Published in final edited form as:

ACS Chem Biol. 2018 September 21; 13(9): 2623–2632. doi:10.1021/acscchembio.8b00555.

Inhibiting Epidermal Growth Factor Receptor Dimerization and Signaling Through Targeted Delivery of a Juxtamembrane Domain Peptide Mimic

Janessa Gerhart, Anastasia F. Thévenin[†], Elizabeth Bloch, Kelly E. King, and Damien Thévenin^{*}

Department of Chemistry, Lehigh University, 6 East Packer Avenue, Bethlehem, Pennsylvania 18015, United States

Abstract

Overexpression and deregulation of the epidermal growth factor receptor (EGFR) are implicated in multiple human cancers and therefore are a focus for the development of therapeutics. Current strategies aimed at inhibiting EGFR activity include monoclonal antibodies and tyrosine kinase inhibitors. However, activating mutations severely limit the efficacy of these therapeutics. There is thus a growing need for novel methods to inhibit EGFR. One promising approach involves blocking the association of the cytoplasmic juxtamembrane (JM) domain of EGFR, which has been shown to be essential for receptor dimerization and kinase function. Here, we aim to improve the selectivity and efficacy of an EGFR JM peptide mimic by utilizing the pH(low) insertion peptide (pHLIP), a unique molecule that can selectively target cancer cells solely based on their extracellular acidity. This delivery strategy potentially allows for more selective targeting to tumors than current methods and for anchoring the peptide mimic to the cytoplasmic leaflet of the plasma membrane, increasing its local concentration and thus efficacy. We show that the conjugated construct is capable of inhibiting EGFR phosphorylation and downstream signaling and of inducing concentration- and pH-dependent toxicity in cervical cancer cells. We envision that this approach could be expanded to the modulation of other single-span membrane receptors whose activity is mediated by JM domains.

Graphical Abstract

^{*}Corresponding Author: damien.thevenin@lehigh.edu.

[†]Present Address

A.F.T.: Department of Biological Sciences, Moravian College, 1200 Main Street, Bethlehem, PA 18018

Author Contributions

J.G., A.F.T., and D.T. designed the project, performed the experiments, analyzed the data and wrote the manuscript. J.G. and K.E.K. synthesized the pHLIP peptide. E.B. cloned the EGFR_TM20 construct.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.8b00555.

Extended methods: maltose complementation test, solid-phase peptide synthesis, preparation of pHLIP-JMA construct, sample preparation of CD and tryptophan fluorescence measurements, tryptophan fluorescence spectroscopy, CD spectroscopy, preparation of POPC liposomes, cell membrane leakage assay; extended figures: synthesis of pHLIP-JMA, maltose complementation test, the interaction of pHLIP-JMA and pHLIPJMA_{Ala} with POPC liposomes, the proliferative response of MDA-MB-231 to EGF, the effect of pHLIP, JMA, and pHLIP-JMA_{Ala} on cell viability, cell migration, and EGFR phosphorylation, the effect of pHLIP-JMA on membrane leakage, purity check, and MALDI-TOF of peptides (PDF)

The authors declare no competing financial interest.

The epidermal growth factor receptor (EGFR) is a known oncogenic driver in numerous cancers¹ and therefore represents an attractive target for drug development.² In normal cells, EGFR undergoes a conformational change upon ligand binding, which stabilizes the active form of the homodimer and induces subsequent tyrosine autophosphorylation.³ Activation of EGFR initiates important signaling pathways that are involved in regulating cell proliferation, metabolism, migration, and angiogenesis.⁴ Dysregulation of mechanisms controlling EGFR activation and expression results in abnormal cell growth and proliferation.¹ Over-expression of EGFR has been implicated in cancers such as lung cancer,⁵ glioblastoma,⁶ and colorectal cancer.⁷ Furthermore, in some cancers, EGFR overexpression serves as an indicator of a poor prognosis, an increased relapse propensity, and a more aggressive disease.⁸

Several strategies have been developed to inhibit aberrant EGFR activity in cancer cells.⁴ For example, monoclonal antibodies such as cetuximab⁹ (Erbix) and panitumumab¹⁰ (Vectibix) block the signaling cascade by binding to the extracellular domain of EGFR. However, when administered as monotherapies, they display a low rate of response with as low as 10% of patients displaying a positive response.¹¹ Further studies showed that the *KRAS* gene, which codes for a small G-protein downstream of EGFR, can harbor activating mutations that render the monoclonal antibodies ineffective.¹² Other therapeutics that target EGFR are tyrosine kinase inhibitors (TKIs), such as gefitinib,¹³ erlotinib,¹⁴ and afatinib.¹⁵ These small molecules, which function by occupying the ATP-binding site of the intracellular catalytic domain, are approved for the treatment of metastatic nonsmall cell lung cancer¹⁶ and pancreatic cancer.¹⁷ However, the clinical efficacy of these TKIs is often limited due to the rapid emergence of drug resistance conferred by EGFR mutants. For instance, patients harboring the L858R mutation who initially respond to gefitinib (Iressa) or erlotinib (Tarceva) frequently develop a second-site mutation (T790M), which prevents the binding of TKIs to the ATP-binding site, lowering their potency.¹⁸ There is therefore a clear need for novel methods to inhibit EGFR.

An alternative approach to combat EGFR dysregulation, including prevalent drug-resistant mutants, is to modulate the activity of EGFR by targeting the cytoplasmic surface, specifically the juxtamembrane (JM) domain, where there is no known resistance mutation. Despite their importance in signal transduction¹⁹ and representing an attractive target for oncogenic intervention via allosteric modulation,^{20,21} JM domains are under-explored as therapeutic targets. A segment of the JM domain of EGFR, denoted as the JMA domain

(residues 645–663), forms a short α -helix that interacts in an antiparallel manner to stabilize the asymmetric dimer.^{22,23} The self-association of the JMA domain has been shown to be necessary for EGFR dimerization and activation, as demonstrated by studies in which EGFR was rendered catalytically inactive when the JMA domain was deleted or mutated.^{21,22,24} Therefore, a peptide mimicking the JMA domain of EGFR could potentially disrupt the antiparallel helical association and interfere with receptor signaling. Iyengar and colleagues showed that such a peptide (EGFR 645–662), when conjugated to the cell-penetrating Tat peptide, displays antitumorigenic effects in multiple human cancer cell types expressing a high level of EGFR by disrupting EGFR signaling.²⁵ Schepartz and colleagues later showed, using a bipartite tetracysteine display,²⁶ that a hydrocarbon-stapled peptide mimic, comprised of the same amino acid residues, inhibits EGFR by disrupting intradimer coiled-coil formation.²⁷

However, the discovery and use of such peptides remain challenging. Indeed, because of their size and polarity, JM peptide mimics are very often cell impermeable and require a strategy to improve their cellular uptake (e.g., cell-penetrating peptide and stapled peptide). Furthermore, even if they could translocate into the cytoplasm, they would be free to diffuse in the cytoplasm, making the targeting of membrane receptors more difficult. Finally, their tumor selectivity relies solely on the overexpression of EGFR in cancer cells. This would likely result in off-target toxicity if used as a treatment modality for, as EGFR is expressed in other tissues.

