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Discrete Coiled Coil Rotamers Form within the EGFRvIII Juxtamembrane Domain

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Abstract

Mutations in the epidermal growth factor receptor (EGFR) extracellular domain (ECD) are implicated in the development of glioblastoma multiforme (GBM), which is a highly aggressive form of brain cancer. Of particular interest to GBM is the EGFR variant known as EGFRvIII, which is distinguished by an in-frame deletion of exons 2–7, which encode ECD residues 6–273. Included within the deleted region is an autoinhibitory tether, whose absence, alongside unique disulfide interactions within the truncated ECD, supports assembly of a constitutively active asymmetric kinase dimer. Previous studies have shown that the binding of growth factors to the ECD of wild-type EGFR leads to the formation of two distinct coiled coil dimers in the cytoplasmic juxtamembrane (JM) segment, whose identities correlate with the downstream phenotype. One coiled coil contains leucine residues at the interhelix interface (EGF-type), whereas the other contains charged and polar side chains (TGF-α-type). It has been proposed that growth-factor-dependent structural changes in the ECD and adjacent transmembrane helix are transduced into distinct JM coiled coils. Here, we show that, in the absence of this growth-factor-induced signal, the JM of EGFRvIII adopts both EGF-type and TGF-α-type structures, providing direct evidence for this hypothesis. These studies confirm that the signals that define JM coiled coil identity begin within the ECD, and support a model in which growth-factor-induced conformational changes are transmitted from the ECD through the transmembrane helix to favor different coiled coil isomers within the JM.

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Author Contributions

A.D. and A.S. designed the experiments. D.M., D.N.R., and J.Y.Z. performed experiments. D.M. and A.S. wrote the paper.

Supporting Information

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Supplemental information, methods, and figures (PDF)

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Epidermal growth factor receptor (EGFR) vIII (EGFRvIII) is the most common EGFR mutation associated with glioblastoma multiforme (GBM),[1–48] which is a high-grade brain tumor with exceptionally high fatality.[9] Multiple studies associate the expression of EGFRvIII with poor survival.[10,11] Unlike EGFR variants with mutations within the kinase domain[12–16] that can be blocked selectively with tyrosine kinase inhibitors (TKIs),[17–21] EGFRvIII contains a wild-type (WT) kinase domain, hindering the development of selective inhibitors.[4,22–25]

The mutations that distinguish EGFRvIII result from an in-frame deletion of 801 base pairs from exons 2–7 of the extracellular domain (ECD) (Figure 1A).[4,26–28] As a result, the receptor lacks residues 6–273, which comprise most of ECD domains I and II. Included in this region is one of three residues (Y246) that interacts with D563 and K585 on domain IV to hold the WT receptor in an autoinhibited conformation (see Figures 1B and 1D).[29–32] The absence of the Y246 autoinhibitory latch renders EGFRvIII constitutively active (Figure 1B).[7,30,31,33] EGFRvIII also lacks the domain II dimerization arm that, in WT EGFR, is necessary for growth-factor-induced ECD dimerization (see Figures 1C and 1D).[30,31] Despite the absence of ECD domains I and II, EGFRvIII forms dimers that are, instead, stabilized by disulfide bonds between cysteine residues exposed as a result of ECD truncation.[34] This disulfide-induced extracellular dimerization event, like growth factor-induced dimerization, supports intracellular formation of an asymmetric kinase dimer[35] that signals constitutively through the MAPK and AKT pathways (among others) to initiate oncogenic activity in the cell.[2,5–7,36]

