SUPPORTING INFORMATION

Inhibiting EGFR Dimerization and Signaling Through Targeted Delivery of a Juxtamembrane Domain Peptide Mimic

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EXTENDED METHODS

Maltose complementation test

Plasmid pAraTM containing the receptor domain of interest were transformed into the MBP-deficient *E coli.* MM39 cells and streaked onto selective lysogeny broth (LB) plates. The following day, one colony was picked from each plate and grown in LB media, from which glycerol stocks were generated. The glycerol stocks were then streaked onto selective M9 minimal plates containing 0.4% (w/v) maltose and incubated at 37°C for three days.

Solid-phase peptide synthesis

pHLIP with a cysteine residue at its C-terminus (H_2N-GGEQNPIYWARYADWLFTTPLLLLDLALLVDADEGTCG-$CONH_2$), JMA (H_2N-TLRRLLQ-$CONH_2$), and JMA_{Ala} (H_2N-TARRAAQ-$CONH_2$) peptides were prepared in our laboratory by Fmoc solid-phase synthesis. A non-cleavable linker (chloro-acetylchloride) was coupled to the JMA/JMA_{Ala} peptide on resin (Scheme S1). Briefly, peptides were purified via reverse-phase high-performance liquid chromatography (RP-HPLC; Phenomenex Luna prep 10 μm 250 x 21.20 mm C8; flow rate 10 ml/min; phase A: water 0.1% TFA; phase B: acetonitrile 0.1% TFA; gradient 60 min from 95/5 A/B to 0/100 A/B). The purity of the peptides was determined by RP-HPLC (Phenomenex Onyx Monolithic 50 x 4.6 mm C18; flow rate 2 ml/min; phase A: water 0.1% TFA; phase B: acetonitrile 0.1% TFA; gradient 45 min from 95/5 A/B to 0/100 A/B), and their identity was confirmed via matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) (Figure S8). pHLIP: purity >95%; calculated ($M+H^+$) = 4212, found ($M+H^+$) = 4212. JMA + Linker: purity >98%; calculated ($M+H^+$) = 976, found ($M+H^+$) = 976. pHLIP-JMA: purity >95%; calculated ($M+H^+$) = 5150, found ($M+H^+$) = 5150. pHLIP-JMA_{Ala}: purity >95%; calculated ($M+H^+$) = 5023, ($M+H^+$) = 5023.

Preparation of pHLIP-JMA construct

Conjugating JMA peptide to pHLIP was achieved by dissolving pHLIP in DMSO followed by the addition of 3 eq. JMA modified with a chloro-acetylchloride linker in DMSO and 50 μL of 1 M Tris buffer, pH 7.0. The solution was mixed at room temperature (RT) for 2–3 hours (Scheme S1). pHLIP-JMA_{Ala} was prepared via the same protocol. The desired pHLIP conjugate was isolated using the same techniques described for the peptides. The purity of the peptide-conjugate was determined by RP-HPLC, as listed and their identity was confirmed by MALDI-TOF MS (Figures S8). The conjugates were quantified at 280 nm by UV/Vis absorbance spectroscopy using the molar absorption coefficient of pHLIP (13940 $M^{-1}{\cdot}cm^{-1}$) and lyophilized in 10^{-8} mole aliquots.

Sample preparation of CD and tryptophan fluorescence measurements

Lyophilized pHLIP-JMA and pHLIP-JMA_{Ala} were resuspended in 5 mM sodium phosphate, pH 8.0, to a concentration of 20 μM and incubated at RT for an hour. The construct were then diluted to 7 μM and, when appropriate, incubated with POPC lipid

vesicles at a peptide-to-lipid molar ratio of 1:300 for 30 minutes at RT. Concentrated HCl was used to adjust the pH to the desired experimental values and the samples were incubated at RT for 30 minutes before spectroscopic experiments.

Tryptophan fluorescence spectroscopy

Fluorescence emission spectra were acquired with Fluorolog-3 Spectrofluorometer (HORIBA) at 25°C. The excitation wavelength was 295 nm to selectively excite Trp residues, and the emission spectrum was measured from 300 to 450 nm. The excitation and emission slits were both set to 10 nm.

CD spectroscopy

Far-UV CD spectra of pHLIP-JMA was recorded on a Jasco J-815 CD spectrometer equipped with a Peltier thermal-controlled cuvette holder (Jasco). Measurements were performed in 0.1 mm quartz cuvette at 25°C with pHLIP-JMA concentration equal to 7 μM. CD intensities are expressed in mean residue molar ellipticity [θ] calculated from the following equation:

$$[\theta]=\frac{\theta_{obs}}{10\times l.c.n}(\text{in degrees cm}^2.\text{dmol}^{-1})$$

where, θ_{obs} is the observed ellipticity in millidegrees, l is the optical path length in centimeters, c is the final molar concentration of the peptides, and n is the number of amino acid residues. Samples were measured in a 0.1 cm path length quartz cuvette and raw data were acquired from 260 nm to 200 nm at 1 nm intervals with a 100 nm/min scan rate, and at least five scans were averaged for each sample. The spectrum of POPC liposomes was subtracted out from all construct samples.

