Supporting Information

Discrete coiled coil rotamers form within the EGFRvIII juxtamembrane domain

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Materials

Unlabeled recombinant human epidermal growth factor (EGF) (#CB40052) was purchased from Corning. Unlabeled recombinant human transforming growth factor-α (TGF-α) (#T7924) and mouse monoclonal (M2) anti-FLAG primary antibody (#F1804) were purchased from Sigma. 2,3-dimercapto-1-propanol (BAL) (#AC115300250) was purchased from Acros Organics. Rabbit monoclonal anti-phospho-EGF receptor Tyr1173 (53A5) (#4407), rabbit polyclonal anti-phospho-EGF receptor Tyr1086 (#2220), rabbit polyclonal anti-phospho-EGF receptor Tyr1068 (#2234), rabbit anti-α-tubulin (#2155) primary antibodies as well as goat polyclonal anti-Rabbit, HRP-conjugated (#7074) and goat polyclonal anti-mouse, HRP-conjugated (#7076) secondary antibodies were purchased from Cell Signaling Technology. CHO-K1 cells were purchased from the American type Culture Collection (ATCC). Dulbecco's phosphate buffered saline (dPBS) (#14190), fetal bovine serum (FBS) (#26140079), penicillin-streptomycin (10,000 U/mL) (#15140122), ReAsH-EDT2 (#T34562) and goat polyclonal anti-mouse, AlexaFluor488-conjugated secondary antibodies (#A10667) were purchased from ThermoFisher Scientific. EGFR UniProtKB accession ID: P00533

Plasmids and Cloning

All plasmids used in bipartite tetracysteine display and related assays are derived from a parent plasmid (pcDNA3.1), generously donated by the Kuriyan Group (University of California, Berkeley), which contains the sequence of full-length WT EGFR with an N-terminal FLAG tag.[1] Mutations and deletions were introduced into pcDNA3.1 using Quikchange Lightning site-directed mutagenesis (Agilent) and primers (Integrated DNA Technologies) listed in Table S1.

Cell Culture

CHO-K1 cells were purchased from ATCC and maintained at 37°C, 5% CO_2, in F12K Medium (Corning) supplemented with 10% fetal bovine serum and pen-strep (100 I.U./mL penicillin and 100 mg/mL streptomycin). Cell densities were determined with a Cellometer Auto T4 automated counter. Transient transfection of CHO-K1 cells was performed via use of the Transit-CHO Transfection Kit, according to the manufacturer's instructions (Mirus Bio LLC).

Bipartite Tetracysteine Display

ReAsH labeling was performed as described previously,[2] by treating CHO-K1 cells that were transiently transfected with plasmids containing the appropriate EGFR variants in the presence of an endocytosis/ATP synthesis inhibition cocktail in F12-K media (10 mM NaN_3, 2 mM NaF, 5 mM 2-deoxy-D-glucose), for 1 h at 37°C. Cells were then stimulated without/with 100 ng/mL of EGF (16.7 nM) or TGF-α (16.7 nM) for 30 min at 4°C prior to labeling. Cells were then washed once with endocytosis/ATP synthesis inhibition cocktail in F12K media before incubation with ReAsH labeling solution (2 mM ReAsH, 20 mM BAL in F12K media) for 60 min at 37°C. Cells were then washed and incubated with endocytosis inhibitor-containing F12K media supplemented with 100 mM BAL for 10 min at 37°C. The media was removed, and the cells were fixed using 4% paraformaldehyde for 25 min at room temperature. Cells were then washed with dPBS and blocked with 10% BSA in dPBS for 30 min at 37°C. Cells were then labeled with primary antibody (mouse anti-Flag, 1:1000 dilution in 10% BSA in dPBS) for 1 h at 37°C, washed three times with

10% BSA in dPBS, then incubated with secondary antibody (AlexaFluor488-conjugated goat anti-mouse, 1:2000 dilution in 10% BSA in dPBS) for 1 h at 37°C. Cells were then washed two times with 10% BSA in dPBS, washed once with dPBS, then nuclear stained with Hoescht 33342 (1.62 mM in dPBS) for 5 min at 37°C. Cells were then washed once with dPBS and stored in dPBS at 4°C prior to imaging. Labeled cells were then analyzed *via* TIRF microscopy, conducted on a Leica Microsystems AM TIRF MC DMI6000B fitted with an EM-CCD camera (Hamamatsu) with HCX PL APO 63x/1.47 oil corrective objectives, as described previously.[2] Images were analyzed as described previously[2]. Briefly, raw data from TIRF microscopy were analyzed using ImageJ 64[3]. Fluorescence intensities of ReAsH and AlexaFluor 488 (EGFR levels) were quantified, and the fold increase of ReAsH fluorescence relative to background was normalized for EGFR expression levels. Normalized values of ReAsH fold-increases were plotted with Prism version 7.0 (for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com), where n represents number of cells quantified, and error bars represent the standard error of the mean. One-way ANOVA followed by a Dunnett multiple comparisons test was performed.