The EGFR extracellular domain does more than simply bind growth factors and promote dimerization: it is an essential component of an allosteric pathway that links growth factor binding to kinase activation.[37,38] Previous studies have shown that the binding of growth factors by WT EGFR leads to the formation of two distinct coiled coil structures in the cytoplasmic juxtamembrane (JM) segment, a region of the intact receptor that is separated from the ECD by a transmembrane helix and a bilayer membrane.[39–41] One coiled coil, favored when WT EGFR is activated by epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-EGF), is distinguished by a hydrophobic, leucine-rich coiled coil interface observed by NMR (EGF-type coiled coil).[42] The other coiled coil, favored when WT EGFR is activated by transforming growth factor-α (TGF-α), epigen, epiregulin, and amphiregulin, is distinguished by electrostatic interactions at the helical interface with leucines decorating the outside surface[40] (Figure 1E). Formation of EGF- and TGF-α-type coiled coils within EGFR correlate with distinct intracellular phenotypes, including the direction of endocytic trafficking, receptor lifetime, and the relative flux through alternative downstream signaling pathways.[40] Given that EGFRvIII is constitutively activated in the absence of growth-factor-induced ECD rearrangements, it provides a unique opportunity to interrogate the relative stabilities of the EGFR juxtamembrane segment in the absence of signals emanating from a WT ECD. Here, we make use of bipartite tetracysteine display[43,44] to demonstrate that, in the absence of this growth factor-induced signal, the JM of EGFRvIII adopts both EGF-type and TGF-α-type structures. Our studies provide further evidence for an allosteric pathway that links growth-factor-induced binding to the extracellular domain to JM coiled coil structure and kinase activation.

Our initial experiments sought to probe the existence and structure of the JM coiled coil formed by intact EGFRvIII dimers within living cells. To do so, we made use of the chemical biology tool bipartite tetracysteine display (Figure 1F).[43,44] This tool exploits the bis-arsenical dye ReAsH[45] as a fluorogenic sensor that lights up only when bound to four cysteine (Cys) side chains in a discrete molecular array.[46] In previous work, we identified a set of CysCys-containing WT EGFR variants whose dimers bind ReAsH and fluoresce only when the JM is assembled into either an EGF-type coiled coil (CC_H-1) or the isomeric TGF-α-type coiled coil (CC_H-10) (Figure 2A).[39–41] The resulting ReAsH fluorescence, detected using total internal reflectance fluorescence microscopy (TIRF-M), provides an in-cell readout of JM structure within intact EGF receptors.[39–41] Visualizing ReAsH fluorescence using TIRF-M restricts fluorophore excitation and emission to a small (100–200 nm) cell surface plane and diminishes the signal from nonspecific cytosolic ReAsH staining.[39]

To probe for formation of the EGF-type or the TGF-α-type coiled-coil within the JM of intact EGFRvIII receptors, we integrated the CC_H-1 or CC_H-10 CysCys mutations into the EGFRvIII sequence to generate vIII-CC_H-1 and vIII-CC_H-10, respectively (Figure 2B). Control experiments confirmed that vIII-CC_H-1 and vIII-CC_H-10 (each carrying an N-terminal FLAG-tag) were expressed in CHO-K1 cells, localized to the cell surface, and underwent the expected phosphorylation at C-terminal tail residues Y1068 and Y1086 in the absence and presence of saturating (16.7 nM) EGF or TGF-α (Figure S1 in the Supporting Information).