Preparation of POPC liposomes

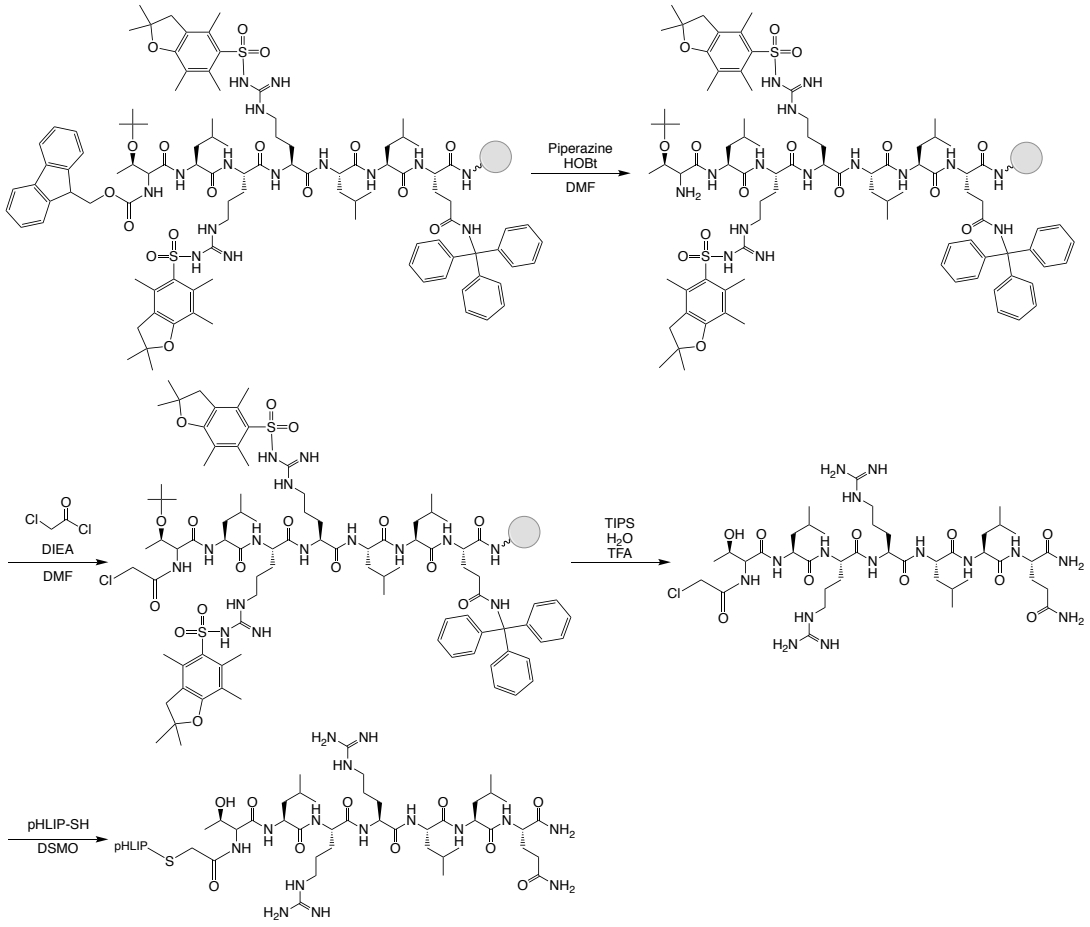
Ten milligrams of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was dried as a thin film and held under vacuum for at least 24 hours. The lipids were then rehydrated in 1 ml of 5 mM sodium phosphate, pH 8.0, for at least 30 minutes with periodic gentle vortexing. The resulting large multilamellar vesicles were freeze-thawed for seven cycles and subsequently extruded through a polycarbonate membrane with 100 nm pores using a Mini-Extruder (Avanti Polar Lipids) to produce large unilamellar vesicles. The Marshall's assay was used to quantify lipid concentration, and the size distribution was verified by dynamic light scattering at a scattering angle of 90° using ALV/GSC-3 goniometer system equipped with ALV/ALV-7004 correlator (ALV-GmbH).

Cell membrane leakage assay

HeLa, H1650, and H1975 cells were plated in 24 well plates at 200,000 cells/well and incubated overnight to reach 100% confluency. Constructs were prepared as previously described for cell viability assays, added to the appropriate wells, and incubated at 37°C for 5–10 minutes. The pH was then adjusted (final volume=200 μL) as described in the Methods section (Cell viability assay). The plate was incubated at 37°C for 1–2 hours.

Following the treatment, the LDH cytotoxicity assay (ThermoFisher, #88953) was performed according to the manufacturer protocol. Briefly, cell media supernatant was transferred to a 96 well plate in triplicate and an equal volume of LDH reaction mixture was added to each well. The plate was incubated for 30 minutes at RT, and then stop solution was added to each well. The absorbance was read at 490 nm and 680 nm on an Infinite® 200 PRO Plate Reader (Tecan). The data was normalized to cells treated with the provided lysis buffer as 100% LDH release.

SUPPORTING FIGURES:



Scheme S1. Synthesis of pHLIP-JMA. The JMA peptide (H_2N-TLRRLLQ-$CONH_2$) was prepared by standard Fmoc solid-phase synthesis on H-Rink amide resin. After deprotection of the C-terminus Fmoc protecting group, a chloro-acetylchloride linker was coupled to the free amine for 3 hours with DIEA to form a stable amide bond. Subsequently, the JMA peptide modified with the linker was cleaved from the resin, purified, and conjugated to pHLIP via the C-terminal cysteine residue in the presence of DIEA for 2–3 hours. pHLIP-JMA_{Ala} was prepared with the same procedure.

