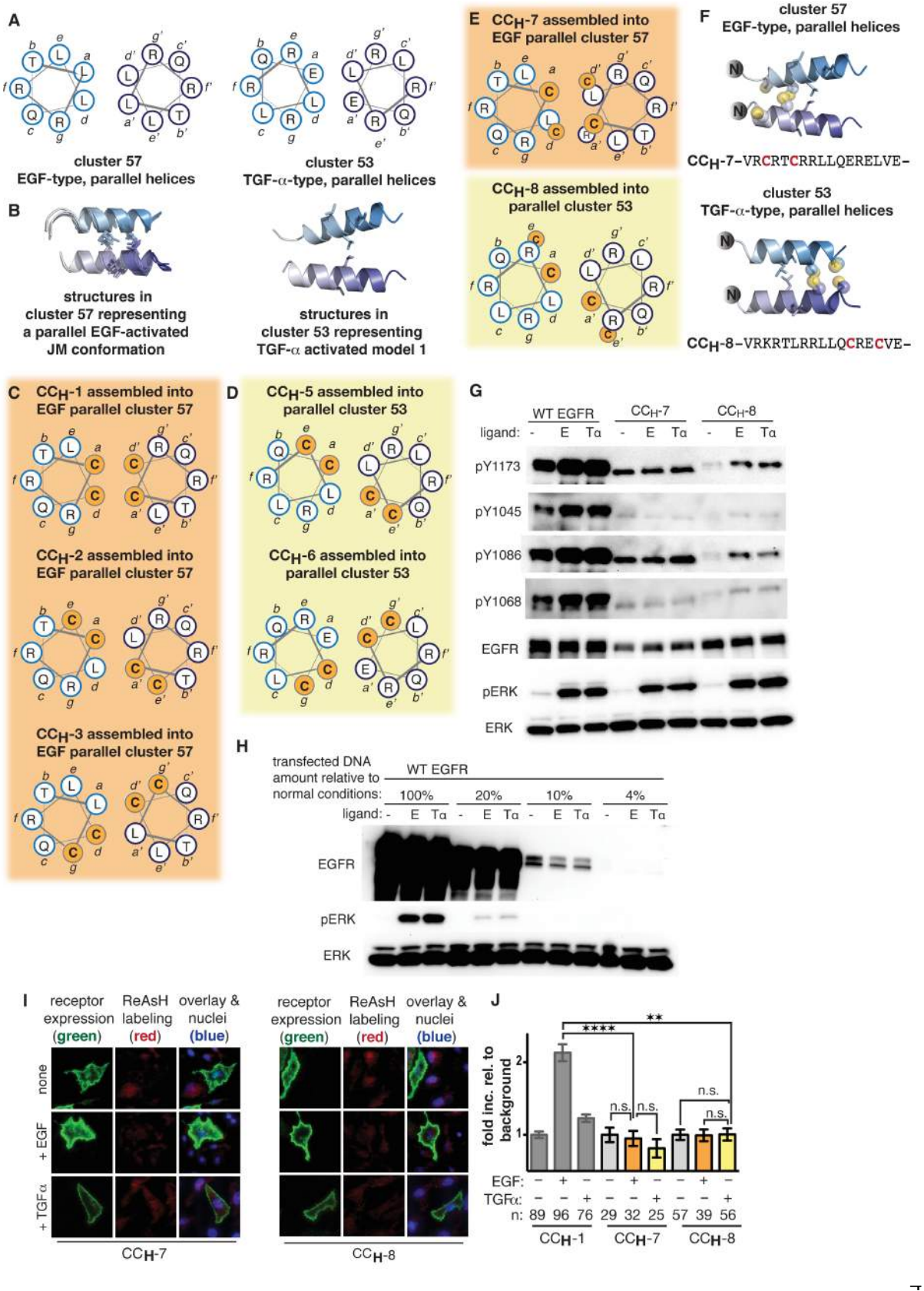

A
cluster 57
EGF-type, parallel helices
cluster 53
TGF-α-type, parallel helices
B
structures in
cluster 57 representing
a parallel EGF-activated
JM conformation
structures in
cluster 53 representing
TGF-α activated model 1
C
CCH-1 assembled into
EGF parallel cluster 57
CCH-2 assembled into
EGF parallel cluster 57
CCH-3 assembled into
EGF parallel cluster 57
D
CCH-5 assembled into
parallel cluster 53
CCH-6 assembled into
parallel cluster 53
E
CCH-7 assembled into
EGF parallel cluster 57
CCH-8 assembled into
parallel cluster 53
F
cluster 57
EGF-type, parallel helices
CCH-7-VRCRTCRRLLQERELVE-
cluster 53
TGF-α-type, parallel helices
CCH-8-VRKRTLRRLLQCRECVE-
G
WT EGFR
CCH-7
CCH-8
ligand: - E Tα - E Tα - E Tα
pY1173
pY1045
pY1086
pY1068
EGFR
pERK
ERK
H
transfected DNA
amount relative to
normal conditions:
WT EGFR
100%
20%
10%
4%
ligand: - E Tα - E Tα - E Tα - E Tα
EGFR
pERK
ERK
I
receptor
expression
(green)
ReAsH
labeling
(red)
overlay &
nuclei
(blue)
none
+ EGF
+ TGFα
CCH-7
CCH-8
J
fold inc. rel. to
background
****
**
n.s.
EGF:
TGFα:
n: 89 96 76 29 32 25 57 39 56
CCH-1
CCH-7
CCH-8

Figure S3. Related to Fig 3. Bipartite display provides no evidence for parallel coiled coils within the EGFR JM in the presence of either EGF or TGF-α. (*A*) Helical wheel diagrams illustrating the helix interfaces of two parallel structures potentially adopted in the EGF or TGF-α activated JM-A region of EGFR. (B) Overlay of structures in cluster 57 and 53 (cluster 53 only had one structure). (*C*) CC_H-1 and CC_H-2 variants that bind ReAsH upon EGF treatment assembled into the cluster 57 conformation corresponding to the predicted parallel EGF-activated JM model. (*D*) CC_H-5 and CC_H-6 variants variants that bind ReAsH upon TGF-α treatment assembled into the cluster 53 conformation corresponding to the predicted parallel TGF-α-activated JM model. (*E*) CC_H-7 and CC_H-8 assembled into the coiled coil diagrams defined by parallel cluster 57 and parallel cluster 53, respectively. (*F*) Models illustrating the relative orientation of Cys-Cys motifs (yellow spheres) in CC_H-7 and CC_H-8 when assembled into the parallel coiled coils defined by clusters 57 and 53, respectively. (*G*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-7, or CC_H-8 and treated with indicated ligand (1 ng/mL) for 5 minutes. Autophosphorylation was detected with α-phosphotyrosine 1173, 1045, 1068, 1086. Total EGFR was detected with α-EGFR and downstream pathway activation was probed with α-pERK and α-ERK. (*H*) Control showing that ERK activity is dependent on EGFR in CHO-K1 cells. Western blot analysis of CHO-K1 cells transfected with 18 µg (100%), 3.6 µg (20%), 1.8 µg (10%), 0.72 µg (4%) of FLAG-tagged WT EGFR and treated with indicated ligand (1 ng/mL) for 5 minutes. Total EGFR levels were detected with α-FLAG and ERK activation was detected with α-pERK and α-ERK. (*I*) TIRF microscopy images of CHO-K1 cells expressing either CC_H-7 or CC_H-8 EGFR (green fluorescence) and treated with EGF or TGF-α (1 ng/mL). Bars represent 10 µm. (*J*) Quantified fold increase in expression-corrected ReAsH fluorescence over background of cells expressing CC_H-7 or CC_H-8 EGFR and treated with or without EGF or TGF-α (1 ng/mL). Error bars represent standard error. ** $p < 0.01$, **** $p < 0.0001$ from one way ANOVA with Bonferroni post-test.