To perform bipartite tetracysteine display, CHO-K1 cells expressing vIII-CC_H-1 or vIII-CC_H-10 were stimulated with growth factor (or not), incubated with ReAsH, washed, and immuno-stained to detect and quantify EGFRvIII expression. Receptor expression was monitored using a fluorescently labeled antibody to an N-terminal FLAG epitope. Using TIRF-M, we quantified the level of both cell surface ReAsH fluorescence (red) and EGFR expression (green) across multiple cells (67–161) that expressed EGFR or EGFRvIII variants. The cell-surface ReAsH fluorescence detected (over background) was normalized to the surface EGFR-expression detected (over background) to calculate the fold-increase in ReAsH fluorescence (Figures 3A and 3B). Cells expressing WT-CC_H-1 or WT-CC_H-10 displayed levels of normalized ReAsH fluorescence relative to background that mirrored previous reports (Figures 3A and 3B).[39–41] By contrast, cells expressing vIII-CC_H-1 or vIII-CC_H-10 showed an almost 2-fold increase in normalized ReAsH fluorescence, relative to background in the absence of any growth factor (1.86 ± 0.09 and 1.79 ± 0.09, respectively) as well as when treated with EGF (1.81 ± 0.10 and 1.70 ± 0.08) or TGF-α (1.80 ± 0.08 and 1.84 ± 0.10) (Figures 3A and 3B). The fold-increase in ReAsH fluorescence observed for cells expressing vIII-CC_H-1 or vIII-CC_H-10 was comparable to that observed when WT-CC_H-1 or WT-CC_H-10 are activated with EGF and TGF-α, respectively.[39–41] Previous work that examined the JM coiled coil status of WT EGFR activated with different growth factors,[39–41] or of constitutively active EGFR kinase domain mutants,[47] has always revealed a preference for one coiled coil over the other. The absence of this preference in the case of EGFRvIII is consistent with two fundamentally different scenarios. One is that the structure of intact and constitutively active EGFRvIII is best-represented as a mixture of dimers containing the EGF-type and TGF-α-type JM coiled coils (if not others). The second possibility is that the JM of constitutively active EGFRvIII can easily assume multiple

different conformations, including, but not limited to, the EGF-type and TGF-α-type JM coiled coils; the associated increased flexibility could also support ReAsH binding and induced fluorescence (Figure 2C).

To differentiate between these two possibilities, we designed a third CysCys-containing EGFRvIII variant, vIII-CC_H-4 (see Figures 2A and 2B). In vIII-CC_H-4, the four Cys residues within the JM are located too far apart to bind ReAsH in either the EGF-type or TGF-α-type conformation.[39] In the EGF-type structure, the Cys residues are located at positions g and e (as well as g' and e'); in the TGF-α-type structure, the Cys residues are located at positions g and b (as well as g' and b'). If the EGFRvIII JM adopts either the EGF-type or the TGF-α-type antiparallel coiled coil, then cells expressing vIII-CC_H-4 should show little or no ReAsH fluorescence. Conversely, if the EGFRvIII JM flexibly adopts multiple different conformations, then cells expressing vIII-CC_H-4 should show high ReAsH fluorescence. Control experiments verified that vIII-CC_H-4 could be expressed in CHO-K1 cells and was constitutively phosphorylated at C-terminal tail positions Y1068 and Y1086 (see Figure S1 in the Supporting Information).

Using bipartite tetracysteine display, we evaluated the fold increase of ReAsH fluorescence of CHO-K1 cells expressing variants WT-CC_H-4 and vIII-CC_H-4 in the absence and presence of EGF and TGF-α. As expected, with cells expressing WT-CC_H-4, no significant fold increase in ReAsH fluorescence, is observed when the cells are activated with either EGF (1.09 ± 0.05) or TGF-α (1.06 ± 0.06) or not stimulated with any growth factor (1.01 ± 0.04) (Figures 3A and 3B).[39] Interestingly, cells expressing vIII-CC_H-4 also showed little or no ReAsH fluorescence both without growth-factor activation (1.19 ± 0.06) and when the cells were stimulated with either EGF (1.17 ± 0.06) or TGF-α (1.19 ± 0.09). These results favor a model in which the EGFRvIII JM assembles constitutively into a mixture of two different antiparallel coiled coils of roughly equal stability. The data are less consistent with a model in which the JM segment of EGFRvIII can easily assume multiple different conformations.

