TRAF4 binds to the juxtamembrane region of EGFR directly and promotes kinase activation

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Contributed by George R. Stark, September 20, 2018 (sent for review June 4, 2018; reviewed by Shao-Cong Sun and Nico Tjandra)

The activation of the epidermal growth factor receptor (EGFR) is crucial for triggering diverse cellular functions, including cell proliferation, migration, and differentiation, and up-regulation of EGFR expression or activity is a key factor in triggering the development of cancer. Here we show that overexpression of a scaffold protein, tumor necrosis factor receptor (TNF-R)-associated factor 4 (TRAF4), promotes EGF-induced autophosphorylation of EGFR (activation) and downstream signaling, whereas TRAF4 deficiency attenuates EGFR activation and EGF-driven cell proliferation. Using structure-based sequence alignment and NMR spectroscopy, we identified a TRAF4 binding site in the C-terminal half of the juxtamembrane (JM) segment of EGFR, a region known to promote asymmetric dimerization and subsequent activation. Deletion of the TRAF4 binding site led to dramatic defects in EGFR activation and EGF-driven cell proliferation. Specific point mutations in the TRAF4 binding site also resulted in significant attenuation of EGFR activation. Detailed structural examination of the inactive versus active forms of EGFR suggests that TRAF4 binding probably induces a conformational rearrangement of the JM region to promote EGFR dimerization. These results identify a novel mechanism of TRAF4-mediated EGFR activation and signaling.

TRAF4 | EGF | EGFR | asymmetric

The epidermal growth factor receptor (EGFR), a member of the ErbB family of transmembrane cell-surface receptor tyrosine kinases (PTKs) (1–4), plays an important role in regulating many different cellular functions, including proliferation, motility, and differentiation (5, 6). Aberrant EGFR activation is a hallmark of many human cancers, including cervical cancer (4–6). EGFR consists of an extracellular domain, a single transmembrane helix, a juxtamembrane (JM) segment, an intracellular kinase domain, and a regulatory C-terminal tail (7). When EGF binds to the extracellular domains, two EGFR molecules associate, and their tyrosine kinase domains are allosterically activated by asymmetric dimerization between the C lobe of one kinase domain, called the "activator," and the N lobe of another kinase domain, called the "receiver" (8, 9). Specifically, while the N-terminal half of the juxtamembrane segment (JM-A) potentiates dimerization by forming an antiparallel helical dimer, the juxtamembrane segment (JM-B) of the receiver forms a clamp over the C lobe of the activator, stabilizing dimerization (10, 11). The activator then destabilizes autoinhibitory interactions of the C-terminal tail of EGFR, together with the activation loop of the receiver (8), resulting in kinase activation. While the formation of the asymmetric dimer has been shown to be critical for EGFR activation, the mechanisms that mediate ligand-induced dimerization are not completely understood.

Tumor necrosis factor receptor (TNF-R)-associated factor 4 (TRAF4) is a member of the TRAF family of scaffold proteins, which is involved in regulating inflammation, antiviral responses, and apoptosis (12). TRAF4 was first identified by differential screening of human metastatic lymph nodes from a breast cancer cDNA library (13). TRAF4 is overexpressed in 43% of 623 different human tumors, and is one of the 67 genes overexpressed in human carcinomas (14, 15). Notably, TRAF4 is not only a marker but also has been proposed to be a driver oncogene in carcinomas, and thus has been implicated in the initiation and progression of primary cancers and metastases (13–18). Although TRAF4 is overexpressed in various human malignancies (15), how TRAF4 participates in tumorigenesis remains unclear.

Given the importance of both TRAF4 and EGFR in tumorigenesis, we wondered whether TRAF4 might be functionally linked to EGFR-dependent signaling. In a transient overexpression experiment, we found that TRAF4 increased the activation of EGFR kinase and downstream signaling. Using structure-based sequence alignment and NMR spectroscopy, we discovered a TRAF4 binding site in the C-terminal half of the juxtamembrane segment (JM-B, residues 664 to 682) of EGFR (11). A peptide containing the TRAF4 binding site (residues 670 to 682) effectively binds to recombinant TRAF4 in vitro, and removal or point mutation of the TRAF4 binding site attenuates EGF-induced phosphorylation of EGFR, downstream signaling, and EGF-driven cell proliferation. Our data reveal a novel role of TRAF4 in regulating EGFR activation, suggesting that blocking the binding of TRAF4 to EGFR might be an effective cancer therapy.

Results and Discussion

TRAF4 Is Required for EGFR Activation in Response to EGF Stimulation. To test whether TRAF4 can regulate EGFR signaling, we first examined the impact of TRAF4 deficiency on EGFR activation.