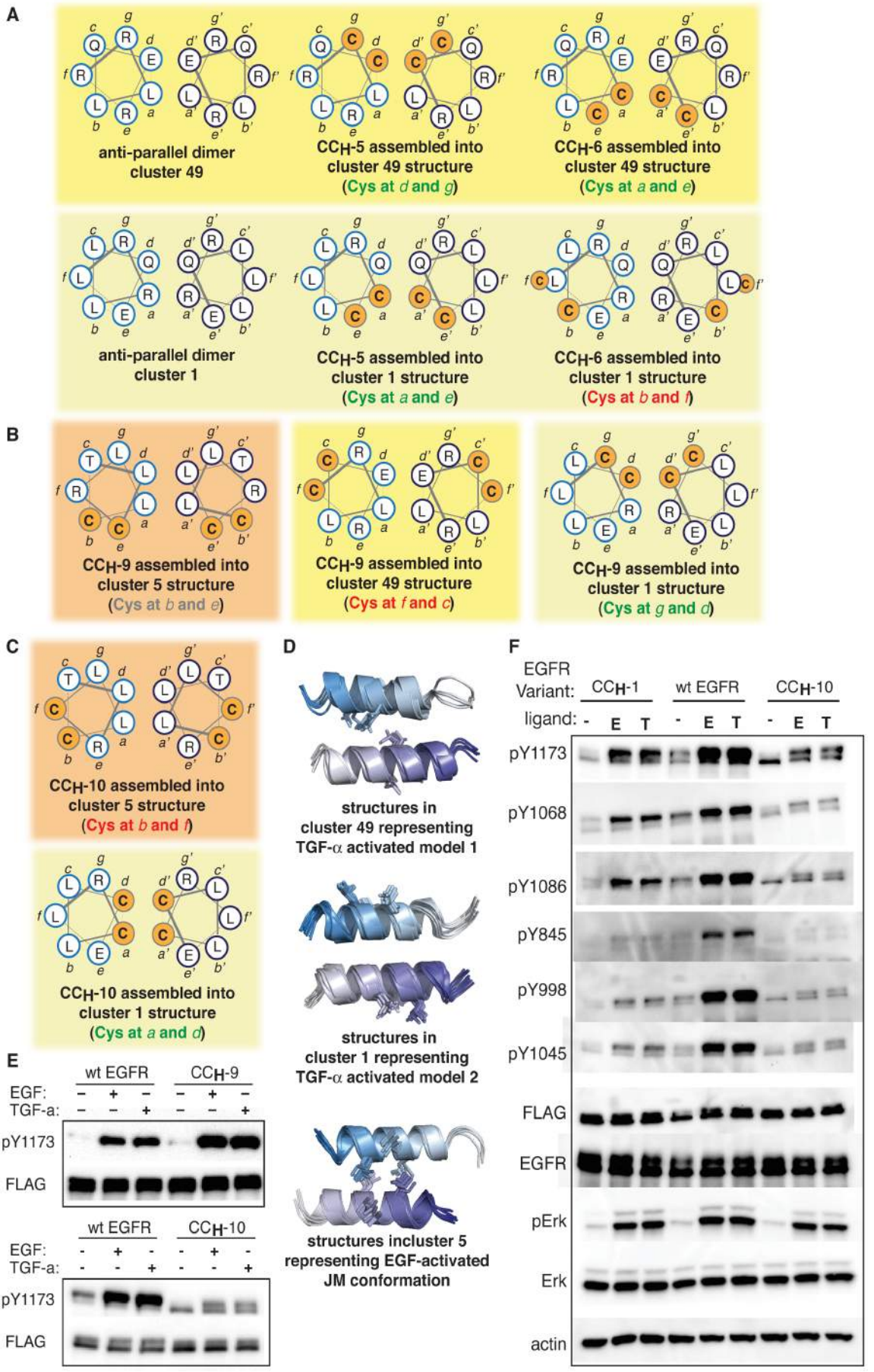

A
anti-parallel dimer cluster 49
CCH-5 assembled into cluster 49 structure (Cys at d and g)
CCH-6 assembled into cluster 49 structure (Cys at a and e)
anti-parallel dimer cluster 1
CCH-5 assembled into cluster 1 structure (Cys at a and e)
CCH-6 assembled into cluster 1 structure (Cys at b and f)
B
CCH-9 assembled into cluster 5 structure (Cys at b and e)
CCH-9 assembled into cluster 49 structure (Cys at f and c)
CCH-9 assembled into cluster 1 structure (Cys at g and d)
C
CCH-10 assembled into cluster 5 structure (Cys at b and f)
CCH-10 assembled into cluster 1 structure (Cys at a and d)
D
structures in cluster 49 representing TGF-α activated model 1
structures in cluster 1 representing TGF-α activated model 2
structures incluster 5 representing EGF-activated JM conformation
E
wt EGFR
CCH-9
EGF:
TGF-a:
pY1173
FLAG
wt EGFR
CCH-10
EGF:
TGF-a:
pY1173
FLAG
F
EGFR Variant:
CCH-1
wt EGFR
CCH-10
ligand: - E T - E T - E T
pY1173
pY1068
pY1086
pY845
pY998
pY1045
FLAG
EGFR
pErk
Erk
actin

