Supporting Information

Hines et al. 10.1073/pnas.1217206110

SI Materials and Methods

Peptide Synthesis. Fibroblast growth factor receptor substrate 2α (FRS2α)-targeting phospho-dependent proteolysis targeting chimeras (phosphoPROTAC) $^{TrkA}PP_{FRS2\alpha}$, phosphatidylinositol-3-kinase (PI3K)-targeting phosphoPROTAC $^{ErbB2}PP_{PI3K}$, their respective phospho-null variants, and the biotinylated pulldown peptides were prepared by solid-phase Fmoc-protected peptide synthesis on a Rainin Symphony Peptide Synthesizer (Rainin Instruments). All of the protected amino acids, including D-arginine [D-Arg; Fmoc-D-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-OH], were from Novabiochem, and the resin used for the amidated C terminus was Fmoc-polyethylene glycol (PEG)-peptide amide linker-polystyrene from Life Technologies. The aminohexanoic acid linkers were added using standard Fmoc coupling techniques; the PEG linkers were from Novabiochem and were added as a single extended (1 h) manual coupling. The percentage of water in the cleavage mixture was 10% to help minimize arginine sulfonation side reactions, and the peptides were typically cleaved a second time to better ensure deprotection of Arg side chains. PhosphoPROTAC peptide purity was measured by analytical reverse-phase HPLC following lypophilization of the product and monitoring of the absorbance at 210 nm: purity of the cleaved peptide varied between 81–87% (representative traces showing the purity of $^{TrkA}PP_{FRS2\alpha}$ and of $^{ErbB2}PP_{PI3K}$ are depicted in Figs. S1 and S2, respectively). Reverse-phase HPLC was carried out on a YMC C-18 column (4 mm × 50 mm, 3-μm particle size, 120-Å pore-size support) that eluted at 1 mL/min with a step gradient where buffer A was 0.05% TFA in water and buffer B was 80% acetonitrile in 0.05% TFA in water (Table S2).

Peptide Phosphorylation Assay. The method used here to measure peptide phosphorylation in vitro has been described in detail by Hardie (1). Briefly, PC12 cells in a 60-mm dish were serum-starved overnight and then treated for 5 min with NGF, or EGF, or IGF1 at 100 ng/mL. Cells were then harvested in hypotonic lysis buffer supplemented with protease and phosphatase inhibitors as described in *Immunoblotting* below. Membranes were pelleted by centrifugation for 5 min at 4 °C in a microfuge at 16,000 × *g*. Membrane pellet was then resuspended in 200 μL of hypotonic lysis buffer and homogenized such that final protein concentration was between 200 and 500 μg/mL. For each peptide phosphorylation reaction, 25 μL of prepared membrane fraction was incubated with 25 μg of $^{TrkA}PP_{FRS2\alpha}$ in a reaction buffer adjusted to 25 mM Tris, pH 7.4, 1 mM DTT, 25 mM $MgCl_2$, and 50 μM ATP with 0.15 μCi/μL [γ^{32}P]ATP in a final volume of 30 μL. Reactions were permitted to run for 2 h at 30 °C before being spotted onto a 1-inch square of P81 phosphocellulose ion exchange paper (Whatman) and terminated by immersion in ice-cold 1% phosphoric acid. Paper squares were rinsed three times in ice-cold 1% phosphoric acid solution, once in ice-cold water, and finally in acetone before air drying and scintillation counting.