Here, we hypothesized that higher selectivity toward cancer cells and more controlled intracellular delivery can be achieved by conjugating a JMA peptide mimic to the pH(low) insertion peptide (pHLIP). pHLIP is a unique peptide that can selectively target cancer cells and tumors in mice solely based on their extracellular acidity while sparing healthy cells.^{28–30} Although no specific gene mutation or chromosomal abnormality is common to all cancers, nearly all solid tumors of all sizes (including metastases) have elevated acidosis regardless of their tissue or cellular origin.^{31–33} As a result, the microenvironment surrounding tumor masses are acidic (pH 6.0–6.8) in contrast to healthy tissues (pH 7.2–7.5).^{34–36} In fact, recent studies revealed that cancer cell surfaces are even more acidic (another 0.3–0.7 pH units lower) than the acidic bulk microenvironment.³⁷ For these reasons, acidosis may provide a universal mode of tumor targeting that is not subject to the selection of resistance.^{33,38}

Importantly, the mechanism by which pHLIP translocates cargo across the cell membrane is not mediated by endocytosis, membrane receptor interaction, or the formation of pores within the membrane but rather through the formation of a transmembrane (TM) α helix across the lipid bilayer in which the C-terminus inserts into the cytoplasm and the N-terminus remains in the extracellular region.^{39,40} Therefore, conjugating a modulating peptide to the C-terminus of pHLIP through a nonreleasable linker would allow for its active translocation into the cytoplasm and anchoring to the intracellular leaflet of the plasma membrane, where it could interfere with its target receptor. We have previously shown that a peptide fragment derived from the third intracellular loop of the G protein-coupled receptor protease-activated receptor 1 (PAR1), when conjugated to the C-terminus of pHLIP, induces selective cytotoxicity in breast cancer cells from downregulation of the PAR1 cell signaling

pathway.⁴¹ In the present study, we extend this approach and show that the pHLIP-mediated delivery of a JMA domain peptide mimic can selectively modulate the dimerization state and activity of EGFR in cancer cells (Figure 1). We envision that this strategy could be applied to modulate other oncogenic, single-span membrane receptors whose activity is mediated by JM domains.

RESULTS AND DISCUSSION

Juxtamembrane Mimic Disrupts the Dimerization of an EGFR-JM Construct.

To determine whether a pHLIPJMA conjugate can compete for EGFR homodimerization in native membrane, we first used the dominant negative AraC-based transcriptional reporter assay (DN-AraTM). DN-AraTM allows for the simultaneous measurement of homo- and heterodimerization of receptor domains directly in the cell membrane of *E. coli*.^{42,43} This assay relies on protein chimera containing the receptor TM domain of interest fused to either the *E. coli* transcription factor AraC (which is active at the *araBAD* promoter as a homodimer) or a AraC mutant unable to activate transcription (AraC*). Both chimeras include an N-terminal maltose-binding protein (MBP) fusion that directs chimera insertion in the inner membrane of AraC-deficient *E. coli* (SB1676). Homodimerization of AraC resulting from receptor domain self-association induces the expression of green fluorescent protein (GFP). Importantly, DN-AraTM does not use the TM domain as a surrogate signal peptide for insertion in the membrane, allowing for the study of receptor domains that include not only the TM domain but also extracellular and cytoplasmic regions.^{44,45} Thus, it also allows for the use of pHLIP as the TM domain in combination with the cytoplasmic JM regions of the target receptor. It is worth noting that pHLIP is derived from the third TM helix of the integral membrane protein bacteriorhodopsin and can consequently adopt a TM conformation without the need for lowering pH if part of a larger protein.^{46,47} In this approach, pHLIP is therefore an integral part of the tested construct.

Briefly, the DNA sequence coding for the TM domain and the first 20 cytoplasmic JM residues of EGFR (Figure 2A, EGFR_TM20) was subcloned in-frame with MBP and AraC (or AraC*) into the pAraTMwt (or pAraTMDN) plasmids (kind gifts of Prof. Berger, The University of Virginia). Similarly, the sequence coding for the first 20 JM residues of EGFR was subcloned in-frame at the 3'-end of the sequence coding for pHLIP (Figure 2A, pHLIP_JM_{wt}). The JMA domain of EGFR contains hydrophobic residues in an *i, i + 3, i + 4* pattern within an LRRL motif. The LxxLL motif forms an α -helix in which Leu 655, Leu 658, and Leu 659 form a hydrophobic interface between helices to stabilize the antiparallel interaction.⁴⁸ Because the LxxLL motif is necessary for EGFR dimerization and activity,²² we expected that the EGFR homodimer will be disrupted by pHLIP-JM_{wt} but not by a construct in which the LRRL motif is mutated to AAAAA (Figure 2A, pHLIP_JM_{Ala}).

As expected, when EGFR_TM20 was expressed as a fusion to AraC, a strong homodimer signal was observed (Figure 2B, #2). This increase is higher than that of the Integrin α_{IIb} L980A TM-CYTO construct, which was tested by Su et al. under the same conditions, and was shown to exhibit strong homodimer-forming tendency (Figure 2B, #1).⁴² When the EGFR_TM20-AraC* fusion was coexpressed as a competitor to the EGFR_TM20-AraC fusion, a significant decrease (2-fold) in GFP expression was observed, which is also

consistent with specific homodimer formation (Figure 2B, #3). Conversely, the pHLIP_JM_{wt} construct did not show extensive propensity for self-association, as evident by low GFP fluorescence (Figure 2B, #5 and #6). Low self-association is beneficial to pHLIP_JM_{wt} potential activity, as it would make it more readily available to interact with EGFR_TM20 as a monomer.