Wild-type EGFR interacts through its extracellular domain (ECD) with seven different growth factors.[48] These factors induce different structures within the cytoplasmic juxtamembrane segment (JM) of the dimeric receptor and propagate different growth-factor-dependent signals to the cell interior.[39–41] Previous work has defined a model to explain how EGFR supports growth-factor-dependent signaling. This model begins with small but significant growth-factor-dependent differences in the structure of the bound ECD, especially in domain IV as it tracks into the transmembrane helix.[39,30,31] These differences lead to transmembrane helix dimers that differ in both cross location and cross angle.[41] Transmembrane helix dimers characterized by smaller cross angles at multiple cross locations induce the EGF-type coiled coil in the adjacent JM, whereas helix dimers with larger cross angles at fewer cross locations induce the TGF-α-type coiled coil (Figure 4A).[41] EGFRvIII provides a unique opportunity to test this model, as kinase activation occurs in the absence of either growth-factor binding or kinase domain mutations. Here, we make use of bipartite tetracysteine display to demonstrate that, in the absence of this growth-factor-induced signal, the JM of EGFRvIII adopts both EGF-type and TGF-α-type structures within the juxtamembrane segment. We show that, in the absence of growth-factor-induced

ECD rearrangements, the JM adopts a well-ordered configuration that appears to be a mixture of EGF-type and TGF-α-type structures (Figure 4A). These results suggest that the EGF-type and TGF-α-type JM coiled coils possess roughly equal stability in the context of the intact full-length receptor (Figure 4B). Overall, our work provides further evidence for an allosteric pathway that links growth-factor-induced binding to the extracellular domain to JM coiled coil structure and kinase activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

EGFR	epidermal growth factor receptor
GBM	glioblastoma multiforme
ECD	extracellular domain
TM	transmembrane
JM	juxtamembrane

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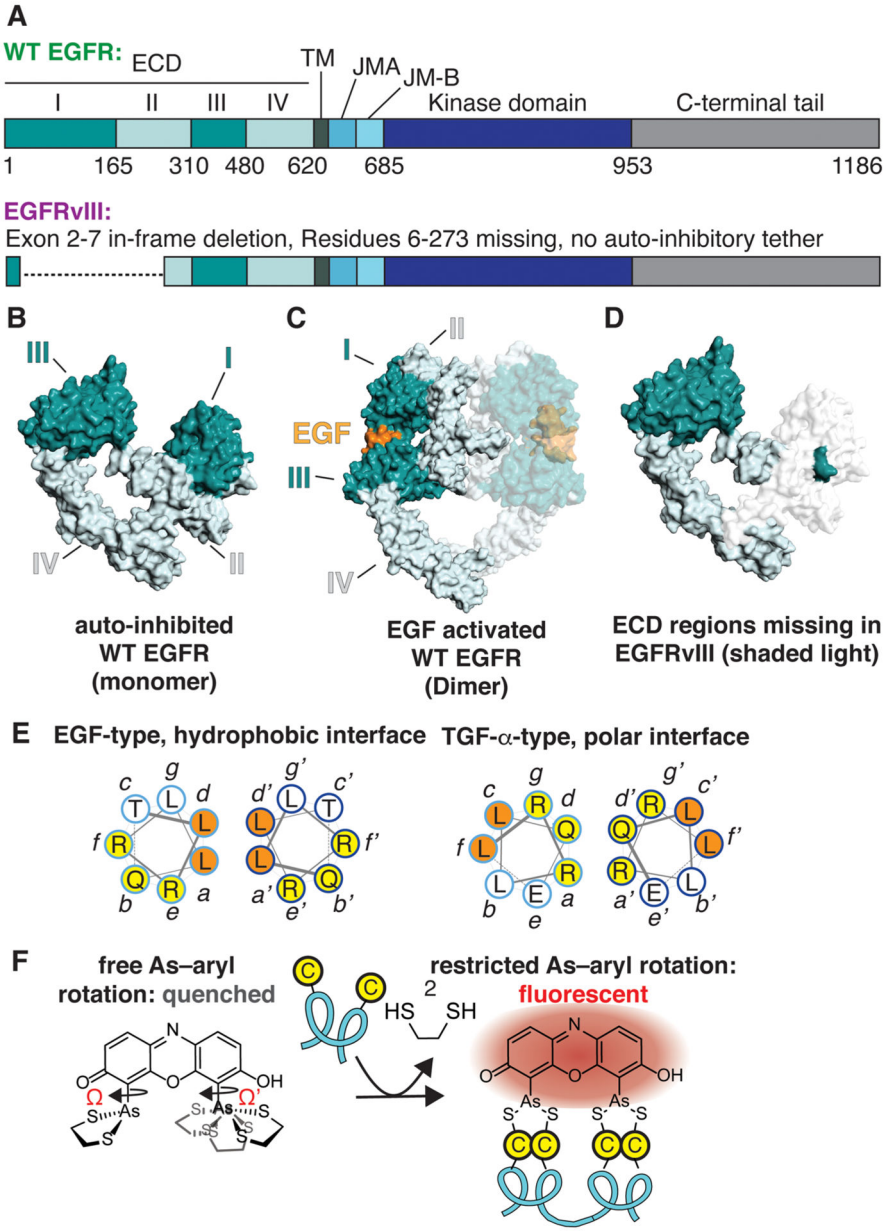

