

Posttranslational protein knockdown coupled to receptor tyrosine kinase activation with phosphoPROTACs

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Posttranslational knockdown of a specific protein is an attractive approach for examining its function within a system. Here we introduce phospho-dependent proteolysis targeting chimeras (phosphoPROTACs), a method to couple the conditional degradation of targeted proteins to the activation state of particular kinase-signaling pathways. We generated two phosphoPROTACs that couple the tyrosine phosphorylation sequences of either the nerve growth factor receptor, TrkA (tropomyosin receptor kinase A), or the neuregulin receptor, ErbB3 (erythroblastosis oncogene B3), with a peptide ligand for the E3 ubiquitin ligase von Hippel Lindau protein. These phosphoPROTACs recruit either the neurotrophic signaling effector fibroblast growth factor receptor substrate 2 α or the survival-promoting phosphatidylinositol-3-kinase, respectively, to be ubiquitinated and degraded upon activation of specific receptor tyrosine kinases and phosphorylation of the phosphoPROTACs. We demonstrate the ability of these phosphoPROTACs to suppress the short- and long-term effects of their respective activating receptor tyrosine kinase pathways both in vitro and in vivo. In addition, we show that activation of phosphoPROTACs is entirely dependent on their kinase-mediated phosphorylation, as phenylalanine-containing null variants are inactive. Furthermore, stimulation of unrelated growth factor receptors does not induce target protein knockdown. Although comparable in efficiency to RNAi, this approach has the added advantage of providing a degree of temporal and dosing control as well as cell-type selectivity unavailable using nucleic acid-based strategies. By varying the autophosphorylation sequence of a phosphoPROTAC, it is conceivable that other receptor tyrosine kinase/effector pairings could be similarly exploited to achieve other biological effects.

protein degradation | SH2 domain | signal transduction

Antagonizing the function of a protein is an effective strategy for determining its role within a cellular context. For some enzymes and/or receptor proteins, this can be accomplished by incubation with a small molecule inhibitor or antagonist. However, specific small molecule inhibitors have not been discovered or developed for many proteins. As alternative strategies, gene deletion and RNAi approaches function at the DNA and mRNA levels to reduce protein expression, thus offering the ability to control function even for those proteins lacking a specific inhibitor (1, 2). However, such nucleic acid-based approaches do not afford the same rapid, direct assessment of protein function as posttranslational intervention; furthermore, in vivo application of these strategies is even today still complicated and relatively cumbersome. Combining the attractive qualities of both RNAi and small molecule inhibitor approaches, we developed a strategy, proteolysis targeting chimeras (PROTACs) for targeted posttranslational knockdown of proteins. Each PROTAC is heterodimeric, consisting of an E3 ubiquitin ligase-binding moiety linked to a ligand that binds to the target protein (3). As such, each PROTAC recruits its target protein to the E3 ubiquitin ligase, resulting in target protein ubiquitination by the E3 ligase and, ultimately, proteasome-mediated degradation of the

target (4–6). Targeting proteins for degradation using PROTACs offers direct intervention at the protein level without the restriction to targets with established small molecule inhibitors because ligands that bind to any site on the target protein can be incorporated into a PROTAC. Moreover, given the irreversibility of protein degradation, PROTAC-mediated control of protein function yields physiological consequences akin to those of RNAi and/or irreversible small molecule inhibitors.

As originally conceived, PROTACs work constitutively to induce targeted protein ubiquitination/degradation irrespective of any intracellular signaling context (4, 6, 7). Here, we advance this paradigm by coupling PROTAC-mediated protein degradation to the activation state of a particular signaling pathway. Specifically, we tested whether a PROTAC can selectively distinguish between an activated versus a quiescent receptor tyrosine kinase-signaling pathway. Because phosphorylated tyrosine residues can serve as ligands for the binding of phosphotyrosine-binding (PTB) and Src homology 2 (SH2) domain-containing proteins (8), a cell-permeable PROTAC molecule that incorporates a specific tyrosine kinase substrate sequence may do the same (9). Thus, phosphorylation of such a PROTAC by a receptor tyrosine kinase (RTK) could generate a binding site for a downstream PTB or SH2 domain-containing protein and its subsequent recruitment to the ubiquitin/proteasome degradation pathway for destruction, thereby disrupting tyrosine kinase signaling (Fig. 1*A* and *B*). In this manner, the phosphoPROTAC would serve as a PROTAC whose activity was inducible, or “conditional” upon activation of a given receptor tyrosine kinase, thus creating an artificial negative feedback mechanism. Our previous study regarding the regulation of nerve growth factor (NGF) activity by small molecules (10) suggested that TrkA (the NGF receptor) signaling would be an interesting and suitable pathway in which to investigate the possibility and/or utility of our signaling context-dependent protein knockdown strategy. The effector protein fibroblast growth factor receptor substrate 2 α (FRS2 α) (also known as SNT-1) (11, 12) emerged as an attractive target protein candidate, given its importance in TrkA signaling and its lack of any enzymatic activity that could be blocked with traditional small molecule inhibitors. Our success in this system subsequently led us to expand the study to the erythroblastosis oncogene (Erb)B2/ErbB3