Importantly, when the pHLIP_JM_{wt}-AraC* fusion was coexpressed as a competitor to the EGFR_TM20-AraC fusion, a 2-fold decrease in GFP level was observed, indicative of heterodimerization (Figure 2B, #2 vs #8). However, when pHLIP_JM_{Ala}-AraC* was coexpressed with EGFR_TM20-AraC, a negligible decrease in fluorescence was observed relative to the EGFR_TM20 homodimer (Figure 2B, #9), confirming the role of the JM_{wt} domain in EGFR_TM20 dimerization and demonstrating that pHLIP_JM_{wt} can disrupt the EGFR dimer through specific interactions with its JM domain.

Immunoblotting and maltose complementation assay showed that all constructs are expressed at similar levels (Figure 2B, inset) and properly inserted in the membrane (Figure S1),⁴² respectively. These verify that the variation in the fluorescence signal was solely due to changes in dimerization state of the constructs.

Synthesis and Interaction of pHLIP-JMA with Lipid Bilayers.

In light of our results with DN-AraTM, we synthesized an EGFR peptide mimic comprising the LRLL motif (H₂N-TLRLLQ-CONH₂, referred subsequently to as JMA) by standard Fmoc solid-phase synthesis and conjugated it to a cysteine at the C-terminal of pHLIP via a nonreducible thioether linkage (Scheme S1, pHLIP-JMA). pHLIP-JMA was purified by RP-HPLC, and its identity was confirmed via MALDI-TOF MS (see Methods). By utilizing this linking strategy, pHLIP can translocate the peptide mimic across the membrane of cells with an acidic extracellular environment found in tumors^{49,50} and persist on the membrane, allowing it to bind to the JM domain of EGFR and disrupt dimerization and signaling. Because the leucines of the LxxLL motif form the binding interface between interacting helices of the JMA domain, a peptide in which the three leucines were replaced with alanines (H₂N-TARRAQ-CONH₂, referred subsequently to as JMA_{Ala}) was prepared and conjugated to pHLIP under the same conditions (pHLIP-JMA_{Ala}) as a control. Fluorescence and far-UV circular dichroism (CD) spectroscopy were used to monitor the interactions of pHLIPJMA and pHLIP-JMA_{Ala} with lipid bilayers. Results show that conjugation of the peptide mimic at the C-terminus of pHLIP does not significantly disrupt the pH-mediated insertion of pHLIP and that pHLIP-JMA and pHLIP-JMA_{Ala} adopt a stable TM α -helix conformation upon pH reduction (Figure S2).

pHLIP-JMA Inhibits Cancer Cell Viability.

One key cellular function mediated by the activity of EGFR is cellular proliferation.⁵¹ Therefore, inhibiting EGFR activation should result in a decrease in cell viability in only EGFR-dependent cell lines. Moreover, because the hypothesized mechanism, by which pHLIP-JMA inhibits EGFR activity, is through the interaction with the JM domain of EGFR and not with the kinase domain, pHLIP-JMA should evoke a decrease in cell viability of cells expressing wild-type EGFR as well as cells harboring EGFR activation and resistance

mutations. Therefore, we tested pHLIP-JMA against three EGFR-dependent cancer cell lines: (1) H1650 lung cancer cells that express EGFR with the activating in-frame deletion mutation (delE746-A750) but are sensitive to TKI, (2) H1975 lung cancer cells that express EGFR with the double mutation (L858R/T790M), resulting in receptor activation and resistance to TKI,⁵² and (3) HeLa cervical cancer cells that express wild-type EGFR. MDA-MB-231 breast cancer cells served as a control because they lack a proliferative response to the ligand epidermal growth factor (EGF)⁵³ (Figure S3) and thus should not be susceptible to an EGFR-dependent drug.

Cells were treated with concentrations ranging from 2.5 nM to 10 μ M of pHLIP-JMA at pH 7.4 or 5.0. The cells were then washed once and cultured for 72 h in complete media at physiological pH at 37 °C, after which cell viability was assessed with the MTT assay. Remarkably, despite the short treatment time,^{27,54} a concentration-dependent decrease in cell viability was observed in all EGFR-dependent cell lines (Figure 3A–C). This suggested that the JM peptide mimic was being selectively translocated across the plasma membrane in a pH-mediated manner by pHLIP. As hypothesized, pHLIP-JMA was effective in decreasing cell viability of cells expressing WT EGFR (HeLa) as well as cells expressing EGFR with either activation (H1650) or resistance mutations (H1975), suggesting that the pHLIP-JMA mechanism of action is through an interaction of the JM domain. In addition, the cytotoxic effect was pH-selective, as no decrease in cell viability was observed when cells were treated with pHLIP-JMA at pH 7.4 (Figure 3A–C, blue lines). The low pH treatment had only a minor effect on the cell viability of the various cell lines, as shown by treatment without pHLIP-JMA (Figure 3, 0 μ M controls). This is consistent with what we observed previously.^{30,41,55,56} The decrease in cell viability was not due to pHLIP insertion itself^{30,55} as no toxicity was observed when the cells were treated with 10 μ M pHLIP (Figure S4A) treated under the same conditions. The JMA peptide alone did not have any inhibitory effects on cell viability either, which is consistent with expectations that the JMA peptide would be unable to cross the plasma membrane on its own (Figure S4B). As hypothesized, when EGFR-dependent cell lines were treated with 10 μ M pHLIP-JMA_{Ala}, no decrease in cell viability was observed (Figure S4C), demonstrating the necessity of the LxxLL motif in the JMA sequence for the activity of the pHLIP conjugate. We also evaluated whether pHLIP-JMA may cause cytotoxicity through the disruption of the plasma membrane by monitoring the release of the intracellular enzyme lactate dehydrogenase (LDH) from damaged cells.^{57,58} Neither pHLIP-JMA nor low pH treatment cause significant release of LDH, as compared to spontaneous LDH release, indicating that the conjugate does not cause dramatic disruption of membrane integrity, which is consistent with our previous studies (Figure S5).^{30,41,55,56}

pHLIP-JMA Inhibits Cancer Cell Migration.