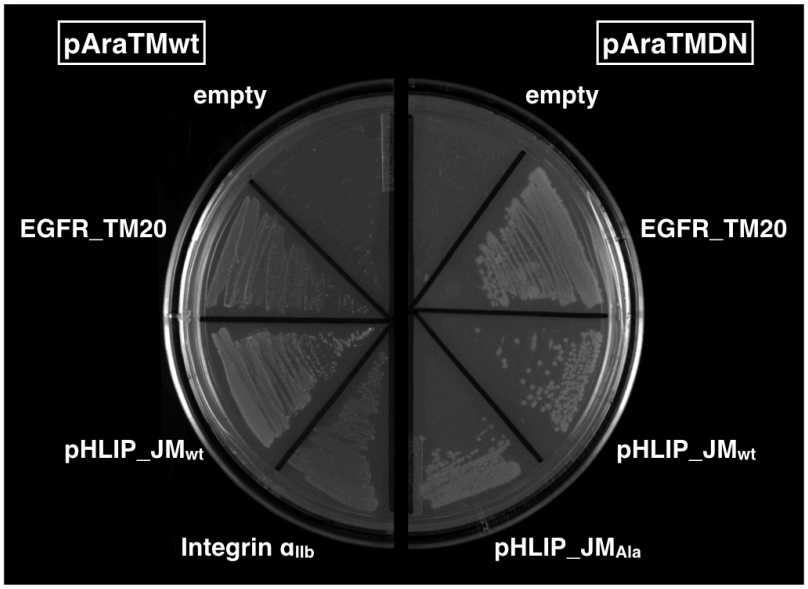


Figure S1. Maltose complementation test. In MM39 cells, which lack endogenous MBP, only chimera that is oriented properly will grow on minimal media. As expected, no growth was observed for the negative control in which no AraC fusion is expressed, but recovery of growth is observed for EGFR_TM20, pHLIP_JM_{wt}, and pHLIP_JM_{Ala} AraC fusion constructs, indicating that they were correctly oriented in the membrane.