Immunoblotting. Cells were treated as indicated for 8 h and then lysed in hypotonic lysis buffer (25 mM Tris·HCl, pH 7.5, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mM phenylarsine oxide, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 0.3 trypsin inhibitor units/mL aprotinin, 1 mM PMSF) using a Dounce homogenizer. Following ultracentrifugation at 100,000 × *g* for 45 min, the pelleted membrane fraction ("P100") was solubilized in hypotonic lysis buffer further supplemented with 1% Nonidet P-40. The solubilized P100 was clarified by centrifugation at 16,000 × *g* in a microfuge at 4 °C for 10 min. Protein concentrations of different samples were normalized by bicinchoninic acid protein assay. Due to the low levels of endogenous FRS2α in PC12 cells, it was necessary to first concentrate it by immunoprecipitation from the solubilized P100 using a polyclonal antibody (Sigma; catalog no. F9052) before immunoblotting with a monoclonal antibody (abcam; catalog no. ab14474). Before immunoprecipitation, the solubilized P100 from all treatment groups was normalized, and an aliquot of the input was reserved for probing of other proteins of interest. For probing for ubiquitin conjugates of FRS2α, PC12 cells were lysed in boiling RIPA buffer (25 mM Tris·HCl, 150 mM NaCl, 1% Nonidet P-40, and 0.5% deoxycholic acid supplemented with protease and phosphatase inhibitors as above) to inactivate deubiquitinases before immunoprecipitation. Cell Signaling Technologies supplied antibodies used to immunoblot for suppressor of cytokine signaling 3 (catalog no. 2923), PI3K p85 (catalog no. 4292), CD54 (catalog no. 4915), activated (phosphorylated) Akt (catalog no. 9271), total Akt (catalog no. 9272), activated (phosphorylated) Erk1/2 (catalog no. 9106), GAPDH (catalog no. 2118), and ubiquitin (catalog no. 3933); antibodies to total Erk1/2 (catalog no. sc-93) and CD40 (catalog no. sc-1731) were purchased from Santa Cruz Biotechnology. Monoclonal antibody for medium-chain neurofilament was from Upstate Biotechnology (catalog no. 05–744).

Cytotoxicity Assay. Following phosphoPROTAC treatment of cells as indicated, culture medium was supplemented with 330 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) and 25 μM phenazine methosulfate (Sigma) and incubated at 37 °C. Mitochondrial reduction of MTS to the formazan derivative was monitored by measuring the medium's absorbance at 490 nm using a Wallac Victor^2 platereader (Perkin-Elmer Life Sciences). Data analysis and statistics were performed using Prism v4.0 software (GraphPad Software).

Xenograft Tumor Growth in Mice. Female athymic (nu/nu) mice were obtained from Charles River. At 6 wk of age, the mice were first sedated by i.p. injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) and then s.c. injected into the dorsal flank near the hip with 2 million human ovarian cancer OVCAR8 cells in 50% RMPI medium 1640:50% Matrigel (Invitrogen); the total s.c. injection volume was 100 μL. After allowing 24 h for recovery from tumor cell implantation, mice were given daily i.p. injections of either $^{ErbB2}PP_{PI3K}$ or $^{ErbB2}NP_{PI3K}$ (10 mg/kg) in 100 μL of PBS or an equal volume of vehicle PBS alone ("control"). At the end of 47 d, mice were killed and the tumors were surgically removed and individually weighed on a Mettler balance (Table S1). Average tumor weight for each treatment group was determined, and statistical analysis of the results consisted of one-way ANOVA followed by Newman–Keuls post hoc test (Prism v4.0, GraphPad Software).

1. Hardie DG (2000) Peptide assay of protein kinases and use of variant peptides to determine recognition motifs. *Methods Mol Biol* 99:191–201.