Figure 1.

Comparison between wild-type (WT) epidermal growth-factor receptor (EGFR) and EGFRvIII. (A) Schematics illustrating the distinct regions and domains of WT EGFR and EGFRvIII. EGFRvIII lacks amino acid residues 6–273 of the extracellular domain (ECD) (dashed line). (B) Surface model of the autoinhibited conformation of the WT EGFR ECD (PDB ID: 1NQL). Subdomains are color-shaded for visualization (I, III: deep teal; II, IV: pale cyan). The ECD is held in an autoinhibited conformation by intramolecular interactions between residues in domains II and IV. (C) Surface model of the WT EGFR ECD dimer bound to EGF (PDB ID: 3NJP). Domains are color-shaded as previously indicated, to visualize subdomain movement upon EGF binding. One of the dimeric partners is shaded light for clearer visualization. (D) Surface model of the autoinhibited EGFR ECD with the

portions missing in lightly shaded EGFRvIII. The ECD deletion prevents formation of autoinhibitory intramolecular interaction between domain II and IV. (E) Helical wheel diagrams showing axial views of interhelix juxtamembrane segment packing in EGF- and TGF-α-type coiled coils. (F) The fluorogenic dye ReAsH is quenched when bound to two ethanedithiol ligands, because of free rotation about the C–S bond. When bound to proteins containing four proximal Cys thiols, rotation is inhibited and the fluorescence is dequenched.