Significance

EGFR is a transmembrane cell-surface receptor tyrosine kinase crucial for regulating diverse physiological and pathological responses, but how protein function is turned on has remained elusive. Our results indicated that EGF stimulation recruits a key scaffold protein called TRAF4, which in turn recognizes the C-terminal half of the juxtamembrane segment of EGFR and promotes EGFR activation. Specific mutations in the TRAF4 binding site of EGFR resulted in significant attenuation of EGF-induced EGFR activation. These findings provide important insight into the mechanism of EGFR activation, and implicate TRAF4 as a potential therapeutic target for treating EGF/EGFR-driven cancers.

Author contributions: G.C., L.Z., X.C., J.Q., and X.L. designed research; G.C., L.Z., X.C., K.S., and C.L. performed research; G.C., L.Z., X.C., K.S., C.L., G.R.S., J.Q., and X.L. analyzed data; and G.C.S., G.R.S., J.Q., and X.L. wrote the paper.

Reviewers: S.-C.S., University of Texas MD Anderson Cancer Center; and N.T., National Heart, Lung, and Blood Institute.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1809599115/-/DCSupplemental.

Published online October 23, 2018.

EGF-induced phosphorylation of EGFR (on Tyr1068 and Tyr992) and AKT were substantially reduced in TRAF4-deficient HeLa cells (Fig. 1 *A* and *C*). In support of this result, EGFR and AKT phosphorylation in response to EGF were enhanced by doxycycline in HeLa cells carrying a doxycycline-inducible TRAF4 construct (Fig. 1 *B* and *D*). Consistently, TRAF4 deficiency attenuated EGF-driven cell proliferation (Fig. 1 *E* and *F*) and expression of cyclin D1, c-Myc, and IL-6 (Fig. 1 *G* and *H*). These results reveal that TRAF4 plays an important role in EGFR-dependent signaling and cell proliferation. We then examined the impact of TRAF4 deficiency on EGF signaling in primary keratinocytes. Interestingly, TRAF4 deficiency substantially reduced EGF-induced AKT and ERK5 phosphorylation, but with little impact on ERK1/2, JNK, or p38 phosphorylation (*SI Appendix*, Fig. S1).

TRAF4 Is Recruited to EGFR via the TRAF Domain. We next examined whether EGFR recruits TRAF4 upon EGF stimulation. Coimmunoprecipitation experiments indeed showed an EGF-induced interaction of TRAF4 and EGFR in HeLa (Fig. 2*A*), HT29 (Fig. 2*B*), A431 (Fig. 2*C*), and melanoma D4M cells (Fig. 2*D*). Deletion of the TRAF domain, but not the Ring or zinc finger domain of TRAF4, failed to restore the EGF-induced recruitment of TRAF4 to EGFR in TRAF4 KO HeLa cells (Fig. 2 *E* and *F*). Deletion of the Ring domain of TRAF4 impairs AKT phosphorylation but not EGFR phosphorylation, whereas the TRAF domain is required for both EGFR and AKT phosphorylation (Fig. 2 *G* and *H*). These data indicate that TRAF4 uses its TRAF domain to regulate EGFR activation, a process independent of its E3 ligase activity (19) but required for triggering downstream AKT phosphorylation.

TRAF4 Is Recruited to EGFR by Binding to the C-Terminal JM-B Domain of EGFR. As suggested by the data in Fig. 2*F*, TRAF4 probably interacts with EGFR directly via its TRAF domain. To confirm this point, we searched for an intracellular portion of EGFR that binds to the TRAF domain. A previous study indicated that the