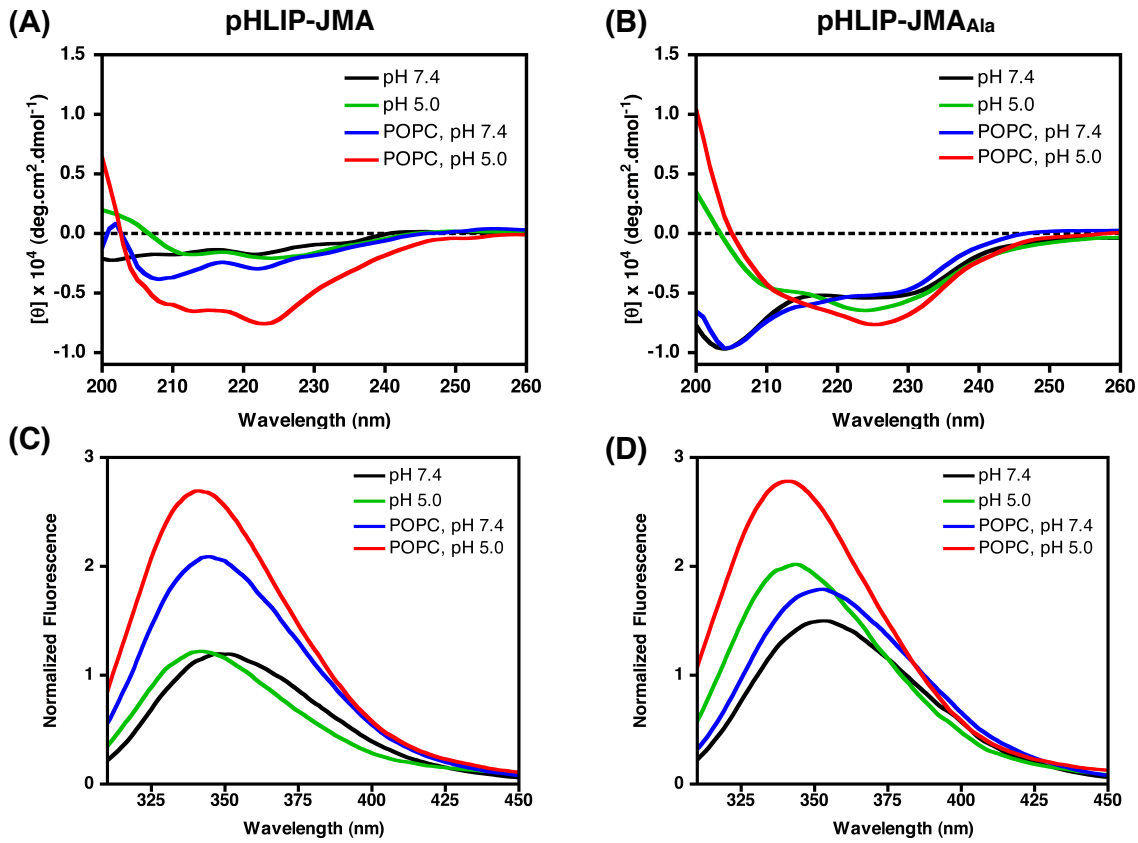


Figure S2. Interaction of pHLIP-JMA and pHLIP-JMA$_{Ala}$ with lipid bilayers. (A,B) CD spectroscopy was used to determine the secondary structure of pHLIP-JMA and pHLIP-JMA$_{Ala}$ (7 μM) in the presence or absence of large unilamellar POPC lipid vesicles. At pH 7.4 (blue line), the conjugates adopt an unstructured configuration, but adopt an α-helical configuration typical of pHLIP upon pH decrease to pH 5.0 (red line). Tryptophan (Trp) fluorescence emission measurements were used to determine whether pHLIP-JMA inserts into the vesicle membrane based on the sensitivity of Trp fluorescence emission to the polarity of the environment (pHLIP has two Trp residues in its sequence). At pH 7.4 (blue line) the emission maximum of (C) pHLIP-JMA is centered at 343 nm indicating that Trp residues are in a relatively polar, aqueous environment. Whereas at pH 5.0 (red line), the emission is blue shifted with a maximum around 339 nm, demonstrating that the Trp residues are in a more hydrophobic environment, indicating that pHLIP-JMA inserts into vesicles. Similar shifts are observed for (D) pHLIP-JMA$_{Ala}$ with an emission maximum centered at 352 nm at pH 7.4 (blue line), and a shift to 341 nm at pH 5.0 (red line). Both constructs also exhibit slight α-helical content and blue-shifted Trp fluorescence emission when incubated at pH 5.0 in the absence of liposomes (green line), suggesting that they may partially aggregate in solution. These results were expected, as it has been previously shown that in the absence of membranes, an acidic environment causes protonation of the aspartic acid residues of pHLIP leading to aggregation.[1,2] Most importantly, we have recently shown that addition of liposomes to aggregated pHLIP causes the dissipation of the aggregates and formation of the inter-facially bound state.[2] These results indicate that, despite its tendency to aggregate in solution, pHLIP can disaggregate after binding to bilayer interfaces and become

available for productive pH-mediated insertion. Finally, it is important to note that, in our experiments, pHLIP conjugates are never present in low pH solution without the presence of membrane (liposomes or cells): They are always pre-incubated in presence of membranes before lowering the pH. Taken together, these results indicate that the presence of JMA and JMA_{Ala} at the C-terminus of pHLIP does not significantly disrupt the pH-mediated insertion of pHLIP, and that the pHLIP conjugates can adopt a stable TM α-helix upon pH reduction. [Peptide]= 7 μM and peptide:lipid ratio = 1:300 with POPC liposomes.