Enhanced cell migration is another feature of aberrant EGFR activity in cancer cells.⁵⁹ For assessing the ability of pHLIP-JMA to inhibit cellular migration, a scratch assay⁶⁰ was performed on the same three EGFR-dependent cell lines: HeLa, H1650, and H1975 cells. Cells were treated with pHLIP-JMA (10 μ M) and then incubated in media containing EGF at pH 7.4 and 5.0. The cells were washed once, scratched, and imaged ($t = 0$ h). After 24 h in complete media containing EGF (except –EGF control), the scratch was imaged again ($t =$

24 h). The EGFR-dependence of HeLa and H1650 was evident, as normalized closure was increased when the cells were stimulated with the EGF ligand (Figure 4, +EGF) as compared to in the absence of the EGF ligand (Figure S6, -EGF). H1975 cells harboring the double mutation L858R/T790M showed no difference in closure in the presence of EGF. This result was expected as the mutation is known to make EGFR constitutively active in the absence of ligand.^{61,62} Figure 4 shows that pHLIP-JMA treatment at low pH inhibits migration of all three cell lines. This indicated that pHLIP-JMA inhibited the cellular migration response through an interaction with the JMA domain of EGFR and not through the kinase domain, consistent with the results obtained with cell viability assays (Figure 3). The inhibitory effect on cellular migration was pH-selective as treatment with pHLIP-JMA at pH 7.4 had no effect (Figure 4, blue bars) and the low pH environment did not impact cell migration (Figure 4, +EGF, red bar). Consistent with cell viability assays, treatment with 10 μ M of the control peptides JMA, pHLIP, or pHLIP-JMA_{Ala} had no inhibitory effect on cell migration (Figure S6). Taken together, these results indicate that pHLIP-JMA inhibited the activity of EGFR through an interaction with the JM domain, as demonstrated by a decrease in cell viability and cell migration.

pHLIP-JMA Inhibits EGFR Phosphorylation and Downstream Signaling.

For probing whether the effect of pHLIP-JMA on the viability and migration of EGFR-dependent cancer cells correlated with EGFR activity, immunoblotting was used to measure the differences in EGFR phosphorylation upon treatment with pHLIP-JMA. HeLa cells (WT EGFR) were serum starved for 2 h and then treated with pHLIP-JMA (10 μ M) for 10 min followed by EGF treatment for (10 ng/mL) for 7.5 min. After treatment, cell lysates were collected and analyzed by immunoblotting. A change in phosphorylation at tyrosine 1068 (Y1068) was observed (Figure 5A and B). As expected, the activity of EGFR is downregulated in the absence of the EGF ligand, as evident by minimal phosphorylation (Figure 5B; -EGF) (Figure 5B; +EGF). However, when EGF ligand was added, EGFR became activated, and a large increase in phosphorylation occurred. The phosphorylation levels remained relatively unchanged between pH 5.0 and 7.4 treatments, indicating that the low pH treatment had minimal effect on EGFR activity. Neither the low pH environment nor the peptide treatment influenced total EGFR levels, which remained unchanged under every condition (Figure 5A; +EGF). Notably, a pH-selective reduction of EGFR phosphorylation occurred only when cells were treated with pHLIP-JMA; a 1.5-fold reduction in phosphorylation was observed at pH 5.0 as compared to pH 7.4 (Figure 5B). In addition, the inhibition of EGFR phosphorylation is not due to pHLIP insertion itself nor the JMA peptide alone because levels remain unchanged at both pH 5.0 and 7.4 when cells were treated with pHLIP or JMA alone (Figure S7A and B). The significance of the JMA sequence was demonstrated as no change in phosphorylation at Y1068 was observed at pH 5.0 as compared to treatment at pH 7.4 when the cells were treated with pHLIP-JMA_{Ala} (Figure S7B).

Activation of EGFR initiates multiple signaling cascades, including activation of the serine/threonine kinase Akt, which mediates several downstream responses, such as angiogenesis, tumorigenesis, and inhibition of apoptosis.⁵⁹ For assessing whether pHLIP-JMA inhibits phosphorylation of downstream targets, samples were analyzed by immunoblotting against

Akt and phospho-Akt at serine 473 (S473) (Figure 5A and C). HeLa cells treated with pHLIP-JMA showed the same pattern of phosphorylation changes as observed for EGFR: a decrease in phosphorylation at S473 was observed at pH 5.0, as compared to treatment at pH 7.4, whereas total Akt levels were unaffected (Figure 5C). When the cells were treated under the same conditions with pHLIP, JMA, and pHLIP-JMA_{Ala}, no pH-dependent change in phosphorylation was observed (Figure S7C). Taken together, these results indicate that pHLIP-JMA decreases EGFR autophosphorylation and inhibits downstream signaling by selectively interacting with the JM domain of EGFR.

Conclusions.

In conclusion, we have shown that pHLIP provides a vehicle to selectively deliver a cell-impermeable JM peptide mimic of EGFR into cells. The pHLIP-JMA conjugate reduces cancer cell proliferation and migration in a pH-dependent manner by inhibiting EGFR dimerization and phosphorylation. This strategy allows the translocation of a polar peptide mimic and offers the considerable advantage of being selective toward tumors, providing a highly efficacious, low side-effect treatment. We envision that our approach could be extended beyond EGFR and be applied to other single-span membrane receptors in which the JM domain plays a role in receptor activity.

METHODS

DN-AraTM Assay.

The DNA sequence coding for the TM domain of EGFR and the first 20 cytoplasmic JM residues of EGFR (EGFR_TM20) were cloned into the unique plasmids (kind gifts of Bryan Berger, The University of Virginia) containing the receptor domains in AraC (pAraTMwt) and AraC* (pAraTMDN). For pHLIP_JM_{wt}, the sequence coding for the first 20 JM residues of EGFR was subcloned in-frame at the 3'-end of the sequence coding for pHLIP. As a control, the sequence coding for the first 20 JM residues of EGFR in which the LRLL motif was mutated to AAAAA was subcloned in-frame at the 3'-end of the sequence coding for pHLIP (pHLIP_JM_{Ala}). The constructs, and the reporter plasmid (pAraGFPCDF), were cotransformed into the AraC-deficient *E. coli* strain SB1676 and streaked onto selective plates. One colony was picked from each construct and grown in 1 mL of LB media for 16 h at 30 °C and 250 rpm. Each culture was diluted to A₆₀₀ ≈ 1.3 into three wells, each containing 300 μL of selective LB media. The culture was induced with 1 mM isopropyl β-D-thiogalactoside and grown for an additional 6 h at 30 °C and 250 rpm. A black 96-well, clear-bottom plate was used to prepare a series 2-fold dilutions of the cultures with a final volume of 100 μL. Absorption at 580 (10) nm and GFP fluorescence emission spectra (excitation maximum 485 (20) nm and emission maximum at 530 (30) nm) were collected using an Infinite 200 PRO Plate Reader (Tecan). The results are reported as the ratio of fluorescence emission at 530 nm to absorbance at 580 nm and normalized to the negative control (empty plasmids and reporter plasmid). Immunoblotting was performed using HRP-conjugated antimaltose binding protein (MBP) monoclonal antibody (New England Biolabs, #E8038) to verify equal expression levels of each construct.