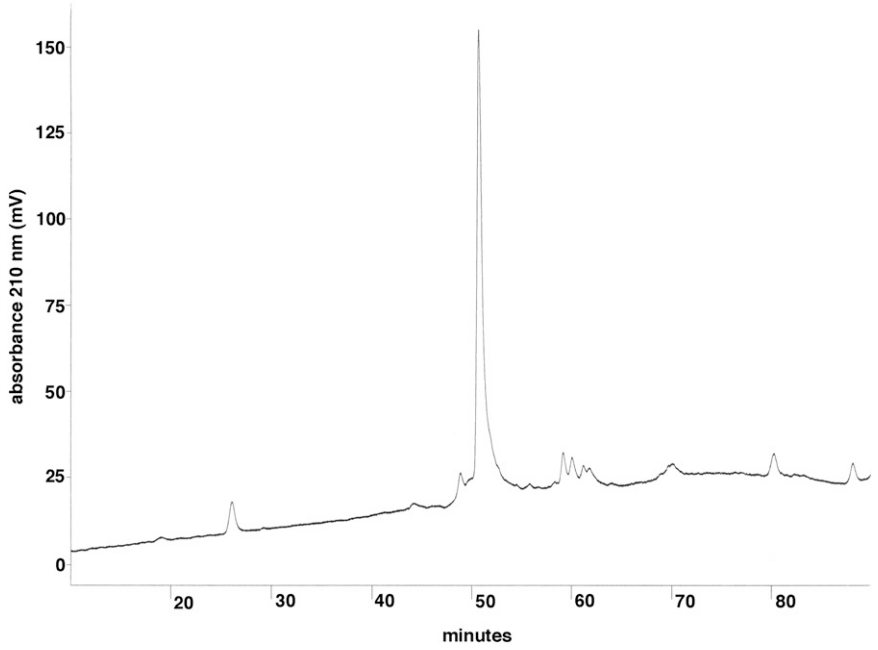

Fig. S1. Analytical reverse-phase HPLC trace of phosphoPROTAC $^{TrkA}PP_{FRS2\alpha}$. Lyophilized peptide was run on a YMC C-18 column that eluted at 1 mL/min with a step gradient as described in Table S2. In this representative trace, $^{TrkA}PP_{FRS2\alpha}$ eluted at 51 min with a purity of 82% as determined by measurement of the area under curve.

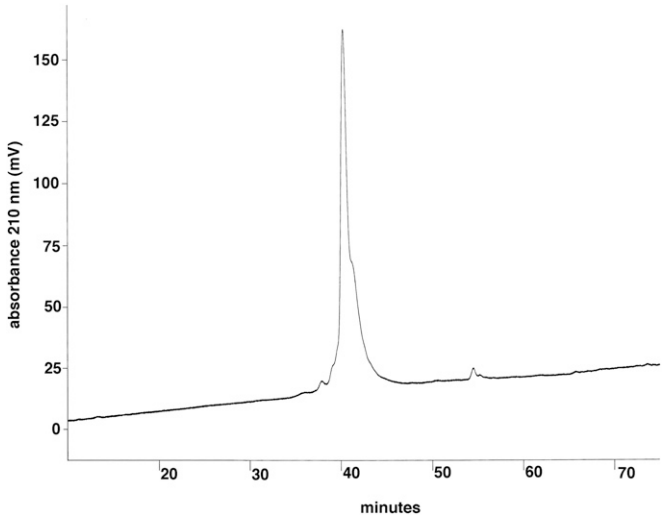

Fig. S2. Analytical reverse-phase HPLC trace of phosphoPROTAC $^{ErbB2}PP_{PI3K.}$ Lyophilized peptide was run on a YMC C-18 column that eluted at 1 mL/min with a step gradient as described in Table S2. In this representative trace, $^{ErbB2}PP_{PI3K}$ eluted at 40 min with a purity of 87% as determined by measurement of the area under curve.

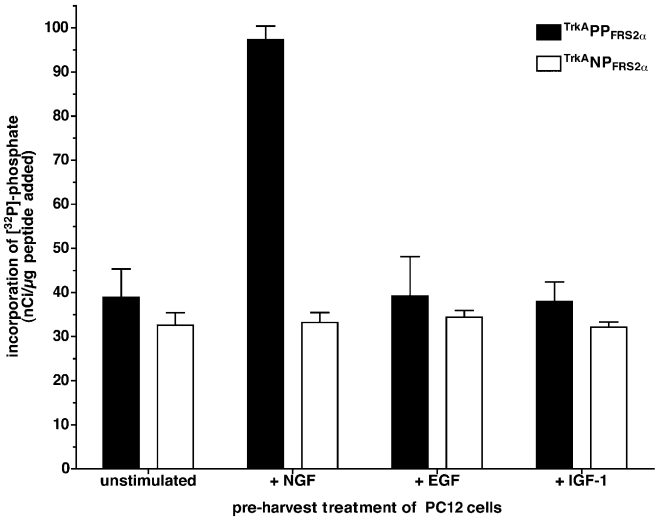

Fig. S3. Incorporation of [^{32}P]-phosphate into $^{TrkA}PP_{FRS2\alpha}$ by nerve growth factor (NGF)-stimulated PC12 cell membranes. [γ-^{32}P]ATP was incubated with 25 μg of $^{TrkA}PP_{FRS2\alpha}$ in membrane fraction from PC12 cells that had been treated with 100 ng/mL NGF (or EGF or IGF1) for 5 min before harvest. Phosphorylated peptides were isolated as described in *SI Materials and Methods* and subjected to scintillation counting. A representative experiment is shown.