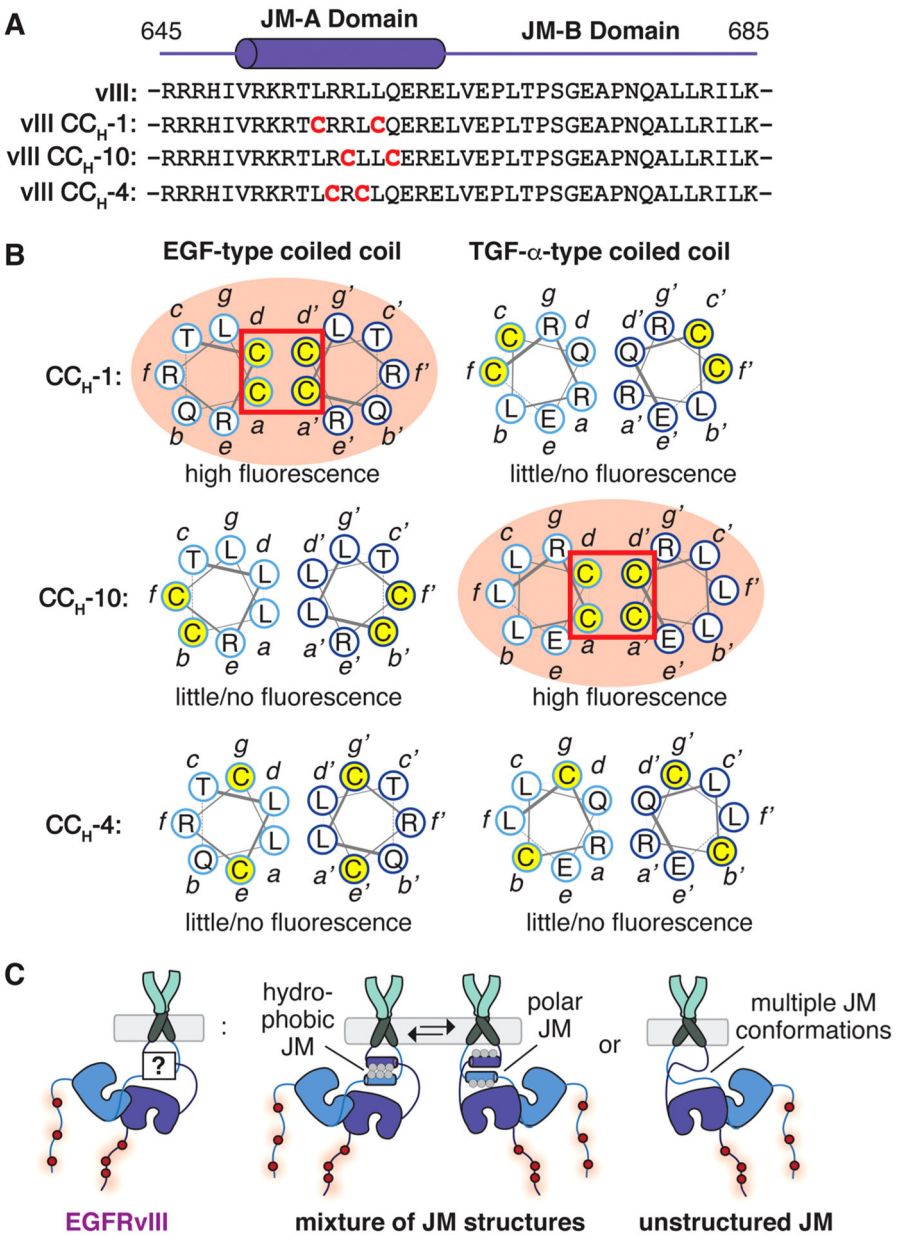

Figure 2.

Probing EGFRvIII JM structure using bipartite tetracysteine display. (A) Sequence of the juxtamembrane (JM) regions of vIII (WT EGFR numbering) alongside those of vIII CC$_H$-1, CC$_H$-10, and CC$_H$-4. JM residues in the vIII sequence that are mutated to Cys in vIII-CC$_H$-1, vIII-CC$_H$-10, and vIII-CC$_H$-4 are colored red. (B) Helical wheel diagrams illustrating axial views of idealized interhelix packing in EGF- and TGF-α-type coiled coils. The helical diagrams shaded with a red background indicate a conformation that is suitable for ReAsH binding with the individual vIII variants. (C) Two models to account for ReAsH binding by both vIII-CC$_H$-1 and vIII-CC$_H$-10.