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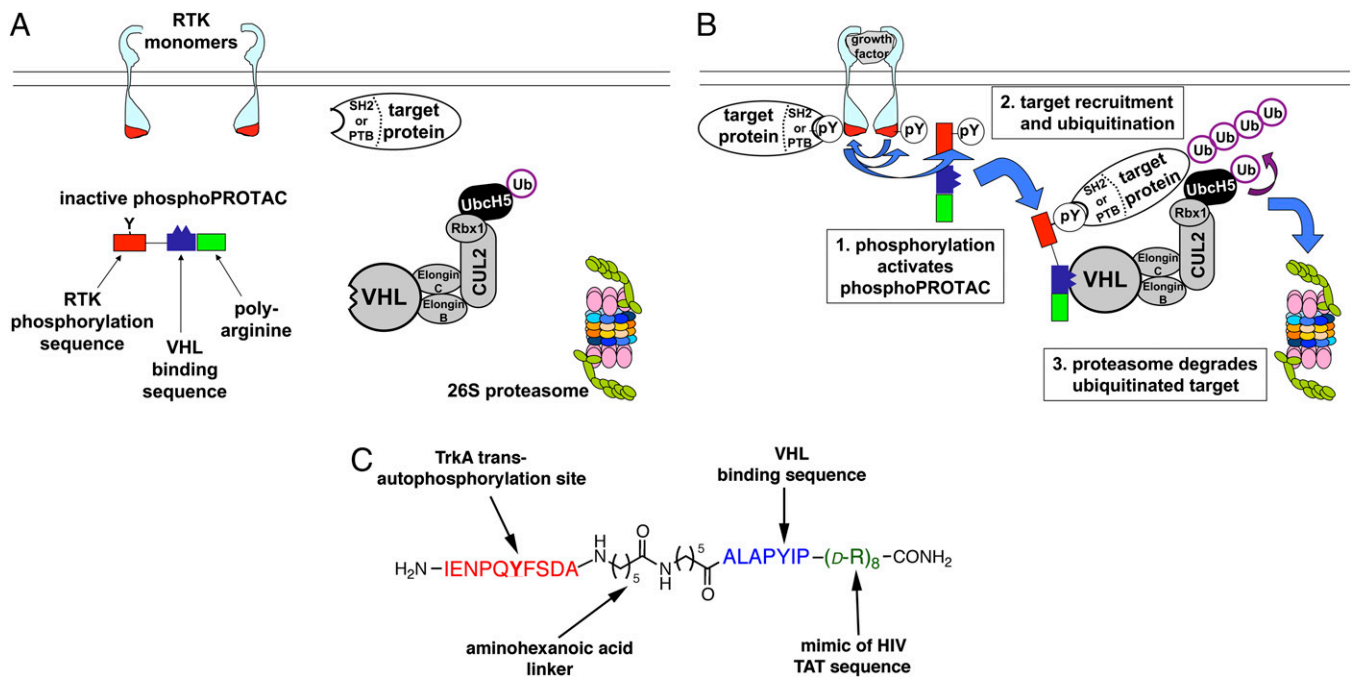


Fig. 1. (A and B) Schematic diagram of conditional postranslational target protein knockdown by a phosphoPROTAC. (A) In the absence of an activated RTK, the phosphoPROTAC is not phosphorylated and fails to bind to the target protein, sparing it from degradation. (B) Upon activation of the RTK, the phosphoPROTAC is phosphorylated, creating a binding site for the SH2- or PTB-domain-containing effector protein and its subsequent recruitment for ubiquitination by VHL and proteasomal degradation. (C) Schematic of phosphoPROTAC^{TrkA}PP_{FRS2 α} : the red peptide sequence corresponds to the TrkA autophosphorylation site; the blue peptide sequence enables binding to the E3 ubiquitin ligase VHL; and the green poly-D-arginine motif permits cell permeability of the peptide.

RTK pathway to show that the phosphoPROTAC strategy can be adapted for other receptor tyrosine kinase pathways and may even hold therapeutic potential.