Cell Culture.

Human cervical adenocarcinoma HeLa and human breast adenocarcinoma MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% FBS, 100 units/ml penicillin, and 0.1 mg/mL of streptomycin. Human lung adenocarcinoma H1975 cells and human lung adenocarcinoma H1650 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented 10% FBS, 100 units/ml penicillin, and 0.1 mg/mL of streptomycin. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

Cell Viability Assay.

HeLa, H1650, H1975, and MDA-MB-231 cells were plated in 96-well plates at a cell density necessary to reach confluency after 72 h. Peptide constructs were solubilized in an appropriate volume of serum-starved media (media without FBS, pH 7.4) so that upon pH adjustment the desired treatment concentration (10 μ M) is obtained. The samples were then gently sonicated for 30–60 s using a bath sonicator (Branson Ultrasonics). After removal of cell media, the constructs were added to the appropriate wells and allowed to incubate for 5–10 min at 37 °C. Then, the media was adjusted to the desired pH (final volume = 50 μ L) using a pre-established volume of media buffered with citric acid, pH 2.0, and incubated for 1–2 h depending on the cell line. Following the treatment, the plate was washed once with 100 μ L of complete media and then recovered for 72 h at 37 °C in 100 μ L of complete media. Cell viability was assessed with the MTT colorimetric assay. Briefly, MTT was solubilized in PBS (10 mg/mL) with brief sonication, and 10 μ L was added to each well. After incubation for 2 h at 37 °C, the formazan crystals were solubilized in 200 μ L of dimethyl sulfoxide (DMSO), and the absorbance at 580 nm was measured using an Infinite F200 PRO microplate reader (Tecan). Cell viability was normalized to control cells treated with media at pH 7.4.

Scratch Assay.

HeLa, H1650, and H1975 cells were seeded in 6-well plates at a cell density necessary to reach confluency after 24 h. Constructs were prepared as previously described for cell viability assays, added to the appropriate wells, and incubated at 37 °C for 5 min. The pH was then adjusted (final volume = 500 μ L) as described for the cell viability assay, and the constructs were incubated for 1–2 h (depending on cell line), after which a scratch was made in the confluent cell monolayer with a 200 μ L pipet tip to create a thin gap. The cells were immediately washed once with complete media, and then phase contrast images were taken ($t = 0$ h). The cells were incubated with complete media containing EGF (100 ng/mL) to stimulate scratch closure or lacking EGF (control) at 37 °C for 24 h at which point phase contrast images were taken ($t = 24$ h) with a 10 $\times$ objective using an Eclipse Ti-S microscope. Scratch areas were quantified with ImageJ using the wound healing tool, and the closure percent was found by calculating the percent change in area between 0 and 24 h. Percent scratch closure was normalized to control cells treated with media containing EGF (100 ng/mL) at pH 7.4.

Cell Treatment for Immunoblot Analyses.

HeLa cells were plated in 24-well plates at 200,000 cells/well and incubated overnight. Two hours before treatment, cell media was replaced with serum-starved media. Constructs were prepared as previously described for cell viability assays, added to the appropriate wells, and incubated at 37 °C for 5 min. The pH was then adjusted as previously described (final volume = 200 μ L). After 10 min at 37 °C, treatment medium was removed and replaced with 500 μ L of serum starvation medium with EGF (10 ng/mL) and incubated for 7.5 min at 37 °C. Cells were solubilized by the addition of 200 μ L of SDS-PAGE sample loading buffer (1 $\times$), then removed and analyzed by immunoblot analysis.

Immunoblot Analyses.

Samples were boiled for 10 min at 95 °C and resolved by SDS-PAGE on a 10% tris-glycine gel. Subsequently, the samples were transferred onto a 0.45 μ m nitrocellulose membrane (GE Healthcare #1060002) at 100 V for 1 h at 4 °C. Membranes were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline Tween 20 (TBS-T) for 1 h at RT and then blotted for phosphorylated proteins (Cell Signaling Technology; phospho-EGFR Y1068 #3777, phospho-Akt S473 #4058). When blotting for total protein levels (Cell Signaling Technology; EGFR #4267, Akt (pan) #2920, β -Actin #3700), the membranes were blocked with 5% dry milk in TBS-T for 1 h at RT. Following blocking, the cells were incubated with primary antibodies in 5% BSA TBS overnight at 4 °C (pEGFR and pAkt at 1:1000 dilution, total EGFR and total Akt 1:2000, β -Actin 1:4000). Cells were washed 4 times with TBS-T and then incubated with the appropriate secondary antibodies in TBS-T for 30 min at RT at 1:4000 dilution (Cell Signaling Technology, antirabbit #7074, antimouse #7076). Following 5–6 washes with TBS-T, the immunoblot was visualized by chemiluminescence after incubation with Clarity Western ECL Substrate (Bio-Rad #1705061). Images were quantified using ImageJ 1.49v and plotted as the normalized (ratio of phosphorylated to total intensity) mean values. Phosphor-ylation was normalized to control cells treated with media containing EGF (10 ng/mL) at pH 7.4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Health [Grant R21CA181868] and start-up funds from Lehigh University to D.T.