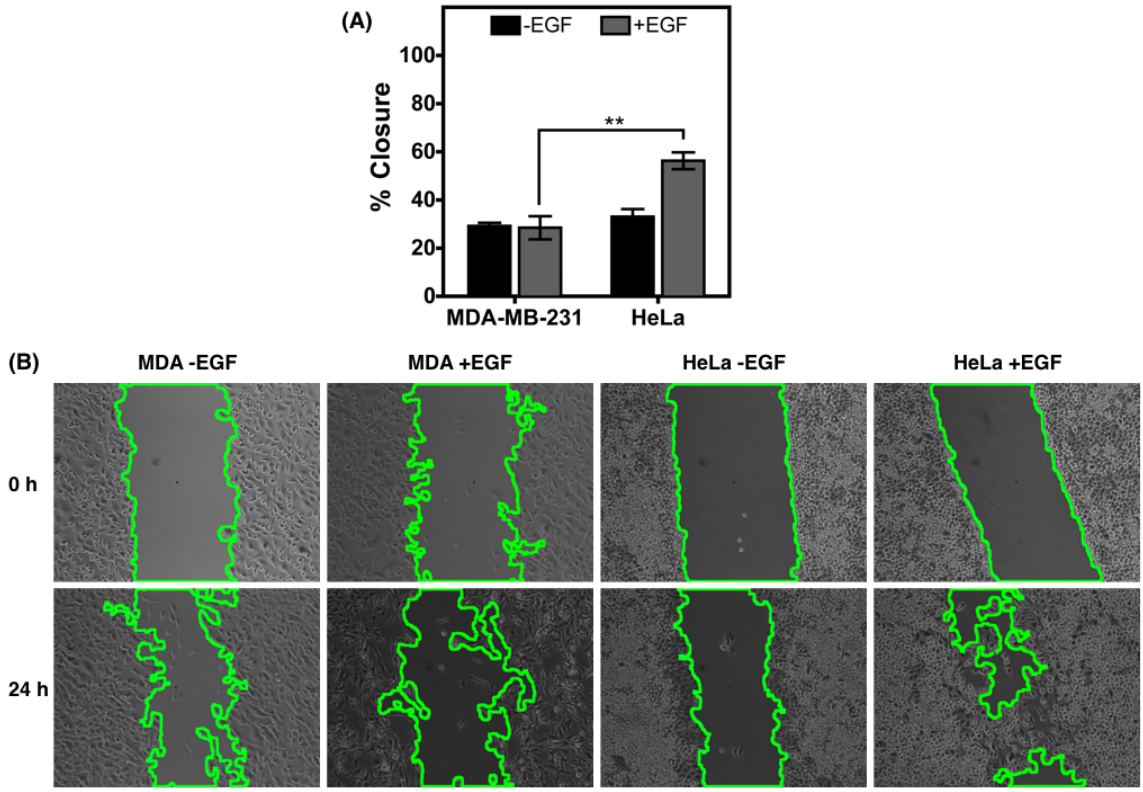


Figure S3. MDA-MB-231 have low EGFR dependence. (A) To assess the ability of EGF to promote cellular migration of MDA-MB-231 a scratch healing assay was performed. HeLa cells were used as a comparison because they are EGFR-dependent. HeLa and MDA-MB-231 cells were grown to confluency in a 6-well plate and starved for 2 hours before being scratched with a 200 μL pipet tip. The cells were washed with PBS and treatment media either containing EGF (100 ng/mL) or lacking EGF (control) was added. Phase contrast images were taken immediately after the initial scratch and after 24 hours of recovery. Scratch areas were quantified with ImageJ using the wound healing tool, and the wound closure % was found by calculating the % change in area between 0 hour and 24 hours. Results are shown as mean ± standard error of the mean (n=4). MDA-MB-231 cells did not display enhanced closure rates upon the addition of EGF, indicating they have low dependence on EGFR. Conversely, EGFR-dependent HeLa showed a ~1.7-fold increase in the presence of EGF as compared to cells incubated in treatment media lacking EGF. Statistical significance was assessed by two tailed Student's *t*-test analyses with 95 % confidence. Asterisks represent statistically significant differences, where $^{**}p < 0.005$. (B) Representative phase contrast images are shown, with tracings added to identify open scratch areas.

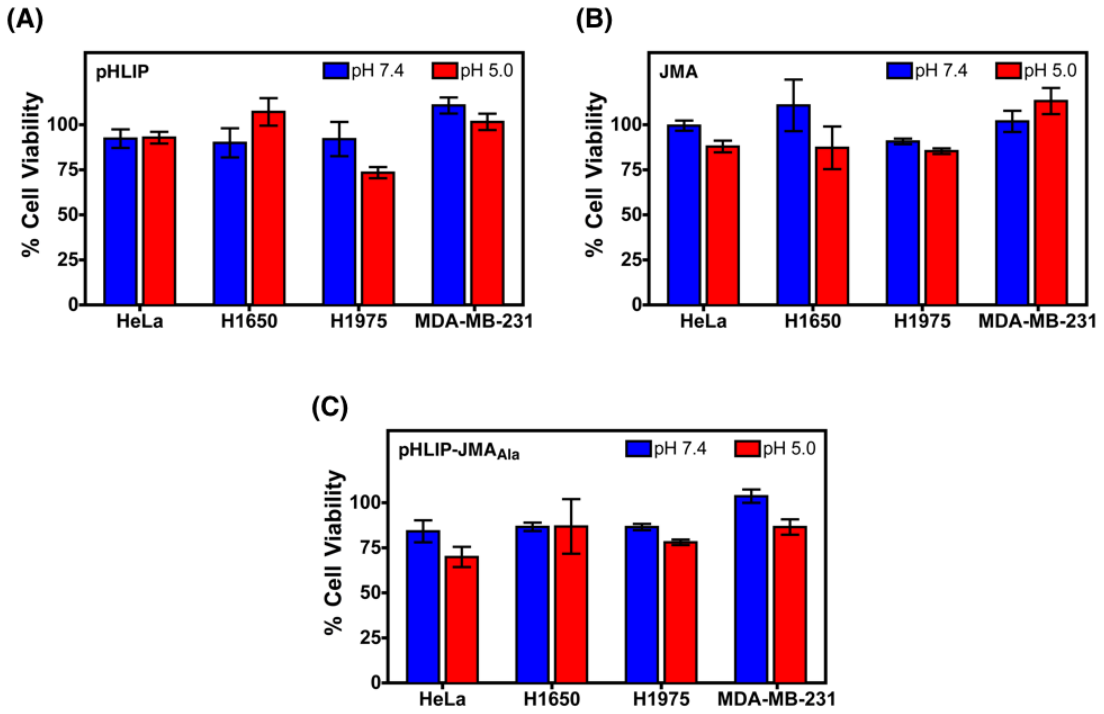


Figure S4. pHLIP, JMA, and pHLIP-JMA$_{Ala}$ are not cytotoxic. (A–C) HeLa, H1650, H1975, and MDA-MB-231 cells were plated in 96 well plates at a cell density necessary to reach confluency after 72 hours. Cell media was removed and replaced with 10 μM of (A) pHLIP, (B) JMA, or (C) pHLIP-JMA$_{Ala}$ and incubated at pH 7.4 or pH 5.0. Cells were washed once with media and grown for 72 hours in complete media at physiological pH, at which point viability was assessed with the MTT assay. All measurements were normalized to the media control (0 μM, pH 7.4), as 100% cell viability. Error bars represent standard error of the mean (n=9–12). No statistically significant ($p > 0.05$) differences were observed between pH 7.4 and pH 5.0 for any peptide, indicating that alone pHLIP, JMA, and pHLIP-JMA$_{Ala}$ are not cytotoxic against all the cell lines tested.