Figure S4. Related to Fig 3. (*A*) Cys-Cys variants that bind ReAsH upon TGF-α-treatment assembled into either cluster 49 or 1 conformations corresponding to the two predicted anti-parallel TGF-α-activated JM models. (*B*) CC_H-9 assembled into the two clusters that represent the TGF-α-activated model, cluster 49 and 1, and the cluster 5 conformation representing the EGF-activated model. (*C*) CC_H-10 assembled into the coiled coil diagrams defined by anti-parallel clusters 5 and 1. (*D*) Overlay of structures in cluster 49, 1, and 5. (*E*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-9, or CC_H-10 and treated with indicated ligand (1 ng/mL) for 5 minutes. Autophosphorylation was detected with α-phosphotyrosine 1173 and total transfected receptor levels were detected with α-FLAG. (*F*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-1, or CC_H-10 and treated with indicated ligand (1 ng/mL) for 5 minutes. C-terminal phosphorylation was detected with α-phosphotyrosine (pY)1173, α-pY1068, α-pY1086, α-pY845, α-pY998, and α-pY1045 antibodies. Total transfected receptor levels were detected with α-FLAG and α-EGFR, downstream signaling was detected with α-p42/44 MAPK (Erk1/2) and α-phospho-p42/44 MAPK(pErk1/2) (pT202/pY204), and α-β-actin was used as a loading control.