ABBREVIATIONS

BSA	bovine serum albumin
CD	circular dichroism
DNArATM	dominant negative AraC-based transcriptional reporter assay
DMEM	Dulbecco's modified Eagle's medium

DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GFP	green fluorescent protein
JM	juxtamembrane
LB	lysogeny broth
LDH	lactate dehydrogenase
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MBP	maltose-binding protein
MS	mass spectroscopy
PAR1	protease-activated receptor 1
pHLIP	pH(low) insertion peptide
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
RT	room temperature
RP-HPLC	reverse-phase high-performance liquid chromatography
RPMI	Roswell Park Memorial Institute
S473	serine 473
TBS-T	tris-buffered saline Tween 20
TM	transmembrane
TKIs	tyrosine kinase inhibitors
Trp	tryptophan
Y1068	tyrosine 1068
WT	wild-type

REFERENCES

- (1.) Yarden Y, and Pines G (2012) The ERBB network: at last, cancer therapy meets systems biology. *Nat. Rev. Cancer* 12, 553–563. [PubMed: 22785351]
- (2.) De Luca A, Carotenuto A, Rachiglio A, Gallo M, Maiello MR, Aldinucci D, Pinto A, and Normanno N (2008) The role of the EGFR signaling in tumor microenvironment. *J. Cell. Physiol* 214, 559–567. [PubMed: 17894407]
- (3.) Lemmon MA, Schlessinger J, and Ferguson KM (2014) The EGFR family: not so prototypical receptor tyrosine kinases. *Cold Spring Harbor Perspect. Biol* 6, a020768.

- (4.) Yewale C, Baradia D, Vhora I, Patil S, and Misra A (2013) Epidermal growth factor receptor targeting in cancer: a review of trends and strategies. *Biomaterials* 34, 8690–8707. [PubMed: 23953842]
- (5.) Mok TSK (2011) Personalized medicine in lung cancer: what we need to know. *Nat. Rev. Clin. Oncol* 8, 661–668. [PubMed: 21862980]
- (6.) Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, and Schlessinger J (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 313, 144–147. [PubMed: 2981413]
- (7.) Spano JP, Lagorce C, Atlan D, Milano G, Domont J, Benamouzig R, Attar A, Benichou J, Martin A, Morere JF, Raphael M, Penault-Llorca F, Breau JL, Fagard R, Khayat D, and Wind P (2005) Impact of EGFR expression on colorectal cancer patient prognosis and survival. *Ann. Oncol* 16, 102–108. [PubMed: 15598946]
- (8.) Ang KK, Berkey BA, Tu X, Zhang H-Z, Katz R, Hammond EH, Fu KK, and Milas L (2002) Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 62, 7350–7356. [PubMed: 12499279]
- (9.) Wong S-F (2005) Cetuximab: an epidermal growth factor receptor monoclonal antibody for the treatment of colorectal cancer. *Clin. Ther* 27, 684–694. [PubMed: 16117976]
- (10.) Yang XD, Jia XC, Corvalan JR, Wang P, and Davis CG (2001) Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit. Rev. Oncol. Hematol* 38, 17–23. [PubMed: 11255078]
- (11.) Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, and Van Cutsem E (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N. Engl. J. Med* 351, 337–345. [PubMed: 15269313]
- (12.) Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, Siena S, and Bardelli A (2007) Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 67, 2643–2648. [PubMed: 17363584]
- (13.) Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, and Gibson KH (2002) ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* 62, 5749–5754. [PubMed: 12384534]
- (14.) Moyer JD, Barbacci EG, Iwata KK, Arnold L, Boman B, Cunningham A, DiOrio C, Doty J, Morin MJ, Moyer MP, Neveu M, Pollack VA, Pustilnik LR, Reynolds MM, Sloan D, Theleman A, and Miller P (1997) Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 57, 4838–4848. [PubMed: 9354447]
- (15.) Minkovsky N, and Berezov A (2008) BIBW-2992, a dual receptor tyrosine kinase inhibitor for the treatment of solid tumors. *Curr. Opin Investig Drugs* 9, 1336–1346.
- (16.) Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S, and Nukiwa T (2010) Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N. Engl. J. Med* 362, 2380–2388. [PubMed: 20573926]
- (17.) Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, and Parulekar W (2007) Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J. Clin. Oncol* 25, 1960–1966. [PubMed: 17452677]
- (18.) Yun C-H, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong K-K, Meyerson M, and Eck MJ (2008) The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc. Natl. Acad. Sci. U. S. A* 105, 2070–2075. [PubMed: 18227510]
- (19.) Deng W, and Li R (2015) Juxtamembrane contribution to transmembrane signaling. *Biopolymers* 104, 317–322. [PubMed: 25846274]