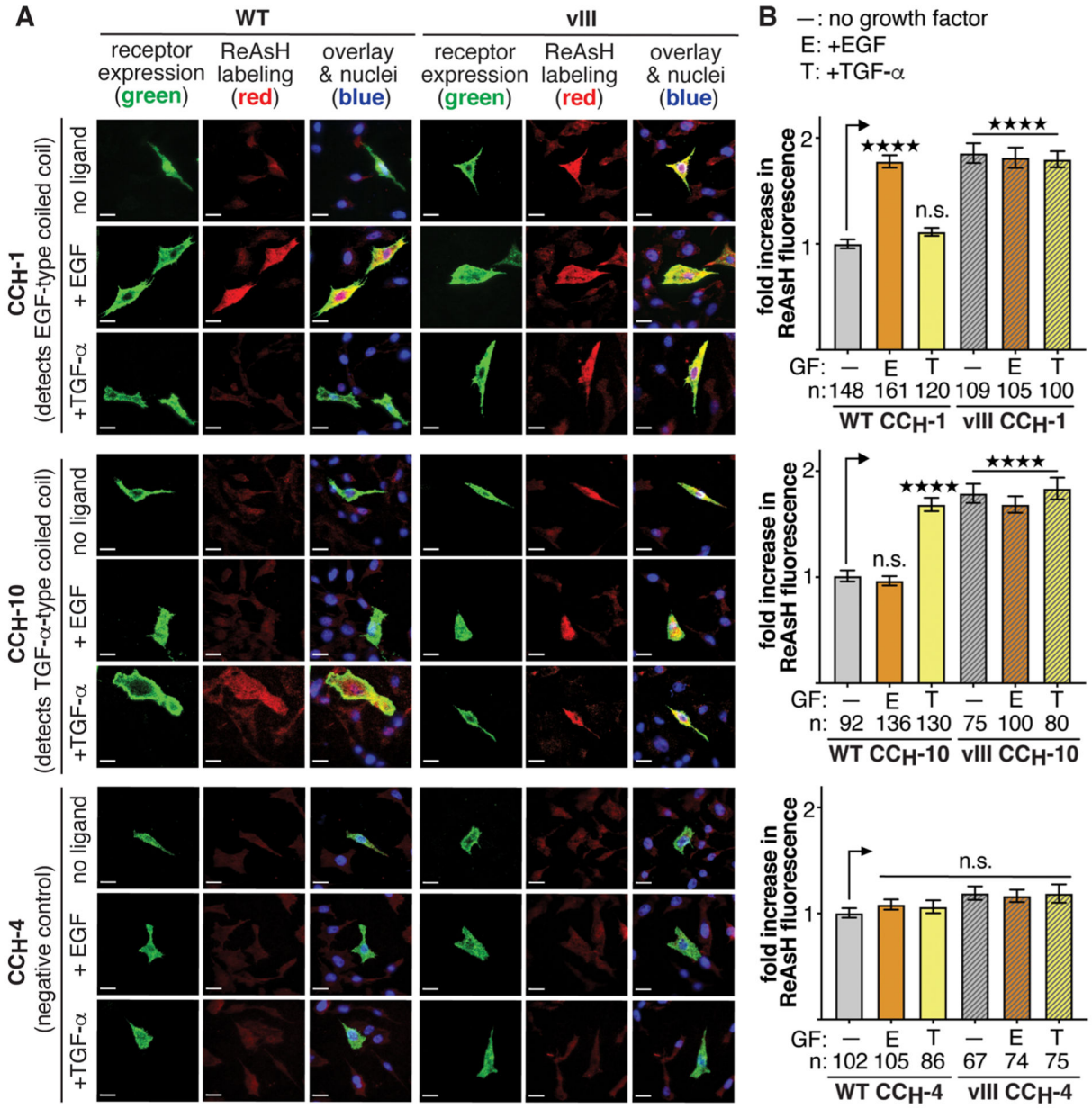

Figure 3.

Juxtamembrane segment of EGFRvIII exists as a mixture of EGF- and TGF-α-type coiled coils. (A) Representative TIRF-M images of CHO-K1 cells illustrating ReAsH labeling (red fluorescence) and expression (green fluorescence) of FLAG-tagged CC_H-1, CC_H-10, and CC_H-4 variants of WT EGFR and EGFRvIII in the absence and presence of EGF or TGF-α stimulation (16.7 nM). Scale bars = 10 μm. (B) Bar plots illustrating the quantification of TIRF-M results from n cells as a fold increase in expression-corrected ReAsH fluorescence over background. Error bars represent the standard error of measurement (sem); (****) $p < 0.0001$, (***) $p < 0.0002$, (**) $p < 0.0021$, (*) $p < 0.0332$ from one-way ANOVA with Dunnett's post-analysis accounting comparison to the WT control for each case without

growth-factor treatment (n.s. = not significant). See also Figure S1 in the Supporting Information.