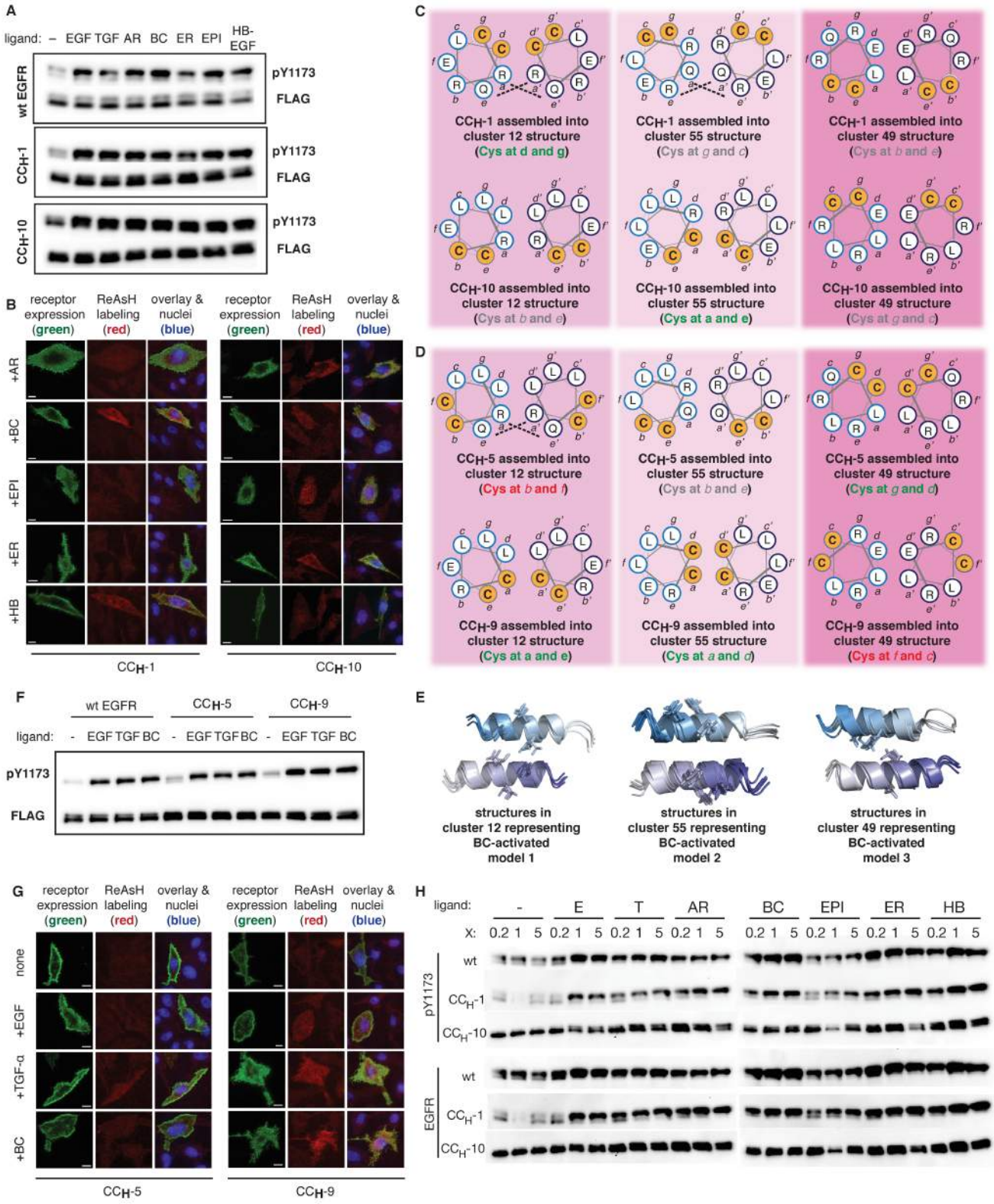

A
ligand: – EGF TGF AR BC ER EPI HB-EGF
wt EGFR
CCH-1
CCH-10
pY1173
FLAG
B
receptor expression (green)
ReAsH labeling (red)
overlay & nuclei (blue)
+AR
+BC
+EPI
+ER
+HB
CCH-1
CCH-10
C
CCH-1 assembled into cluster 12 structure (Cys at d and g)
CCH-1 assembled into cluster 55 structure (Cys at g and c)
CCH-1 assembled into cluster 49 structure (Cys at b and e)
CCH-10 assembled into cluster 12 structure (Cys at b and e)
CCH-10 assembled into cluster 55 structure (Cys at a and e)
CCH-10 assembled into cluster 49 structure (Cys at g and c)
D
CCH-5 assembled into cluster 12 structure (Cys at b and f)
CCH-5 assembled into cluster 55 structure (Cys at b and e)
CCH-5 assembled into cluster 49 structure (Cys at g and d)
CCH-9 assembled into cluster 12 structure (Cys at a and e)
CCH-9 assembled into cluster 55 structure (Cys at a and d)
CCH-9 assembled into cluster 49 structure (Cys at f and c)
E
structures in cluster 12 representing BC-activated model 1
structures in cluster 55 representing BC-activated model 2
structures in cluster 49 representing BC-activated model 3
F
wt EGFR
CCH-5
CCH-9
ligand: - EGF TGF BC - EGF TGF BC - EGF TGF BC
pY1173
FLAG
G
receptor expression (green)
ReAsH labeling (red)
overlay & nuclei (blue)
none
+EGF
+TGF-α
+BC
CCH-5
CCH-9
H
ligand: - E T AR BC EPI ER HB
X: 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5 0.2 1 5
pY1173
EGFR
wt
CCH-1
CCH-10

Figure S5. Related to Fig. 5. (*A*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-1, or CC_H-10 and treated with either EGF, TGF-α, BC, HB-EGF (1 ng/mL) or AR, ER, or EPI (2 µg/mL) for 5 minutes. Autophosphorylation was detected with α-phosphotyrosine 1173 and total transfected receptor levels were detected with α-FLAG. (*B*) TIRF microscopy images of CHO-K1 cells expressing either FLAG-tagged CC_H-1 or