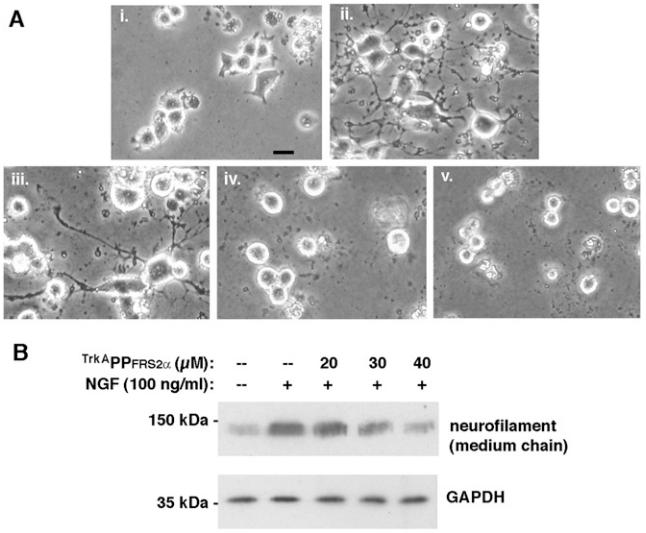

Fig. S4. Knockdown of FRS2α by $^{TrkA}PP_{FRS2\alpha}$ blocks PC12 neuronal differentiation by NGF. (*A*) $^{TrkA}PP_{FRS2\alpha}$ dose-dependently prevents neurite outgrowth from PC12 cells cultured in the presence of NGF: (i) undifferentiated PC12 cells (Scale bar, 10 μm). (ii) PC12 cells differentiated for 48 h with 100 ng/mL NGF. (iii) PC12 cells treated for 48 h with NGF and 20 μM $^{TrkA}PP_{FRS2\alpha}$. (iv) PC12 cells treated for 48 h with NGF and 30 μM $^{TrkA}PP_{FRS2\alpha}$. (v) PC12 cells treated for 48 h with NGF and 40 μM $^{TrkA}PP_{FRS2\alpha}$. (*B*) Blockade of NGF-dependent induction of the neuronal marker neurofilament (medium chain) by $^{TrkA}PP_{FRS2\alpha}$.