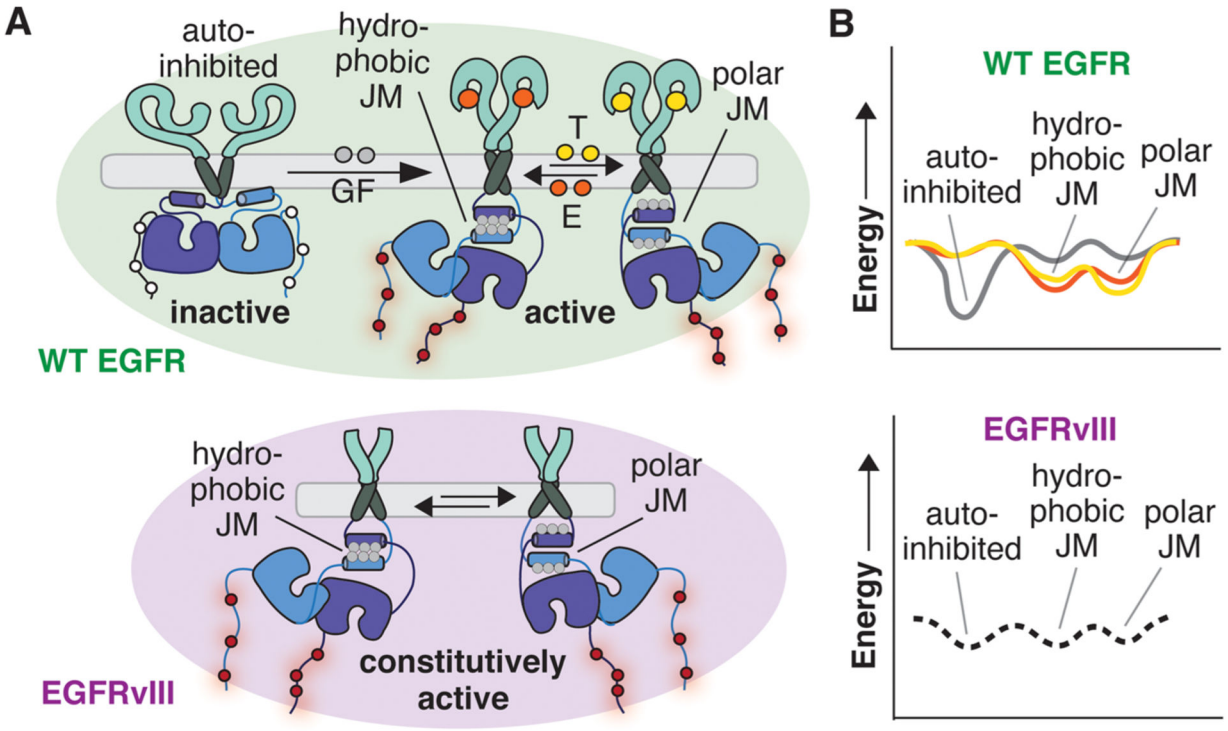

Figure 4.

(A) Cartoon illustrating the preferred conformation of the JM coiled-coil in WT EGFR and EGFRvIII. In WT EGFR, the JM conformation is influenced by the identity of the growth factor bound to the ECD. When bound to EGF, the JM adopts an antiparallel coiled coil characterized by a leucine-rich, hydrophobic interface; when bound to TGF-α, the antiparallel coiled coil is characterized by a polar interface. In the constitutively active EGFRvIII, in the absence of growth factor-induced ECD rearrangements, both EGF-type and TGF-α coiled coil conformations are adopted. (B) Hypothetical energy well diagrams illustrating the different energy landscapes of the JM region in WT EGFR and EGFRvIII.