CC_H-10 (green fluorescence) and treated with EGF, TGF-α, BC, HB-EGF (1 ng/mL) or AR, ER, or EPI (2 µg/mL). ReAsH staining was conducted after an initial ligand pre-treatment of ligand at 4° C because ReAsH labeling must occur before fixation and reliable ReAsH staining was not achieved by co-treatment of cells with ligand and ReAsH. Bars represent 10 µm. (*C*) Cys-Cys variants that bind ReAsH upon BC-treatment assembled into either cluster 12, 55, or 49 conformations corresponding to the three predicted anti-parallel BC-activated JM models. (*D*) CC_H-9 and CC_H-5 assembled into the three clusters that represent the BC-activated model, cluster 12, 55 and 49. (*E*) Overlay of structures in cluster 12, 55, and 49. (*F*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-9, or CC_H-5 treated with indicated ligand (1 ng/mL) for 5 minutes. Autophosphorylation was detected with α-phosphotyrosine 1173 and total transfected receptor levels were detected with α-FLAG. (*G*) TIRF microscopy images of CHO-K1 cells expressing either FLAG-tagged CC_H-5 or CC_H-9 (green fluorescence) and treated with EGF, TGF-α, or BC (1 ng/mL). Bars represent 10 µm. (*H*) Western blot analysis of CHO-K1 cells transfected with wt EGFR, CC_H-1, or CC_H-10 and treated with either EGF, TGF-α, BC, HB-EGF at 0.2X (0.2 ng/mL), 1X (1 ng/mL) or 5X (5 ng/mL) or AR, ER, or EPI at 0.2X (0.4 µg/mL), 1X (2 µg/mL), 5X* (10 µg/mL) for 5 minutes. Autophosphorylation was detected with α-phosphotyrosine 1173 and total transfected receptor levels were detected with α-EGFR. *These samples were treated with ligand in ½ of volume as all other samples. Although the extent of phosphorylation of CC_H-10 decreases at the highest AR and ER concentrations, CC_H-10 is fully active at an AR or ER concentration of 180 nM, the concentration used in the TIRF experiments in Figure 5.