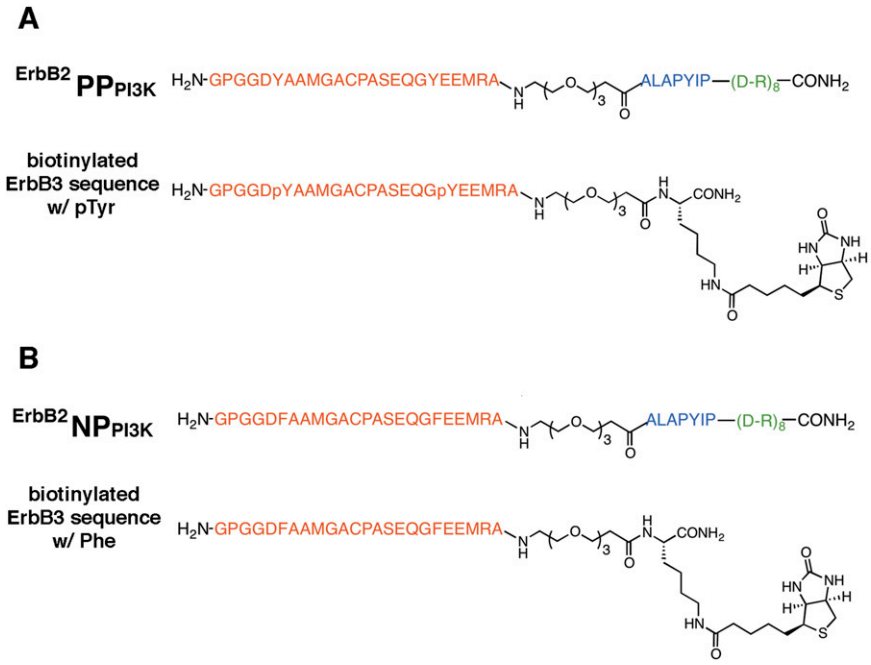

Fig. S5. Schematic of PI3K-targeting phosphoPROTAC, null variant, and biotinylated analogs for pulldown assay. (*A*) Schematic of the PI3K-targeting phosphoPROTAC $^{ErbB2}PP_{PI3K}$ and a biotinylated version of its targeting sequence for streptavidin pulldown from cell lysate. The red peptide sequence corresponds to a 24-amino-acid sequence in ErbB3 that is dual tyrosine phosphorylated by activated ErbB2 (this sequence is already phosphorylated in the biotinylated version). The blue peptide sequence is derived from the transcription factor hypoxia-inducible factor-1α (HIF-1α), which enables binding to the E3 ubiquitin ligase von Hippel Lindau. The green sequence is a poly-D-arginine motif that permits cell permeability of the peptide via the same mechanism used by the structurally related HIV transactivator of transcription (Tat) protein. The PI3K-targeting phosphoPROTAC contains a 13-atom polyethylene glycol linker in place of the aminohexanoic acid linker previously used with the FRS2α-targteting phosphoPROTAC. (*B*) Schematic of the null variant $^{ErbB2}NP_{PI3K}$ and a biotinylated version of its targeting sequence for streptavidin pulldown from cell lysate. The color coding of the functional sequences is the same as that used in *A*. Note that the red targeting sequence in $^{ErbB2}NP_{PI3K}$ and its biotinylated version contains phenylalanine substitutions in place of the tyrosines in $^{ErbB2}PP_{PI3K}$.