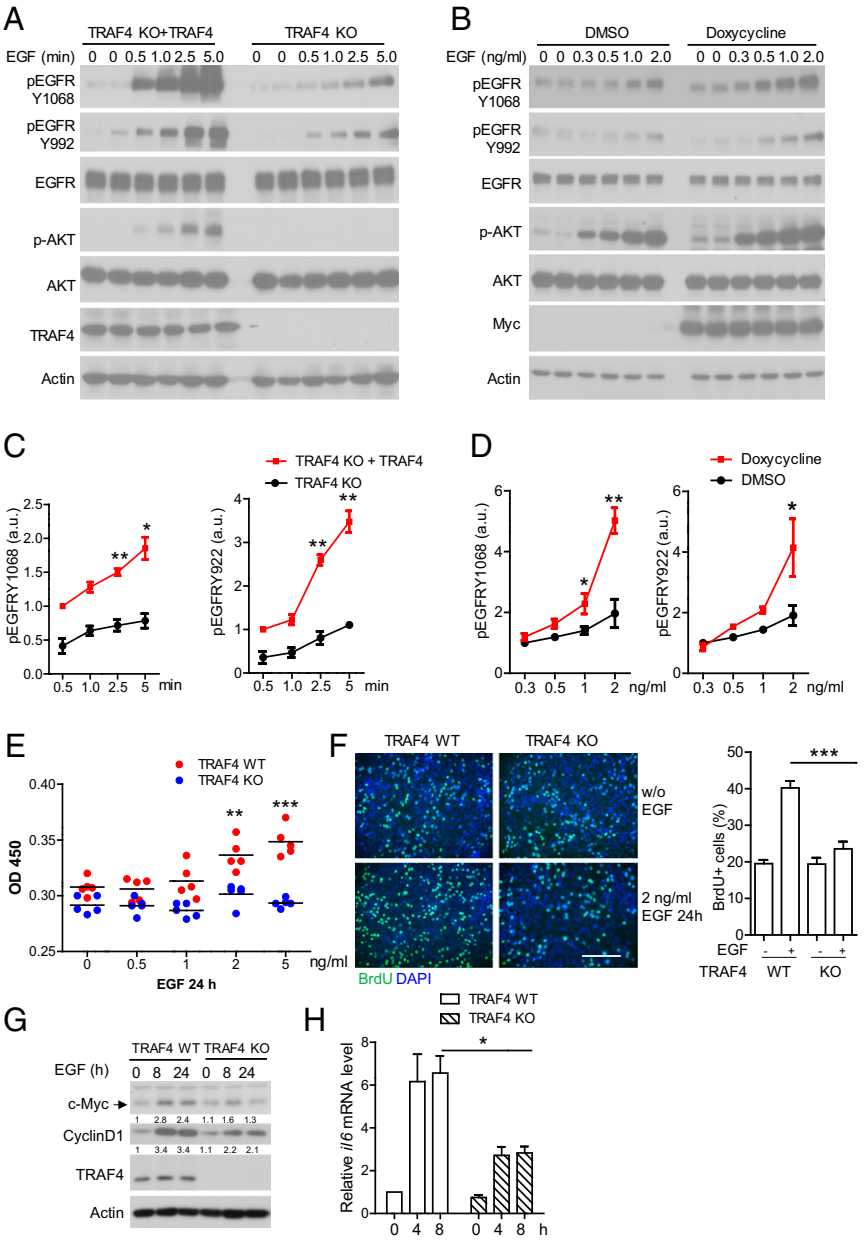

Fig. 1. TRAF4 is required for EGFR activation in response to EGF stimulation. (*A*) TRAF4-deficient (TRAF4 KO) HeLa cells were transfected with TRAF4 WT or empty vector and, 24 h later, the cells were starved of serum overnight and stimulated with 2 ng/mL EGF for various times. Cell lysates were then analyzed by the Western method with the indicated antibodies. (*B*) HeLa cells containing TRAF4-Myc in a Tet-on inducible system were treated with doxycycline for 24 h and then starved overnight, followed by EGF stimulation at the indicated concentrations for 5 min. Cell lysates were then analyzed by the Western method. (*C* and *D*) Phosphorylated EGFR (pY1068 and pY992) as shown (*A* and *B*) was quantified by densitometry, expressed in arbitrary units (normalized to actin). Bar graphs show mean arbitrary units ± SEM from three experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ (*t* test). (*E*) WT and TRAF4 KO HeLa cells were cultured in 96-well plates and stimulated with 2 ng/mL EGF for 24 h. A CCK-8 cell proliferation assay was performed after EGF stimulation. $n = 5$ wells per group. $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed *t* test). Data are representative of at least three independent experiments. (*F*) WT and TRAF4 KO HeLa cells were cultured in 12-well plates and stimulated with 2 ng/mL EGF for 24 h, with BrdU added during the last 3 h, followed by anti-BrdU staining. The graph represents mean percentages of $BrdU^{+}$ cells per 20× magnification field. Five fields were analyzed, ±SEM. $^{***}P < 0.001$ (two-tailed *t* test). (Scale bar, 200 μm.) (*G*) WT and TRAF4 KO HeLa cells were starved overnight, followed by EGF (2 ng/mL) stimulation and Western analysis. Numbers indicate the relative amounts of c-Myc and Cyclin D1, normalized to actin. (*H*) WT and TRAF4 KO HeLa cells were starved overnight, followed by EGF stimulation (2 ng/mL) and real-time PCR analysis. Graphs represent mean fold inductions, ±SEM, from three technical replicates. $^{*}P < 0.05$ (two-tailed *t* test). Data are representative of at least two independent experiments.