Results

Designing a PhosphoPROTAC Activated by NGF-TrkA Signaling. Following sustained NGF treatment, rat pheochromocytoma PC12 cells differentiate into a postmitotic neuronal phenotype (13). Thus, PC12 cells have been used extensively to study NGF-dependent neuronal differentiation and the signaling induced by NGF binding to its receptor tyrosine kinase, TrkA. NGF-induced dimerization results in TrkA transautophosphorylation on specific tyrosine residues in its intracellular domain (14), thereby fully activating the kinase. In addition, transautophosphorylation on other tyrosine residues creates binding sites for SH2 and PTB domain-containing substrate proteins, which, upon recruitment, are themselves phosphorylated by TrkA. One such substrate is FRS2 α , a myristoylated, membrane-tethered protein and a major downstream effector for TrkA-driven neuronal differentiation. The PTB domain-containing FRS2 α binds to phosphorylated TrkA and, following its own phosphorylation, serves as a scaffolding protein to coordinate downstream signaling pathways (15). FRS2 α -mediated signaling includes the B-rapidly accelerated fibrosarcoma (B-Raf)/mitogen-activated erk kinase (MEK)/extracellular signal-regulated kinase 1 and 2 (Erk1/2) pathway, sustained activation of which is essential to drive the changes in protein expression and cell morphology characteristic of neuronal differentiation (16).

We designed a phosphorylation-dependent PROTAC, or phosphoPROTAC (PP) that would knockdown FRS2 α levels upon NGF activation of TrkA. We designated this molecule, “TrkA^{PP}FRS2 α .” This phosphoPROTAC contains a TrkA-derived decapeptide sequence (Fig. 1C) whose central tyrosine is phosphorylated by TrkA following NGF binding and receptor dimerization (12). We hypothesized that this sequence in TrkA^{PP}FRS2 α would also be phosphorylated by NGF-bound TrkA and thus

enable TrkA^{PP}FRS2 α to similarly bind FRS2 α . In turn, to target the FRS2 α :TrkA^{PP}FRS2 α complex for ubiquitination of FRS2 α , the phosphoPROTAC also contains a seven-amino-acid sequence derived from the transcription factor hypoxia-inducible factor-1 α (HIF-1 α), which serves as a recognition sequence for binding to the von Hippel Lindau (VHL) tumor suppressor (17). VHL is the substrate recognition component of a larger E3 ubiquitin ligase complex that ubiquitinates lysine residues of bound proteins, targeting them to the proteasome for proteolytic degradation. In addition, a poly-D-arginine tag was incorporated (Fig. 1C) to enhance cell permeability via the same mechanism used by the HIV transactivator of transcription (Tat) protein (6, 18–20). PhosphoPROTAC peptides were synthesized using standard solid-phase Fmoc coupling, and the cleaved peptide product was typically between 82% and 88% pure (Figs. S1 and S2).

Incubation of this peptide with freshly prepared membrane fraction from NGF-treated PC12 cells supplemented with ATP γ [³²P] revealed the incorporation of [³²P]phosphate into TrkA^{PP}FRS2 α (Fig. S3) compared with incubation of the peptide with membrane fraction from untreated PC12 cells. This increase in ³²P content was not observed when we incubated membrane fraction from NGF-treated cells with a phosphoPROTAC variant possessing a phenylalanine instead of the tyrosine residue within the TrkA transautophosphorylation motif. The variant peptide cannot be phosphorylated, or is a “null” phosphoPROTAC (NP), which we designated as TrkA^{NP}FRS2 α . Furthermore, incubation of TrkA^{PP}FRS2 α with membrane fraction from cells treated with epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF1), neither of which activate TrkA, did not increase ³²P incorporation into TrkA^{PP}FRS2 α . This indicated that activation of the phosphoPROTAC should be specific to NGF treatment.

Efficacy and Selectivity of a FRS2 α -Targeting PhosphoPROTAC. To test the activity of TrkA^{PP}FRS2 α , we incubated PC12 cells with