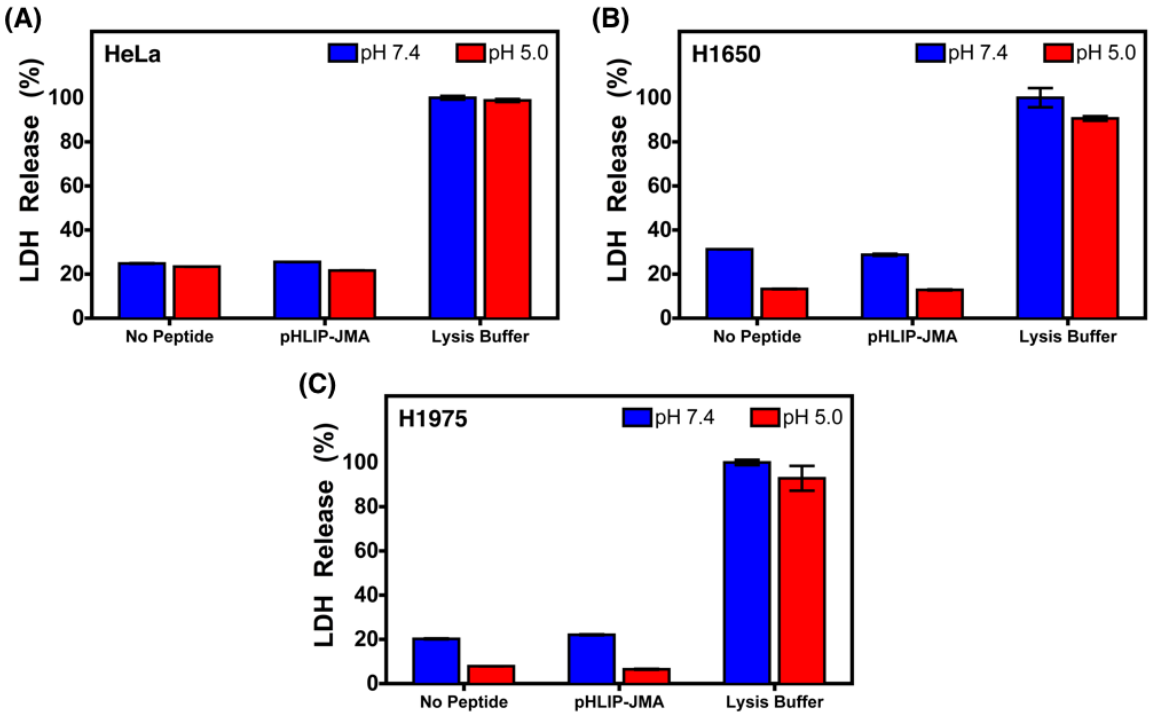


Figure S5. pHLIP-JMA does not cause cell membrane leakage. (A) HeLa, (B) H1650, and (C) H1975 cells were seeded at a density of 200,000 cells/well in a 24 well plate, allowed to adhere overnight, and treated with 10 μM pHLIP-JMA at pH 7.4 (blue bars) or pH 5.0 (red bars). Cell media supernatant was monitored for the presence of LDH following treatment. The data was normalized to cells treated with the provided lysis buffer as 100% LDH release. Error bars represent standard error of the mean (n=3). Neither pHLIP-JMA nor low pH treatment causes statistically significant ($p > 0.05$) release of LDH as compared to cells treated with no peptide, indicating that the conjugate does not cause dramatic disruption membrane integrity.