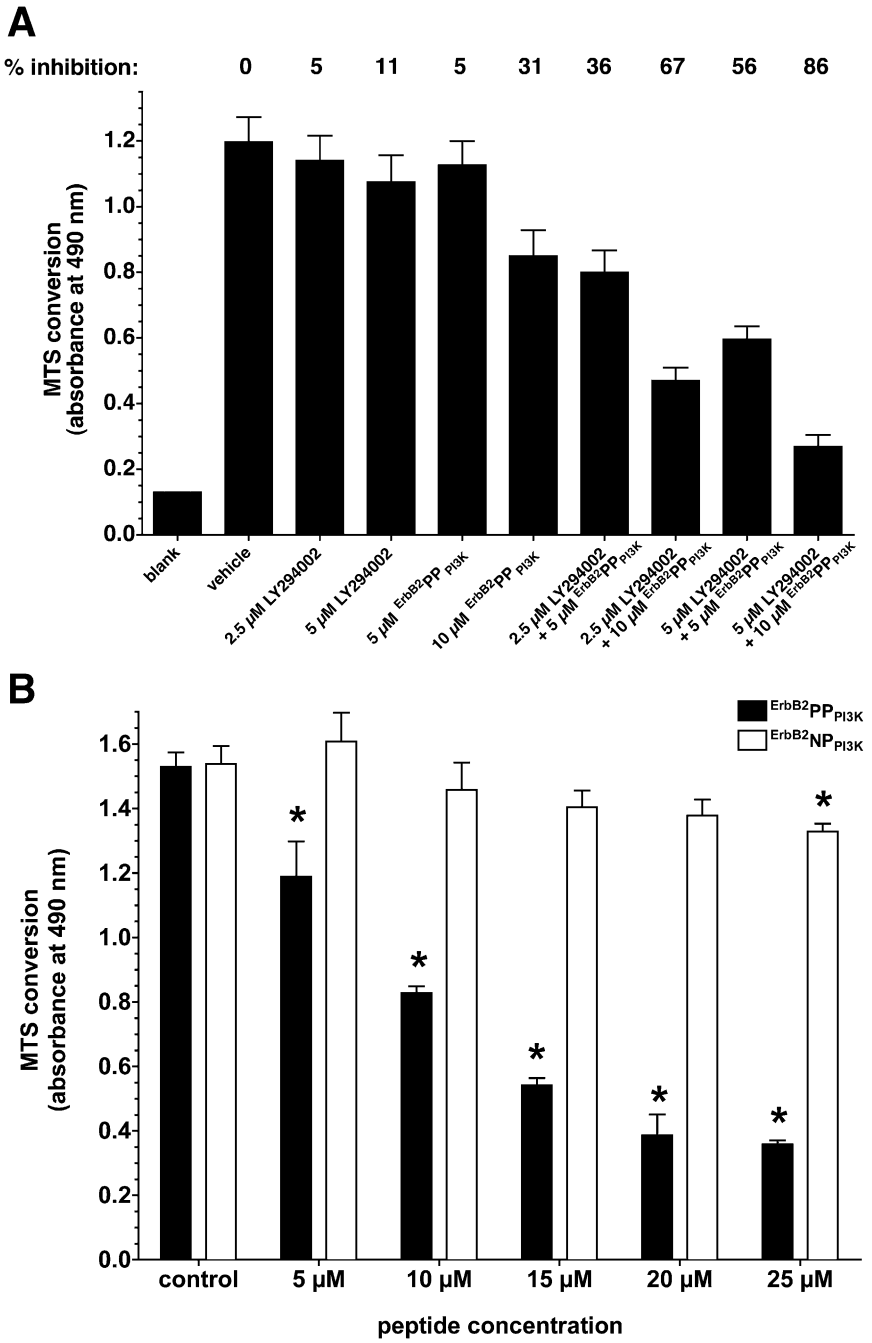

Fig. S6. Inhibition of PI3K signaling due to targeted knockdown by $^{ErbB2}PP_{PI3K}$ is phospho-dependent and reduces cell viability. (*A*) $^{ErbB2}PP_{PI3K}$ and LY294002 share a common molecular target. Loss of human breast cancer MCF-7 cell viability from cotreatment of MCF-7 cells with concentrations of the PI3K inhibitor, LY294002, and $^{ErbB2}PP_{PI3K}$ is synergistic. Bars represent the mean ± SD of three replicate experiments. (*B*) $^{ErbB2}PP_{PI3K}$ reduces viability of MDA-MB-175 breast cancer cells. The activity of this phosphoPROTAC against MDA-MB-175 cells is more potent than MCF-7 cells (Fig. 4). Bars represent the mean ± SD of three replicate experiments. Asterisk denotes significant difference in MTS conversion compared with control (untreated) cells determined by Student *t* test ($P < 0.05$).