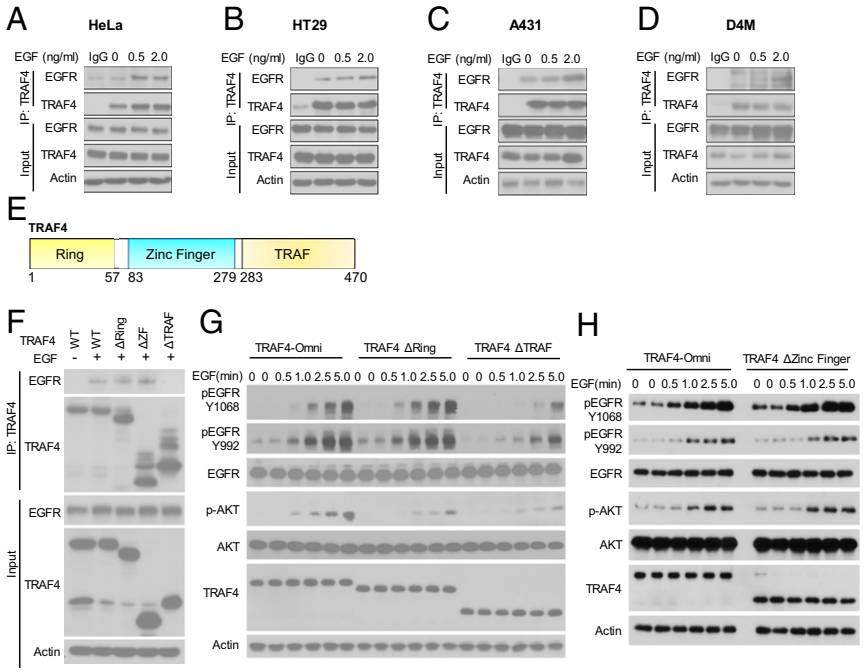

Fig. 2. TRAF4 is recruited to EGFR via the TRAF domain. (*A–D*) Serum-starved HeLa cells (*A*), HT29 cells (*B*), A431 cells (*C*), or D4M melanoma cells (*D*) were stimulated with 2 ng/mL EGF for 5 min. Cell lysates were immunoprecipitated with anti-TRAF4 antibody followed by Western analysis with the indicated antibodies. IP, immunoprecipitation. (*E*) Domain structure of TRAF4. (*F*) TRAF4-deficient (TRAF4 KO) HeLa cells transfected with TRAF4 WT or TRAF4 mutants (ΔRing; ΔZinc Finger, ΔZF; ΔTRAF) were starved overnight and then stimulated with 2 ng/mL EGF for 5 min. Cell lysates were immunoprecipitated with anti-TRAF4 followed by Western analysis with the indicated antibodies. (*G* and *H*) TRAF4 KO HeLa cells transfected with TRAF4 WT or TRAF4 mutants (ΔRing; ΔZinc Finger; ΔTRAF) were starved overnight and then stimulated with 2 ng/mL EGF for various times. Cell lysates were analyzed by the Western method with the indicated antibodies. Data are representative of at least three experiments.

TRAF domain of TRAF4 preferably binds to an RLXA motif (20); however, such a motif was not found in EGFR. Since the TRAF domain of TRAF4 is structurally similar to the TRAF domains of TRAFs 2, 3, and 6, which bind to a PX(Q/E)XXX motif (21), we speculated that the TRAF domain of TRAF4 would also bind to a similar motif, containing a proline residue (*SI Appendix*, Fig. S2). Given that TRAF4 regulates the phosphorylation of EGFR (Fig. 1 *A–D*), we focused our search on the intracellular domain. Nineteen proline residues are present in this region of EGFR. By excluding proline residues that are