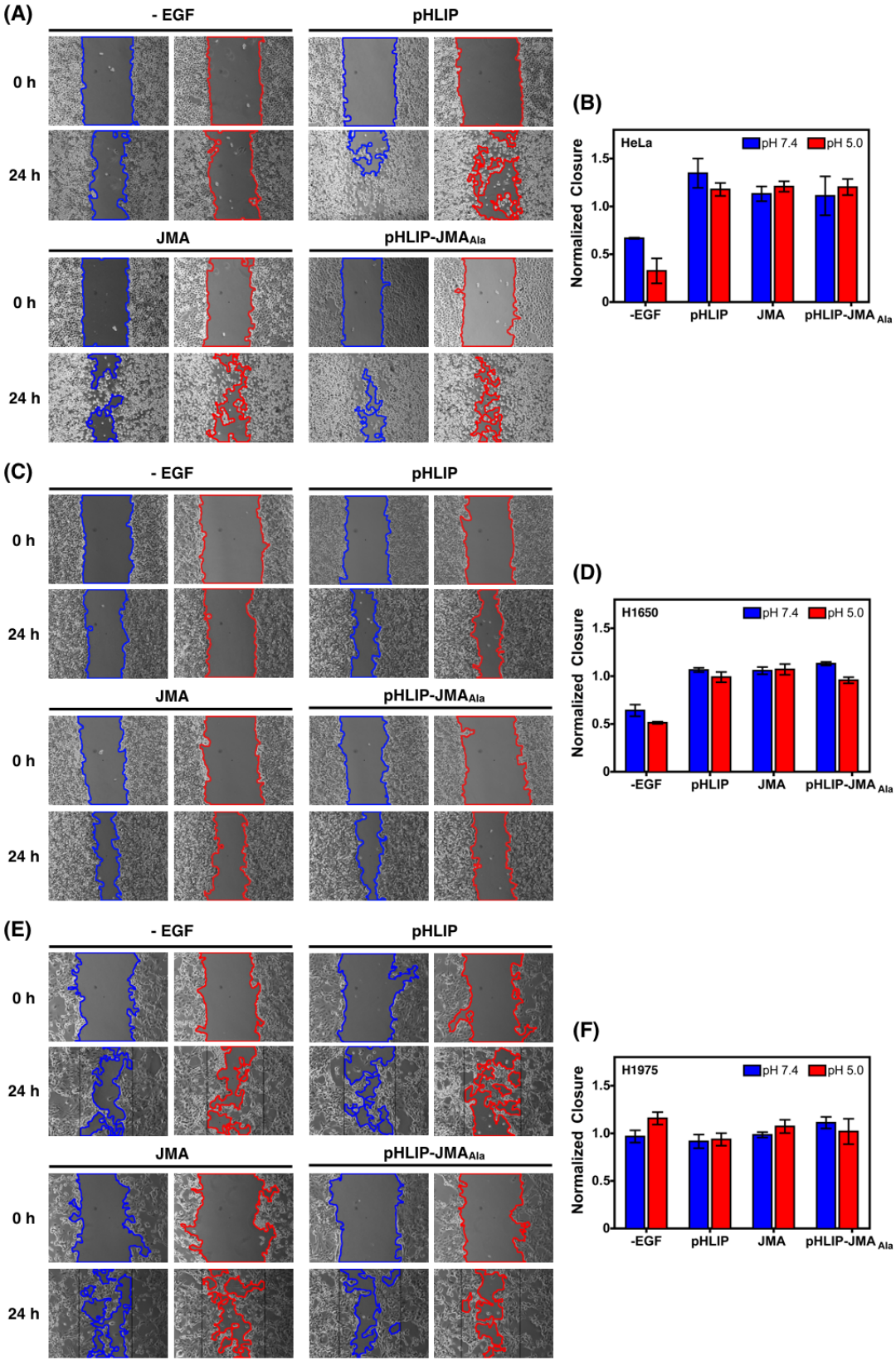

(A)
- EGF
pHLIP
0 h
24 h
JMA
pHLIP-JMAAla
(B)
HeLa
pH 7.4
pH 5.0
Normalized Closure
-EGF
pHLIP
JMA
pHLIP-JMA Ala
(C)
(D)
H1650
(E)
(F)
H1975

Figure S6. pHLIP, JMA, and pHLIP-JMA_{Ala} peptides do not inhibit cell migration. (A,B) HeLa (WT EGFR), (C,D) H1650 (delE746–A750), and (E,F) H1975 (L858R/T790M) cells were treated with pHLIP, JMA, or pHLIP-JMA_{Ala} (10 μM), washed once, and scratched with a 200 μL pipette tip. The cells were incubated in media containing EGF (100 ng/mL) and imaged after 24 hours. Wound closure was found by calculating the percent change in area between 0 hour and 24 hours, 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). 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. No statistically significant ($p>0.05$) differences were observed between pH 7.4 and pH 5.0 for any peptide, indicating that alone pHLIP, JMA, and pHLIP-JMA_{Ala} do not inhibit cell migration in any cell lines tested.

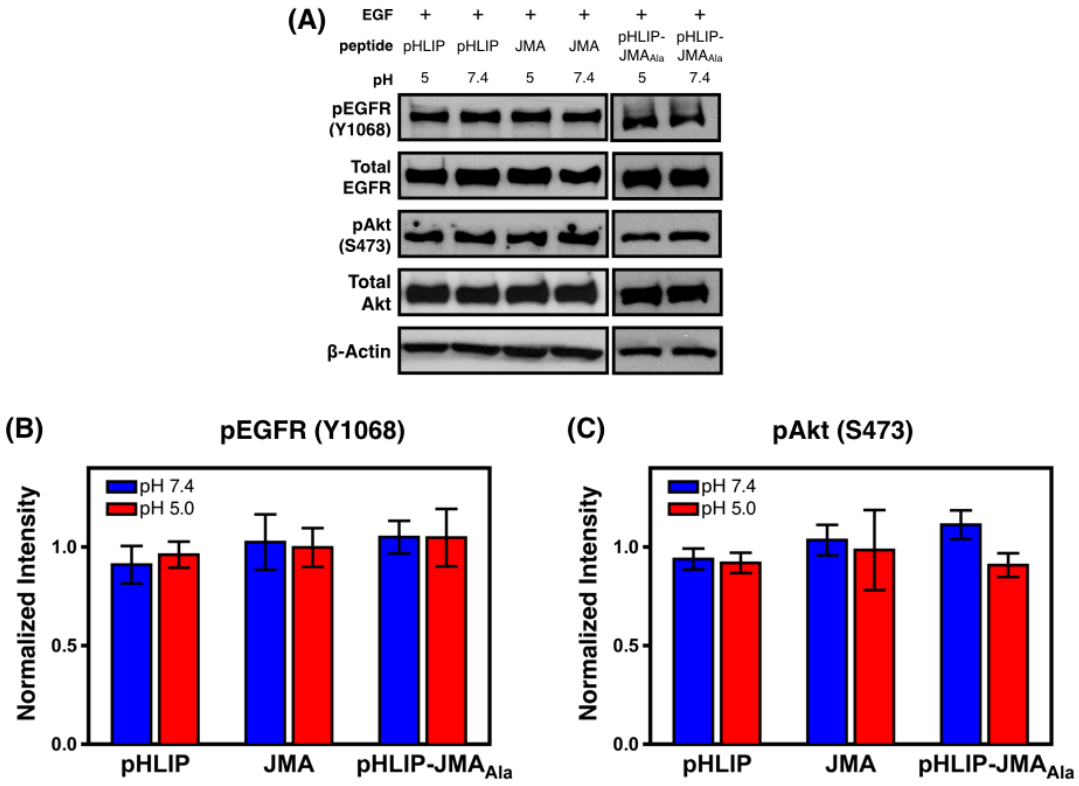


Figure S7. pHLIP, JMA, and pHLIP-JMA$_{Ala}$ do not inhibit EGFR phosphorylation and downstream signaling. (A) Serum starved HeLa (WT EGFR) cells were treated with pHLIP, JMA, and pHLIP-JMA$_{Ala}$ (10 μM) for 10 minutes, followed by EGF treatment for (10 ng/mL) for 7.5 minutes. Cell lysates were collected 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 with error bars representing the standard error of the mean (n=3). No statistically significant ($p > 0.05$) differences were observed between pH 7.4 and pH 5.0 for any peptide for (B) pEGFR (Y1068) and (C) pAkt (S473), indicating that alone pHLIP, JMA, and pHLIP-JMA$_{Ala}$ do not inhibit EGFR activity in any cell lines tested.