- (20.)) Macdonald-Obermann JL, and Pike LJ (2009) The intracellular juxtamembrane domain of the epidermal growth factor (EGF) receptor is responsible for the allosteric regulation of EGF binding. *J. Biol. Chem* 284, 13570–13576. [PubMed: 19336395]
- (21.)) Thiel KW, and Carpenter G (2007) Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. *Proc. Natl. Acad. Sci. U. S. A* 104, 19238–19243. [PubMed: 18042729]
- (22.)) Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, Wemmer DE, Zhang X, and Kuriyan J (2009) Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. *Cell* 137, 1293–1307. [PubMed: 19563760]
- (23.)) Red Brewer M, Choi SH, Alvarado D, Moravcevic K, Pozzi A, Lemmon MA, and Carpenter G (2009) The juxtamembrane region of the EGF receptor functions as an activation domain. *Mol. Cell* 34, 641–651. [PubMed: 19560417]
- (24.)) He L, and Hristova K (2012) Consequences of replacing EGFR juxtamembrane domain with an unstructured sequence. *Sci. Rep* 2 (2), 854. [PubMed: 23152945]
- (25.)) Boran ADW, Seco J, Jayaraman V, Jayaraman G, Zhao S, Reddy S, Chen Y, and Iyengar R (2012) A potential peptide therapeutic derived from the juxtamembrane domain of the epidermal growth factor receptor. *PLoS One* 7, No. e49702. [PubMed: 23166750]
- (26.)) Luedtke NW, Dexter RJ, Fried DB, and Schepartz A (2007) Surveying polypeptide and protein domain conformation and association with FIAsh and ReAsH. *Nat. Chem. Biol* 3, 779–784. [PubMed: 17982447]
- (27.)) Sinclair JKL, Denton EV, and Schepartz A (2014) Inhibiting epidermal growth factor receptor at a distance. *J. Am. Chem. Soc* 136, 11232–11235. [PubMed: 25075632]
- (28.)) Thévenin D, An M, and Engelman DM (2009) pHLP-mediated translocation of membrane-impermeable molecules into cells. *Chem. Biol* 16, 754–762. [PubMed: 19635412]
- (29.)) An M, Wijesinghe D, Andreev OA, Reshetnyak YK, and Engelman DM (2010) pH-(low)-insertion-peptide (pHLP) translocation of membrane impermeable phalloidin toxin inhibits cancer cell proliferation. *Proc. Natl. Acad. Sci. U. S. A* 107, 20246–20250. [PubMed: 21048084]
- (30.)) Burns KE, Robinson MK, and Thévenin D (2015) Inhibition of cancer cell proliferation and breast tumor targeting of pHLP-monomethyl auristatin E conjugates. *Mol. Pharmaceutics* 12, 1250–1258.
- (31.)) Helmlinger G, Sckell A, Dellian M, Forbes NS, and Jain RK (2002) Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism. *Clin. Cancer Res* 8, 1284–1291. [PubMed: 11948144]
- (32.)) Seyfried TN, and Shelton LM (2010) Cancer as a metabolic disease. *Nutr. Metab* 7, 7.
- (33.)) Hanahan D, and Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674. [PubMed: 21376230]
- (34.)) Zhang X, Lin Y, and Gillies RJ (2010) Tumor pH and its measurement. *J. Nucl. Med* 51, 1167–1170. [PubMed: 20660380]
- (35.)) Wike-Hooley JL, Haveman J, and Reinhold HS (1984) The relevance of tumour pH to the treatment of malignant disease. *Radiother. Oncol* 2, 343–366. [PubMed: 6097949]
- (36.)) Vaupel P, Kallinowski F, and Okunieff P (1989) Blood Flow, Oxygen and Nutrient Supply, and Metabolic Microenvironment of Human Tumors: A Review. *Cancer Res* 49, 6449–6465. [PubMed: 2684393]
- (37.)) Anderson M, Moshnikova A, Engelman DM, Reshetnyak YK, and Andreev OA (2016) Probe for the measurement of cell surface pH in vivo and ex vivo. *Proc. Natl. Acad. Sci. U. S. A* 113, 8177–8181. [PubMed: 27382181]
- (38.)) Bailey KM, Wojtkowiak JW, Hashim AI, and Gillies RJ (2012) Targeting the metabolic microenvironment of tumors. *Adv. Pharmacol* 65, 63–107. [PubMed: 22959024]
- (39.)) Reshetnyak YK, Andreev OA, Lehnert U, and Engelman DM (2006) Translocation of molecules into cells by pH-dependent insertion of a transmembrane helix. *Proc. Natl. Acad. Sci. U. S. A* 103, 6460–6465. [PubMed: 16608910]
- (40.)) Zoonens M, Reshetnyak YK, and Engelman DM (2008) Bilayer interactions of pHLP, a peptide that can deliver drugs and target tumors. *Biophys. J* 95, 225–235. [PubMed: 18359793]

- (41).) Burns KE, and Thévenin D (2015) Down-regulation of PAR1 activity with a pHLIP-based allosteric antagonist induces cancer cell death. *Biochem. J* 472, 287–295. [PubMed: 26424552]
- (42).) Su P-C, and Berger BW (2012) Identifying key juxtamembrane interactions in cell membranes using AraC-based transcriptional reporter assay (AraTM). *J. Biol. Chem* 287, 31515–31526. [PubMed: 22822084]
- (43).) Su P-C, and Berger BW (2013) A novel assay for assessing juxtamembrane and transmembrane domain interactions important for receptor heterodimerization. *J. Mol. Biol* 425, 4652–4658. [PubMed: 23876708]
- (44).) Barton R, Palacio D, Iovine MK, and Berger BW (2015) A cytosolic juxtamembrane interface modulates plexin A3 oligomerization and signal transduction. *PLoS One* 10, No. e0116368. [PubMed: 25565389]
- (45).) Wanganu B, and Berger BW (2016) A specific, transmembrane interface regulates fibroblast activation protein (FAP) homodimerization, trafficking and exopeptidase activity. *Biochim. Biophys. Acta, Biomembr* 1858, 1876–1882.
- (46).) Hunt JF, Earnest TN, Bousché O, Kalghatgi K, Reilly K, Horváth C, Rothschild KJ, and Engelman DM (1997) A biophysical study of integral membrane protein folding. *Biochemistry* 36, 15156–15176. [PubMed: 9398244]
- (47).) Hunt JF, Rath P, Rothschild KJ, and Engelman DM (1997) Spontaneous, pH-dependent membrane insertion of a transbilayer alpha-helix. *Biochemistry* 36, 15177–15192. [PubMed: 9398245]
- (48).) Endres NF, Das R, Smith AW, Arkhipov A, Kovacs E, Huang Y, Pelton JG, Shan Y, Shaw DE, Wemmer DE, Groves JT, and Kuriyan J (2013) Conformational coupling across the plasma membrane in activation of the EGF receptor. *Cell* 152, 543–556. [PubMed: 23374349]
- (49).) Rofstad EK, Mathiesen B, Kindem K, and Galappathi K (2006) Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 66, 6699–6707. [PubMed: 16818644]
- (50).) Gillies RJ, Robey I, and Gatenby RA (2008) Causes and consequences of increased glucose metabolism of cancers. *J. Nucl. Med* 49 (Suppl 2), 24S–42S. [PubMed: 18523064]
- (51).) Starbuck C, and Lauffenburger DA (1992) Mathematical model for the effects of epidermal growth factor receptor trafficking dynamics on fibroblast proliferation responses. *Biotechnol. Prog* 8, 132–143. [PubMed: 1368006]
- (52).) Sordella R, Bell DW, Haber DA, and Settleman J (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 305, 1163–1167. [PubMed: 15284455]
- (53).) Davidson NE, Gelmann EP, Lippman ME, and Dickson RB (1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol. Endocrinol* 1, 216–223. [PubMed: 3502607]
- (54).) Sinclair JKL, and Schepartz A (2014) Influence of macrocyclization on allosteric, juxtamembrane-derived, stapled peptide inhibitors of the epidermal growth factor receptor (EGFR). *Org. Lett* 16, 4916–4919. [PubMed: 25207804]
- (55).) Burns KE, Hensley H, Robinson MK, and Thévenin D (2017) Therapeutic Efficacy of a Family of pHLIP-MMAF Conjugates in Cancer Cells and Mouse Models. *Mol. Pharmaceutics* 14, 415–422.
- (56).) Burns KE, McCleerey TP, and Thévenin D (2016) pH-Selective Cytotoxicity of pHLIP-Antimicrobial Peptide Conjugates. *Sci. Rep* 6, 28465. [PubMed: 27334357]
- (57).) Chan FK-M, Moriwaki K, and De Rosa MJ (2013) Detection of Necrosis by Release of Lactate Dehydrogenase Activity, in *Immune Homeostasis*, pp 65–70, Humana Press, Totowa, NJ.
- (58).) Chan FK-M, Moriwaki K, and De Rosa MJ (2013) Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol. Biol* 979, 65–70. [PubMed: 23397389]
- (59).) Lee J, and Moon C (2011) Current status of experimental therapeutics for head and neck cancer. *Exp. Biol. Med. (London, U. K.)* 236, 375–389.
- (60).) Liang C-C, Park AY, and Guan J-L (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc* 2, 329–333. [PubMed: 17406593]
- (61).) Suda K, Onozato R, Yatabe Y, and Mitsudomi T (2009) EGFR T790M mutation: a double role in lung cancer cell survival? *J. Thorac. Oncol* 4, 1–4. [PubMed: 19096299]