Autophosphorylation analysis

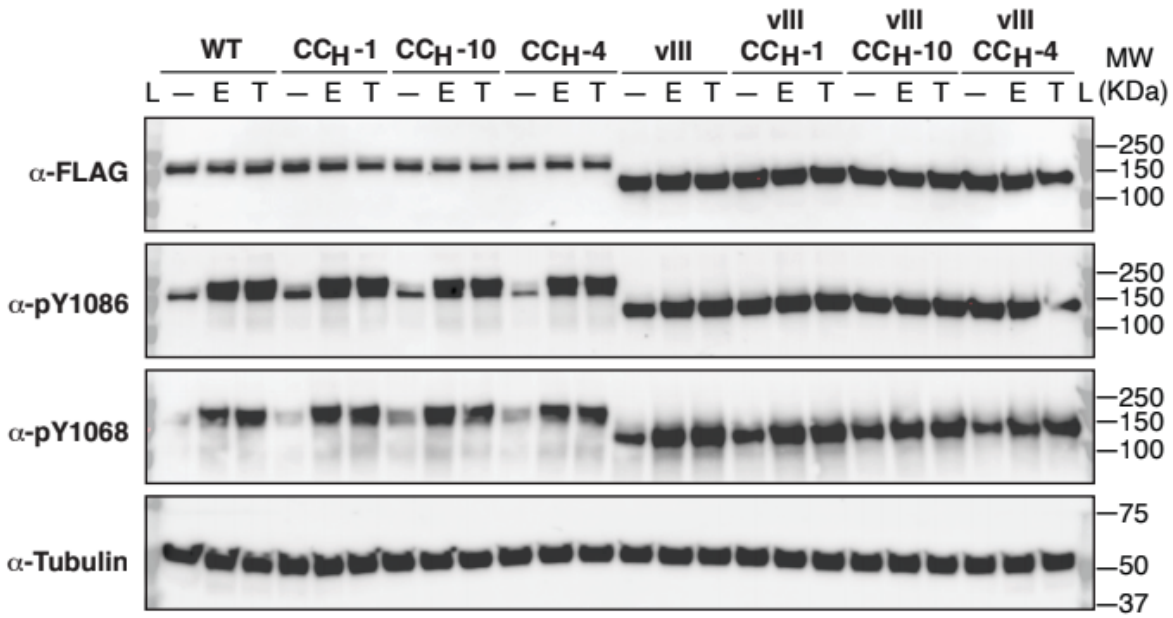
Western blot analysis of EGFR autophosphorylation in transiently transfected CHO-K1 cells was performed as described previously.[2,4] CHO-K1 cells, transiently transfected with a plasmid encoding the appropriate EGFR variant, were collected (5 x 10^5 cells), resuspended in 200 mL of serum free F12K media, stimulated with 100 ng/mL of EGF (16.7 nM) or TGF-α (16.7 nM) for 5 min at 37°C (or not), pelleted, washed with serum-free F12K media, pelleted again, then lysed in 100 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% Triton X-100,

pH 7.5, 1x complete protease inhibitor cocktail (Roche), 1x PhosStop), on ice for 1 hr. Clarified cell lysates were then subjected to reducing 10% polyacrylamide SDS-PAGE electrophoresis and transferred to immuno-blot PVDF membranes. Membranes were blocked with 5% milk in TBS-T Buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.4) for 1 hr followed by an overnight incubation at 4℃ with the indicated primary (rabbit or mouse) antibodies. Blots were then washed with TBS-T and incubated with either anti-rabbit or anti-mouse goat horseradish peroxidase conjugate secondary antibodies (Cell Signaling Technology) for 1 h at room temperature, then washed with TBS-T. Blots were then visualized using Clarity Western ECL reagents (BioRad). Displayed blot images have been adjusted for brightness/contrast using ImageJ[3] and have been cropped to highlight band signal for full-length EGFR (~170 kDa) or EGFRvIII (~145 kDa)

Table S1: Mutagenesis Primers*

S. No.	Primer Name	Sequence
1	EGFRvIII, forward	5'-cgagccgtgatctgtcaccacataattTTTCTTTTCCTC CAGTCCGGAGC-3'
2	EGFRvIII, reverse	5'-gacaagggctccggactggaggaaaagaaaAATTATGTGGT GACAGATCACGGCTC-3'

*Mutagenesis primers for the insertion of cysteines to generate the CC_H-1, CC_H-4, and CC_H-10 variants of EGFRvIII have been previously described.[4,5]



Supplementary Figure S1. CC_H-1, CC_H-10 and CC_H-4 variants of WT EGFR and EGFRvIII are expressed and phosphorylated as expected. Immunoblots comparing the relative expression and activity (as judged by the level of receptor autophosphorylation at Y1068, Y1086) of CC_H-1, CC_H-10 and CC_H-4 variants of WT EGFR and EGFRvIII with the corresponding EGFR variant lacking CysCys substitutions within the JM. In each case, transiently transfected CHO-K1 cells were stimulated with 100 ng/mL (16.7 nM) EGF or TGF-α (or serum free media) for 5 min at 37°C. **For details, see EGFR autophosphorylation analysis above.**

References

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