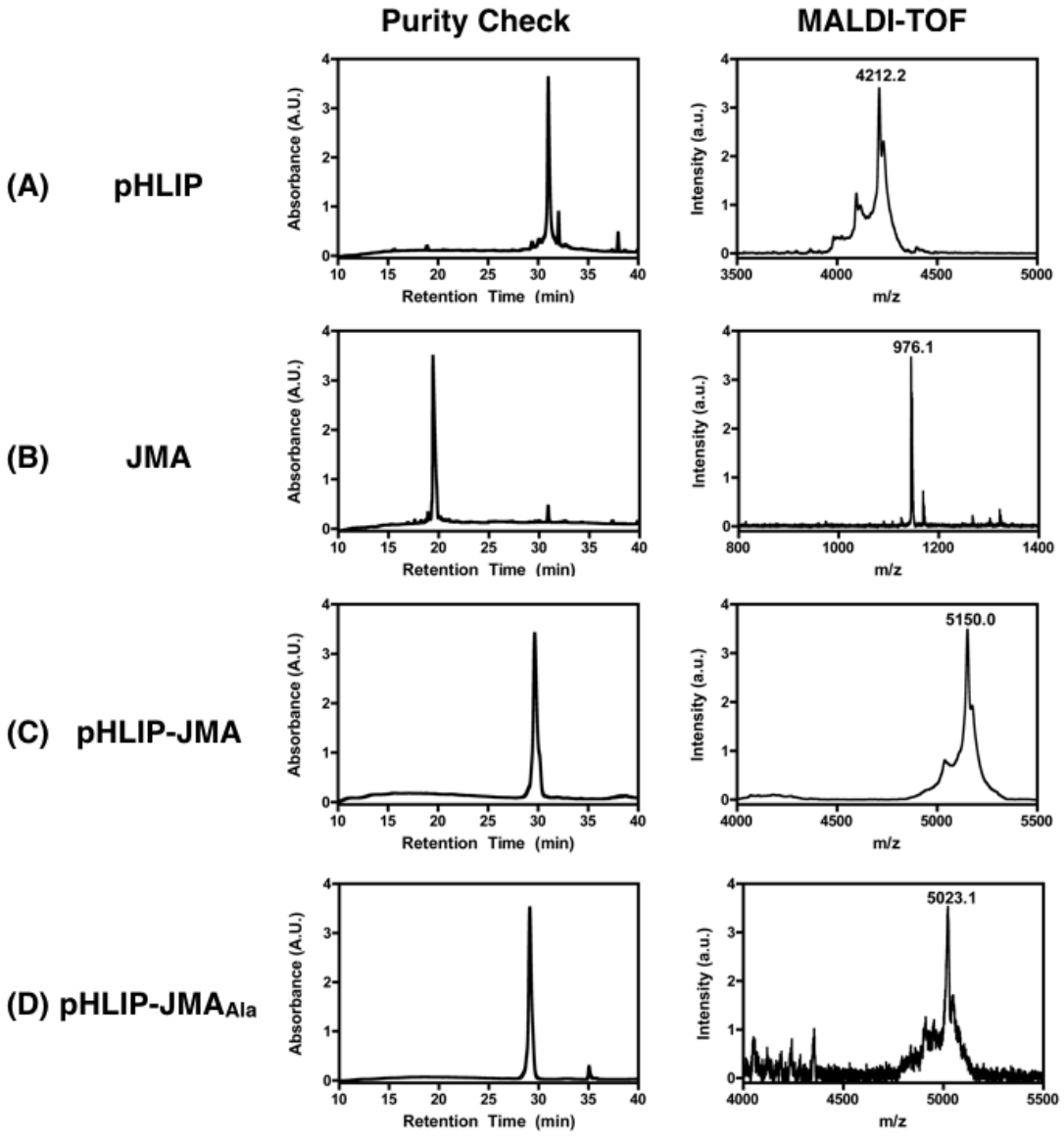


Figure S8. Purity check and MALDI-TOF check of peptides. The purity check and MALDI-TOF of (A) pHLIP, (B) JMA, (C) pHLIP-JMA, and (D) pHLIP-JMA$_{Ala}$ peptides.

(1) Reshetnyak, Y. K., Segala, M., Andreev, O. A., and Engelman, D. M. (2007) A monomeric membrane peptide that lives in three worlds: in solution, attached to, and inserted across lipid bilayers. *Biophysj 93*, 2363–2372.

(2) Vasquez-Montes, V., Gerhart, J., King, K. E., Thévenin, D., and Ladokhin, A. S. (2017) Comparison of lipid-dependent bilayer insertion of pHLIP and its P20G variant. *Biochim. Biophys. Acta 1860*, 534–543.