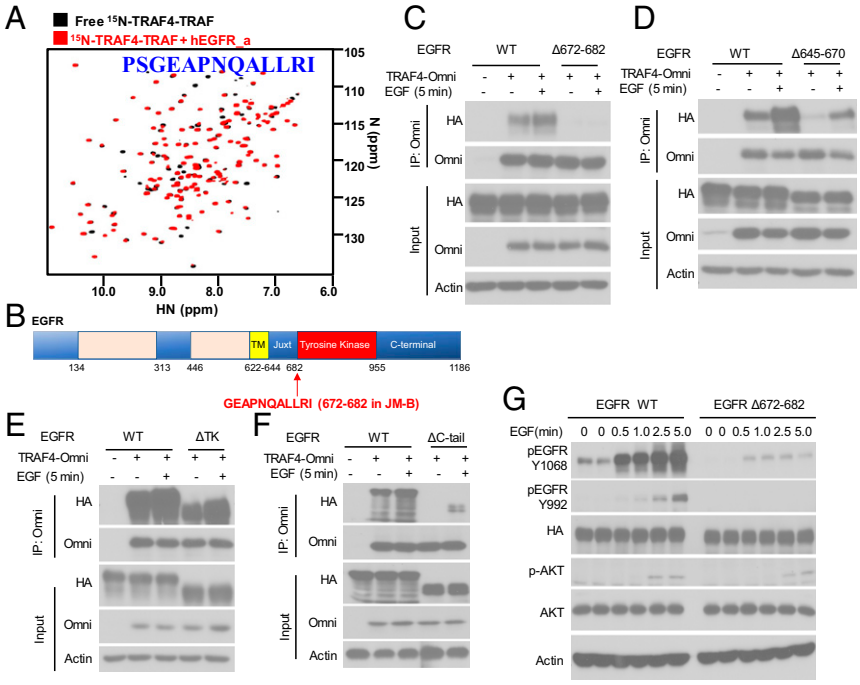

Fig. 3. C-terminal JM-B of EGFR is required for EGF-induced recruitment of TRAF4 to EGFR. (*A*) The HSQC spectra of 100 μM ^{15}N-labeled TRAF4-TRAF in the absence (black) and presence of 1 mM peptide hEGFR_a (red). (*B*) Domain structure of EGFR. The 24-aa signal peptide at the N terminus of EGFR is not listed in the model. TM, transmembrane. (*C–F*) Omni-tagged TRAF4 was cotransfected with HA-tagged EGFR WT or EGFR mutants [Δ672–682 (*C*); Δ645–670 (*D*); ΔTK (690–955) (*E*); ΔC tail (955–1186) (*F*)] into EGFR-deficient (EGFR KO) HeLa cells. The transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for 5 min. Cell lysates were immunoprecipitated with mouse anti-Omni, followed by Western analysis with the indicated antibodies. Data are representative of at least three experiments. (*G*) EGFR KO HeLa cells were transfected with HA-tagged EGFR WT or mutant EGFR (Δ672–682). After 24 h, the transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for the indicated times. Cell lysates were then analyzed by the Western method.

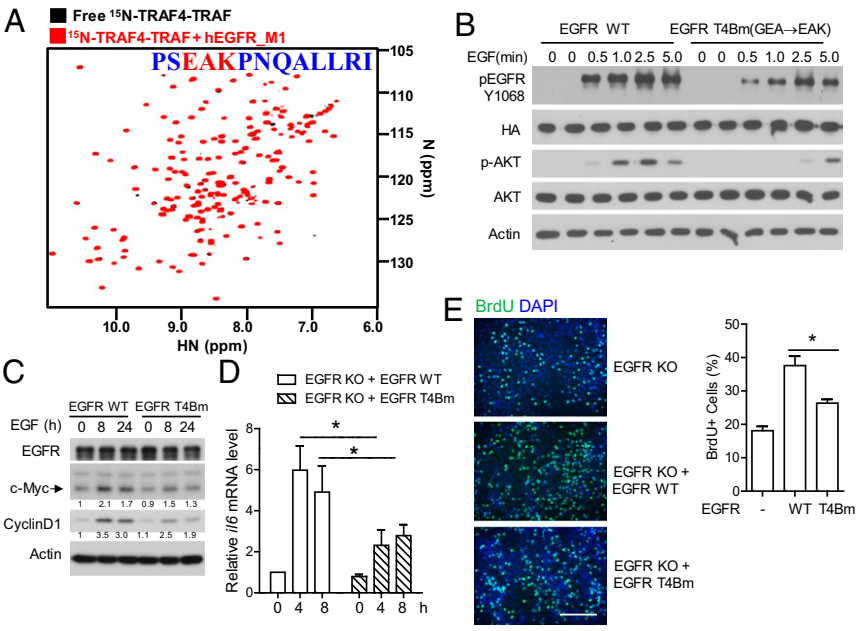

Fig. 4. TRAF4 binding mutation in C-terminal JM-B of EGFR compromises EGFR phosphorylation. (*A*) The HSQC spectra of 100 μM ^{15}N-labeled TRAF4-TRAF in the absence (black) and presence of 1 mM peptide hEGFR_M1 (red). (*B*) HA-tagged EGFR WT or EGFR mutant EGFR (T4Bm, TRAF4 binding mutant, GEA to EAK) was transfected into EGFR-deficient (EGFR KO) HeLa cells. After 24 h, the transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for the indicated times. Cell lysates were analyzed by the Western method. Data are representative of at least three experiments. (*C*) EGFR KO HeLa cells restored with EGFR WT or an EGFR TRAF4 binding mutant (T4Bm, GEA to EAK) were starved overnight, followed by EGF stimulation (2 ng/mL) and Western analysis. Numbers indicate the fold induction of c-Myc and Cyclin D1, quantified by normalization to actin. (*D*) EGFR KO HeLa cells restored with EGFR WT or EGFR T4Bm were starved overnight and then treated with EGF (2 ng/mL), followed by RT-PCR analysis. The graph shows the fold induction of *IL-6* mRNA, ±SEM from three technical replicates. $*P < 0.05$ (two-tailed *t* test). Data are representative of at least two independent experiments. (*E*) EGFR KO HeLa cells restored with EGFR WT or EGFR TRAF4 binding mutant were cultured in 12-well plates and stimulated with 2 ng/mL EGF for 24 h, with BrdU added during the last 3 h, followed by anti-BrdU staining. The graph represents mean percentages of BrdU$^+$ cells per 20× magnification field. Five fields were analyzed, ±SEM. $*P < 0.05$ (two-tailed *t* test). (Scale bar, 200 μm.)