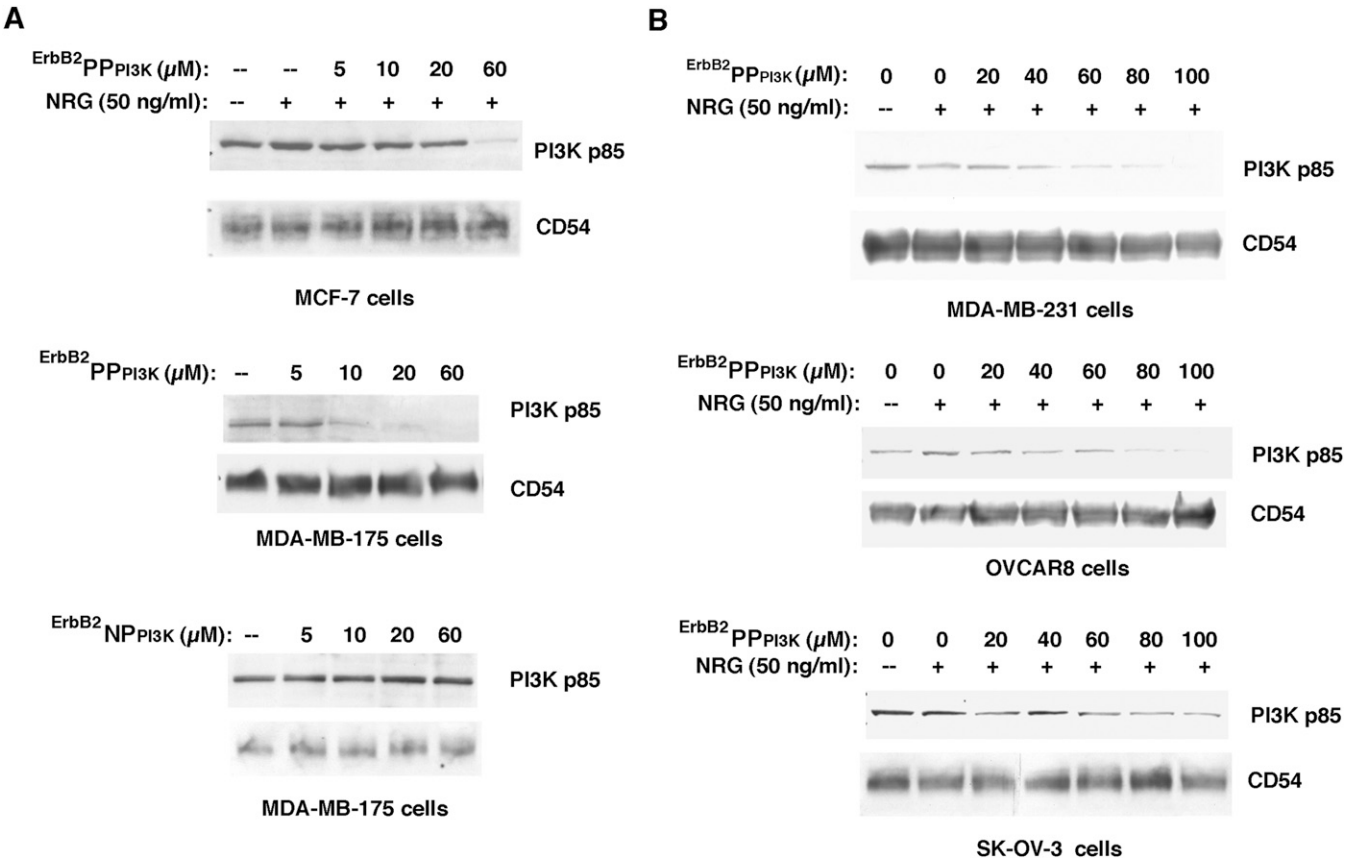

Fig. S7. Knockdown of PI3K by $^{ErbB2}PP_{PI3K}$ in breast and ovarian cancer cell lines. (*A*) PhosphoPROTAC $^{ErbB2}PP_{PI3K}$ more potently knocks down levels of PI3K p85 in MDA-MB-175 than in MCF-7 cells. (*B*) $^{ErbB2}PP_{PI3K}$ knocks down PI3K p85 in MDA-MB-231 breast cancer and OVCAR8 and SK-OV-3 ovarian cancer cells.

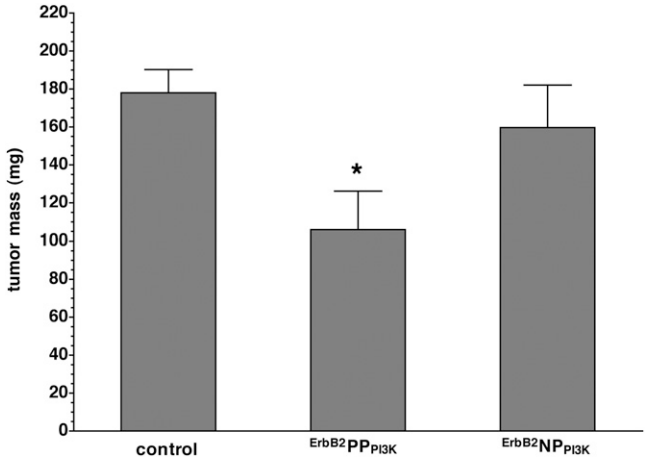

Fig. S8. Inhibition of tumor growth in nude mice by phosphoPROTAC $^{ErbB2}PP_{PI3K}$ significantly reduced growth of implanted OVCAR8 ovarian cell tumors in vivo (asterisk denotes significant difference from control mice as assessed by one-way ANOVA with Newman–Keuls post hoc test ($P < 0.05$).

Table S1. Average weights of athymic mice over OVCAR8 tumor explant experiment

Treatment group	Average body mass day 1 (g)	Average body mass day 23 (g)	% weight increase by day 23	Average body mass day 47 (g)	% weight increase by day 47
Control	17.5 ± 1.1	20.8 ± 1.9	18.9 ± 4.8	24.0 ± 2.4	37.1 ± 8.7
$^{ErbB2}PP_{PI3K}$	20.2 ± 1.3	23.3 ± 1.9	15.3 ± 5.5	25.4 ± 2.5	25.7 ± 7.6
$^{ErbB2}NP_{PI3K}$	19.3 ± 1.0	22.9 ± 1.8	18.7 ± 5.0	25.8 ± 1.6	33.7 ± 8.2

Values listed are the mean ± SD for each of the three treatment groups.

Table S2. Elution step-gradient conditions used in HPLC analyses of synthesized peptide phosphoPROTACs

Time (min)	% buffer A	% buffer B
0	98	2
63	62.5	37.5
95	25	75
105	2	98
112	2	98