- (62).) Lowder MA, Doerner AE, and Schepartz A (2015) Structural Differences between Wild-Type and Double Mutant EGFR Modulated by Third-Generation Kinase Inhibitors. *J. Am. Chem. Soc* 137, 6456–6459. [PubMed: 25973741]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Schematic representation of the hypothesized effect of pHLIP-JMA on the dimerization state of EGFR. Upon the binding of EGF (orange) to the extracellular domain (green), EGFR undergoes a conformational change, which stabilizes the active form of the homodimer and induces subsequent tyrosine autophosphorylation in the kinase domain (blue).³ The JMA domain (residues 645–663 of EGFR) forms a short α -helix (red) that interacts in an antiparallel manner to stabilize the asymmetric dimer.^{22,23} A peptide mimicking the JMA domain of EGFR could potentially disrupt the antiparallel helical association and interfere with receptor signaling when delivered to tumor cells by pHLIP (purple). The peptide sequences of pHLIP and the EGFR JM membrane helix are shown.

(A) pHLIP_JM_{wt}: AAEQNPIYWARYADWLFTTPLL~~LL~~DLALVVDAD~~EGT~~**RRRHIVRKRTLRRLL**QEREL
 EGFR_TM20: KIPSIATGMVGA~~LLLL~~LVVALGIGLF**MRRRHIVRKRTLRRLL**QEREL
 pHLIP_JM_{Ala}: AAEQNPIYWARYADWLFTTPLL~~LL~~DLALVVDAD~~EGT~~**RRRHIVRKRTAAAA**QEREL

Figure 2.

Juxtamembrane mimic disrupts EGFR_TM20 dimerization. (A) Amino acid sequences of EGFR_TM20, pHLIP_JM_{wt}, and pHLIP_JM_{Ala} constructs used in the AraC-TM assays in which the TM regions are underlined and the amino acids corresponding to the JM region are shown in red. The bolded amino acids highlight the LRRL motif of the JM domain, which has been previously shown to be important for the dimerization of the receptor.²² (B) GFP fluorescence measurements from AraC-TM assays. EGFR_TM20 homodimerization signal (#2) is inhibited 50% by pHLIP_JM_{wt} (#8). The pHLIP_JM_{Ala} construct in which the LRRL motif was mutated to AAAAA (#9) was used as a negative control to demonstrate that EGFR_TM20 dimer is indeed disrupted through the JMA domain. Results are shown as mean $\pm$ SEM ($n = 15-27$). For statistical significance to be assessed, two tailed Student's t -test analyses were performed with 95% confidence level (Prism for Macintosh) (ns: nonsignificant; $P = 0.2833$). (B, inset) Representative immunoblot using anti-MBP monoclonal antibody confirming similar expression of mutant constructs.

Figure 3. pHLIP-JMA inhibits cell viability in a pH- and concentration-dependent manner. Three EGFR-dependent cell lines that differ in EGFR expression and mutational state were treated with pHLIP-JMA; HeLa expresses WT EGFR; H1650 (delE746-A750) has amplified EGFR expression, and H1975 expresses double mutant (L858R/T790M) EGFR. Cells were treated with pHLIP-JMA (10 μ M), washed once with media, and cultured for 72 h in complete medium at physiological pH. Cell viability was assessed with the MTT assay. All measurements were normalized to the media control (0 μ M, pH 7.4) as 100% cell viability in which the error bars represent standard error of the mean ($n = 9-12$). Significantly, when (A) HeLa, (B) H1650, and (C) H1975 were treated with pHLIP-JMA, pH- and concentration-dependent cell growth inhibition was observed with no significant decrease in cell viability at pH 7.4. (D) MDA-MB-231 were used as a control because they are not EGFR dependent, and thus, as expected pHLIP-JMA does not reduce cell viability.

Figure 4. pHLIP-JMA inhibits cell migration in a pH-dependent manner. (A,B) HeLa (WT EGFR), (C,D) H1650 (delE746-A750), and (E,F) H1975 (L858R/T790M) cells were treated with pHLIP-JMA (10 μ M), washed once, and scratched with a 200 μ L pipet tip. The cells were incubated in media containing EGF (100 ng/mL) and imaged after 24 h. Scratch closure was found by calculating the percent change in area between 0 and 24 h, which was then normalized to control cells treated with media containing EGF (100 ng/mL) at pH 7.4. Error bars represent standard error of the mean ($n = 4$). Statistical significance was calculated using unpaired t test (at 95% confidence intervals). Asterisks represent statistically significant differences, where $*p < 0.05$. Representative phase contrast images are shown with tracings added to identify open scratch areas. Notably, a pH-dependent decrease in cell

migration was observed in all three EGFR-dependent cell lines when treated with pHLIP-JMA.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. pHLIP-JMA reduces EGFR phosphorylation and downstream signaling in a pH-dependent manner. (A) Serum-starved HeLa (WT EGFR) cells were treated with pHLIP-JMA (10 μ M) for 10 min followed by EGF treatment (10 ng/mL) for 7.5 min. Cell lysates were collected and analyzed by Western blot for EGFR and phospho-EGFR (Y1068) and Akt and phospho-Akt (S473). The normalized (ratio of phospho to total intensity) data were plotted as mean values from which the error bars represent the standard error of the mean ($n = 3$). Statistical significance was calculated using unpaired t test (at 95% confidence intervals). Asterisks represent statistically significant differences where $*p < 0.05$ and $**p < 0.005$. pHLIP-JMA inhibits phosphorylation of (B) pEGFR (Y1068) and (C) pAKT (S473) in a pH-dependent manner.