buried in the structure, we identified four proline-containing candidates, named hEGFR_a, b, c, and d (*SI Appendix*, Table S1). We synthesized these fragments and tested their binding to the TRAF domain of TRAF4 in solution by NMR-based 2D-HSQC analysis. As shown in Fig. 3*A* and *SI Appendix*, Fig. S3, peptide hEGFR_a, which corresponds to EGFR residues 670 to 682, specifically induced chemical shift changes in amino acid residues of the TRAF domain of TRAF4. As a control, this fragment does not interact with the TRAF domain of TRAF6 (*SI Appendix*, Fig. S4). Although hEGFR_b and c do not show any obvious interaction with the TRAF domain of either TRAF4 or 6, hEGFR_d does interact with both, with no apparent selectivity (*SI Appendix*, Figs. S5 and S6 and Table S1). The peptide RRLRARARARA (named TR4), shown to bind the TRAF4-TRAF domain previously (20), also interacts with the TRAF4-TRAF domain in our HSQC experiment (*SI Appendix*, Fig. S7). Notably, hEGFR_a induced a similar chemical shift perturbation pattern as TR4 (Fig. 3*A* and *SI Appendix*, Fig. S7), suggesting that hEGFR_a likely binds to the same conserved pocket of the TRAF4-TRAF domain as TR4 (20). Our HSQC titration also revealed that hEGFR_a and TR4 bind to TRAF4-TRAF with similar affinity (*SI Appendix*, Fig. S8). Altogether, these data highlight a specific interaction between EGFR (residues 670 to 682) and the TRAF domain of TRAF4. Consistently, deletion of the C-terminal region of JM-B (Fig. 3*B*; residues 672 to 682) abolished the EGF-induced recruitment of TRAF4 to EGFR in EGFR KO HeLa cells (Fig. 3*C*), whereas deletion mutants of the N-terminal juxtamembrane domain (ΔJuxta, residues 645 to 670), the kinase domain (ΔTK, residues 690 to 955), and the C-terminal domain of EGFR (ΔC tail, residues 955 to 1186) were still recruited to EGFR in EGFR KO HeLa cells upon EGF stimulation (Fig. 3 *D*–*F*). These results indicate that a C-terminal domain of JM-B (residues 672 to 682) is required for EGF-induced recruitment of TRAF4 to EGFR. In support of this conclusion, deletion of this region failed to restore EGF-induced EGFR signaling and AKT activation in EGFR KO HeLa cells (Fig. 3*G*).

Since hEGFR_a has two proline residues, it has two possible binding motifs, PSGEAP and PNQALL. Therefore, we tested the binding of four mutant peptides (hEGFR_M1, M2, M3, and M4) to the TRAF domain of TRAF4 (*SI Appendix*, Table S1). hEGFR_M1, which bears three mutations (GEA to EAK), but not the other mutations, abolished binding to the TRAF4-TRAF domain (Fig. 4*A* and *SI Appendix*, Figs. S9–S11 and Table S1), indicating that PSGEAP is the major binding site. Such a proline-containing binding sequence was unexpected and is distinctly different from the PXQ/EXXX motif known to bind to TRAF domains such as those of TRAF2, 3, and 6 (21), suggesting its unique ability to recognize TRAF4-TRAF. Consistently, such a sequence does not bind to TRAF6 (*SI Appendix*, Fig. S4). On the other hand, the peptide sequences of hEGFR_a and hEGFR_d are very different from the RLXA motif proposed to recognize TRAF4 (20), suggesting that they may share some structural similarity when recognizing the TRAF4-TRAF domain, a point that remains to be examined via detailed structural characterization. Nevertheless, EGFR bearing GEA-to-EAK mutations (EGFRT4Bm) substantially reduced EGFR activation and cell proliferation in response to EGF (Fig. 4 *B*–*E*). These data demonstrate that the direct interaction between the TRAF4-TRAF domain and EGFR JM-B alone is sufficient to mediate EGFR activation. The functional role of the binding of hEGFR_d to TRAF4 remains to be determined. Since TRAF domains, including that of TRAF4, exist as trimers (20, 21), it is possible that hEGFR_a and hEGFR_d from the same EGFR molecule