Supplementary Experimental Procedures

Materials

TransIT®-CHO Transfection Kits were purchased from Mirus. Epidermal Growth Factor (EGF) was purchased from Fischer. Transforming Growth Factor-α was purchased from Sigma. Amphiregulin (AR), Betacellulin (BC), Epigen (EPI), Epiregullin (ER), Heparin-binding EGF (HB-EGF) were all purchased from RnD Systems. ReAsH-EDT_2 was purchased from Invitrogen. British Anti-Lewisite (BAL) was purchased from Acros Organics. 10% polyacrylamide SDS-PAGE gels and Clarity™ Western ECL reagents were purchased from Bio-Rad. Fibronectin from bovine plasma, non-enzymatic cell dissociation solution (C5914), and monoclonal anti-FLAG® M2 antibodies produced in mouse were purchased from Sigma. Phospho-EGF receptor (Tyr1173) (53A5) Rabbit mAb, Phospho-EGF receptor (Tyr1086) Rabbit mAb, Phospho-EGF receptor (Tyr1068) (1H12) Mouse mAb, Phospho-EGF receptor (Tyr1045) Rabbit mAb, Phospho-EGF receptor (Tyr845) Rabbit mAb, Phospho-EGF receptor (Tyr998) Rabbit mAb, p44/42 MAPK (ERK1/2) (137F5), Phospho-p42/44 MAPK(pErk1/2) (pT202/pY204) (D13.14.4E), EGF receptor (D38B1), β-actin (D6A8) Rabbit mAb, anti-mouse IgG HRP-linked, and anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technologies. Alexa Fluor® 488 goat anti-mouse antibody IgG, IgA, IgM (H+L) was purchased from Invitrogen. Hoechst 33342 trihydrochloride trihydrate was purchased from Molecular Probes. PhosSTOP phosphatase inhibitor and cOmplete mini, EDTA-free protease inhibitor cocktails were purchased from Roche. Glass bottom microwell dishes (P35G-1.5-14-C) were purchased from Mat Tek. Rosetta 3.4 was obtained from rosettacommons.org.

Constructs

All EGFR constructs were cloned from wt EGFR in a pCDNA3.1 vector using a Quikchange Lightning site-directed mutagenesis kit (Agilent) according to the manufacturer's instructions with the following primers:

CC_H-7

Forward `5′-gcgccacatcgttcggtgccgcacgtgccggaggctgctgcagg-3′`

Reverse `5′-cctgcagcagcctccggcacgtgcggcaccgaacgatgtggcgc-3′`

CC_H-8

Forward 5'-tgcggaggctgctgcagtgcagggagtgtgtggagcctcttacac-3'

Reverse 5'-gtgtaagaggctccacacactccctgcactgcagcagcctccgca-3'

CC_H-9

Forward 5'-ggaagcgcacgctgtgcaggctgctgtgcgagagggagcttgt-3'

Reverse 5'-acaagctccctctcgcacagcagcctgcacagcgtgcgcttcc-3'

CC_H-10

Forward 5'-aagcgcacgctgcggtgcctgctgtgcgagagggagcttgtg-3'

Reverse 5'-cacaagctccctctcgcacagcaggcaccgcagcgtgcgctt-3'