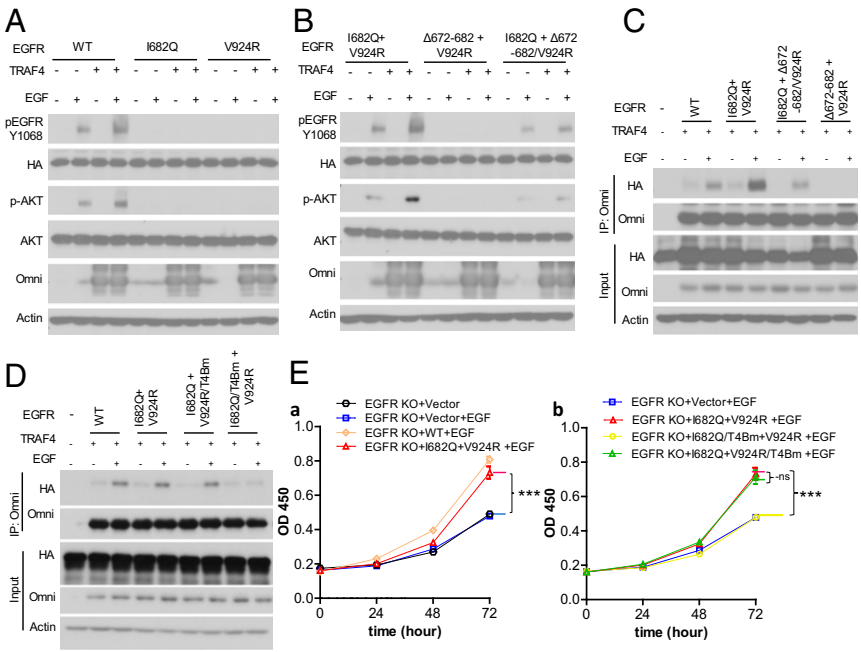

Fig. 5. TRAF4 stimulates EGFR to form asymmetric dimers. (*A*) Omni-tagged TRAF4 was cotransfected with HA-tagged EGFR WT, EGFR activator mutant I682Q, or receiver mutant V924R into EGFR-deficient (EGFR KO) HeLa cells. Transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for 5 min. Cell lysates were then analyzed by the Western method. (*B*) Omni-tagged TRAF4 was cotransfected with HA-tagged, EGFR activator I682Q mutant, receiver V924R mutant, or the TRAF4 binding motif (residues 672–682) deletion mutant into EGFR KO HeLa cells. The transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for 5 min. Cell lysates were then analyzed by the Western method. (*C*) Omni-tagged TRAF4 was cotransfected with HA-tagged, EGFR WT, EGFR activator I682Q mutant, EGFR receiver V924R mutant, or the TRAF4 binding motif deletion (Δ672–682) mutant into EGFR KO HeLa cells. The transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for 5 min. Cell lysates were immunoprecipitated with mouse anti-Omni, followed by Western analysis with the indicated antibodies. Data are representative of at least three experiments. (*D*) Omni-tagged TRAF4 was cotransfected with HA-tagged, EGFR WT, EGFR activator I682Q mutant, EGFR receiver V924R mutant, or EGFR activator and receiver mutant carrying the TRAF4 binding mutation (T4Bm, GEA-EAK) into EGFR KO HeLa cells. The transfected cells were serum-starved overnight and stimulated with 2 ng/mL EGF for 5 min. Cell lysates were immunoprecipitated with mouse anti-Omni, followed by Western analysis with the indicated antibodies. Data are representative of at least three experiments. (*E*) EGFR KO HeLa cells transfected with vector, WT EGFR, EGFR activator I682Q mutant, EGFR receiver V924R mutant, or a mutant carrying the TRAF4 binding site mutation (T4Bm, GEA-EAK) were cultured in 96-well plates and stimulated with EGF. A CCK-8 cell proliferation assay was performed after EGF stimulation. Data are presented in *E*, *a* and *b*. (*E*, *a*) EGFR KO cells restored with WT EGFR or coexpression of EGFR activator I682Q mutant or EGFR receiver V924R mutant. (*E*, *b*) EGFR KO cells restored by coexpression of EGFR activator and receiver mutants or a mutant carrying the TRAF4 binding mutation (T4Bm, GEA-EAK). $n = 5$ wells per group, ±SEM. ***$P <$ 0.001; ns, not significant (two-tailed t test). Data are representative of at least three independent experiments.

bind to different subunits of the TRAF trimer, thereby enhancing the binding between TRAF4 and EGFR. Consistently, the ΔC-tail construct of EGFR, which lacks hEGFR_d but contains hEGFR_a, exhibits reduced binding to TRAF4 (Fig. 3*F*). It should be noted that TRAF4 also bears a membrane binding site (22) which may further strengthen the binding of TRAF4 to EGFR, which is also membrane-anchored, a scenario seen in the interactions of other membrane-associated proteins, such as Rap1 and talin (23).

TRAF4 May Activate EGFR by Promoting Its Asymmetric Dimerization. Activation of the kinase domain of EGFR occurs through an allosteric mechanism involving the formation of an asymmetric dimer (7). The importance of allosteric asymmetric dimerization was shown by both the activator impairment mutation V924R and the receiver impairment mutation I682Q, which inactivate EGFR by disrupting its ability to form asymmetric dimers (8). TRAF4 did not enhance the phosphorylation of either the activator impairment mutant V924R or the receiver impairment mutant I682Q (Fig. 5*A*). On the other hand, in a receiver and activator coexpression system, TRAF4 enhanced activation of the kinase domain of EGFR (Fig. 5*B*). These data support the idea that TRAF4 might promote the asymmetric dimerization of EGFR that is essential for its activation. Consistently, removing the TRAF4 binding motif (residues 672 to 682) from the activator (Fig. 5*B*) or employing the GEA-to-EAK mutation (EGFR T4Bm, GEA-EAK; Fig. 4*B*) attenuated EGF-induced EGFR and AKT activation. Coimmunoprecipitation experiments showed that removing the TRAF4 binding motif or employing the GEA-to-EAK mutation in the activator but not receiver abolished the EGF-induced recruitment of TRAF4 to EGFR (Fig. 5 *C* and *D*), suggesting that TRAF4 may favor binding to the activator. In addition, EGFR KO cells expressing EGFR with a TRAF4 binding-defective mutation in the activator (I682Q/T4Bm, GEA-EAK) did not show enhanced proliferation in response to EGF (Fig. 5*E*).

Consistently, structural examination of the asymmetric dimer of the EGFR kinase domain revealed that residues 672 to 682 in the activator are highly accessible to the TRAF domain of TRAF4 (Fig. 6). Comparison of the asymmetric dimer with the inactive monomeric EGFR kinase domain suggests that TRAF4 binding may facilitate a conformational rearrangement of the JM region, facilitating the formation of an antiparallel helical dimer, leading to asymmetric dimerization and activation (Fig. 6).

In summary, our studies have unraveled a novel mechanism for TRAF4-mediated EGFR dimerization and activation. Previous studies have shown that the juxtamembrane domains [designated JM-A (residues 645 to 664) and JM-B (residues 664 to 682)] of EGFR play a critical role in EGF-induced asymmetric dimerization between the C lobe of one kinase domain, the activator, and the N lobe of a second kinase domain, the receiver. We identified a TRAF4 binding site in the C-terminal half of the juxtamembrane segment JM-B. TRAF4 deficiency or removal or

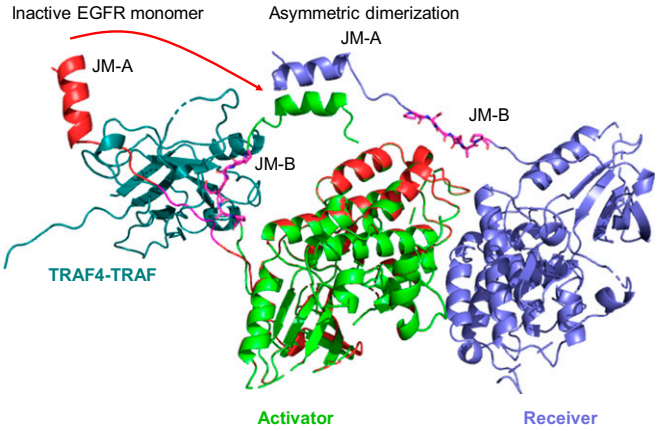

Fig. 6. Model for asymmetric EGFR dimer formation promoted by TRAF4. Schematic illustration of how TRAF4 (dark teal) binds to the JM-B region (the TRAF4 binding site is colored magenta) of an inactive EGFR monomer (red) to facilitate a positional change of JM-A that promotes the formation of an antiparallel helical dimer with another JM-A. Notice that JM-B in the receiver (blue) clamps onto the activator (green) and is not freely accessible for TRAF4 binding, which contrasts to the JM-B of the activator that is surface-exposed and accessible to TRAF4.

mutation of the TRAF4 binding site of EGFR (residues 672 to 682) attenuated the EGF-induced phosphorylation of EGFR and downstream signaling. Moreover, loss of the TRAF4 binding site reduced EGFR-driven cell proliferation. Our studies thus have strong consequences for the design of new EGFR-based cancer therapies, given the central role of EGFR in cancer development and progression. Although many EGFR inhibitors have been developed and are used in the clinic, many patients eventually develop resistance (24, 25). Therefore, it is critical to explore alternative approaches to inhibiting EGF receptor activation and downstream signaling. Increased TRAF4 expression correlates with elevated malignancy in many cancers. Our studies demonstrate that TRAF4 directly interacts with EGFR and promotes EGFR-mediated signaling by facilitating the dimerization of EGFR and downstream AKT activation, as well as promoting cell proliferation. Blocking TRAF4 binding to EGFR may provide a novel approach for cancer therapy. Notably, EGF-induced AKT phosphorylation was reduced in TRAF4-deficient HeLa cells transfected with either TRAF or Ring domain deletion mutants of TRAF4, indicating the importance of E3 ligase activity for EGF-induced AKT activation. Development of small-molecule inhibitors of TRAF4 E3 ligase activity might provide a second new strategy to inhibit cancer cell proliferation. Taken together, our findings demonstrate a novel role of TRAF4 in EGFR signaling and suggest TRAF4 as a potential target for therapeutic intervention in cancer patients.

Materials and Methods

Detailed information on cell lines and cell culture, antibodies and reagents, constructs and cell transfection, Western blot and immunoprecipitation, NMR 2D-HSQC, cell proliferation assay, BrdU staining, RNA isolation and real-time PCR, and quantification and statistical analysis is available in *SI Appendix, Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Constance E. Brinckerhoff for kindly providing the D4M cell line, and Dr. W. Qian for technical support. The study was supported by the US National Institutes of Health Grant (5P01 CA062220-23) (to X.L. and G.R.S.), Grant (5P01 HL103453-08) (to X.L.), and Grant (R01 HL058758) (to J.Q.) at the Lerner Institute at the Cleveland Clinic.

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