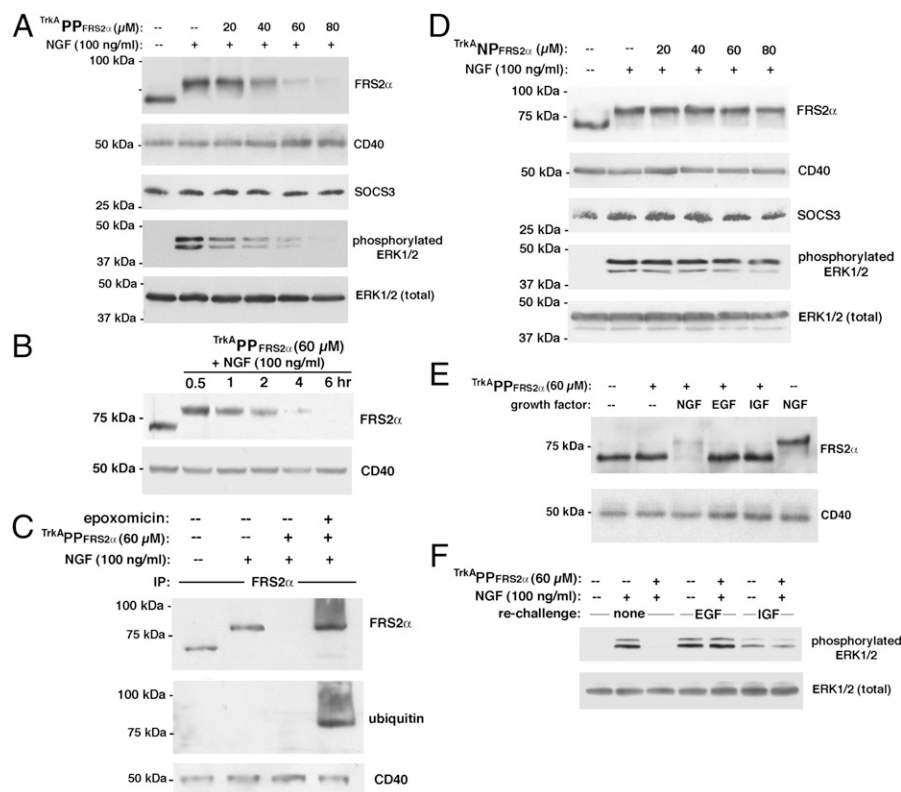


Fig. 2. Function of $TrkA^{PPFRS2\alpha}$, a phosphoPROTAC peptide that conditionally targets FRS2 α for degradation upon TrkA activation. (A) Selective knockdown of FRS2 α by $TrkA^{PPFRS2\alpha}$ and inhibition of NGF-dependent Erk1/2 activation in PC12 cells. Cells were incubated with the phosphoPROTAC for total of 7 h. (B) Knockdown of FRS2 α by $TrkA^{PPFRS2\alpha}$ is time dependent. Cells were incubated with 60 μM $TrkA^{PPFRS2\alpha}$ for 30 min to 6 h. (C) Activated $TrkA^{PPFRS2\alpha}$ recruits FRS2 α to be ubiquitinated. PC12 cells were pretreated with 100 nM epoxomicin for 1 h before incubation with 60 μM $TrkA^{PPFRS2\alpha}$ and with NGF for 7 h before lysis in boiling RIPA buffer. (D) Phosphorylation dependence of FRS2 α knockdown by phosphoPROTAC. Phospho-null variant, $TrkA^{NPFRS2\alpha}$, is inactive in the presence of NGF. (E) Knockdown of FRS2 α by $TrkA^{PPFRS2\alpha}$ is dependent on activation of TrkA but not on other receptor tyrosine kinases. NGF, EGF, and IGF1 were all used at 100 ng/mL. In lanes where NGF was not added, tonic activity of TrkA was blocked by supplementation with TrkA inhibitor (1 μM). (F) Knockdown of FRS2 α by $TrkA^{PPFRS2\alpha}$ does not interfere with signaling of other RTKs through Erk1/2. NGF, EGF, and IGF1 were all used at 100 ng/mL.

increasing concentrations of the phosphoPROTAC in the presence of NGF (100 ng/mL) for a total of 7 h (Fig. 2A). Whereas treatment with NGF alone resulted in the phosphorylation of FRS2 α (as evidenced by the upward band shift), addition of $TrkA^{PPFRS2\alpha}$ at 40 μM reduced FRS2 α levels by ~50%. Furthermore, concentrations ≥ 60 μM of $TrkA^{PPFRS2\alpha}$ were maximally effective and knocked down FRS2 α to <10% of control. We saw no decrease in the levels of two other unrelated proteins—the integral membrane protein CD40 (21) as well as the SH2 domain-containing protein suppressor of cytokine signaling 3 (SOCS3) (22), which suggested that protein knockdown by $TrkA^{PPFRS2\alpha}$ was selective for the target. To confirm the functional significance of FRS2 α protein knockdown, we next investigated the ability of NGF to activate Erk1/2 in the presence of $TrkA^{PPFRS2\alpha}$. Concordant with FRS2 α knockdown, Erk1/2 activation by NGF was inhibited by $TrkA^{PPFRS2\alpha}$. However, this reduction of Erk1/2 activation by NGF was not due to overall reduction of Erk1/2 levels, confirming decreased NGF-mediated signaling in $TrkA^{PPFRS2\alpha}$ -treated cells.

In addition to being concentration dependent, knockdown of FRS2 α was time dependent as well (Fig. 2B). To confirm that $TrkA^{PPFRS2\alpha}$, like our previous PROTACs, causes knockdown of target by recruitment to the ubiquitin-proteasome pathway, we pretreated the PC12 cells with the proteasome inhibitor epoxomicin: whereas immunoblotting for FRS2 α in the presence of NGF and $TrkA^{PPFRS2\alpha}$ alone showed little remaining immunoreactivity, the additional presence of epoxomicin not only stabilized FRS2 α but also revealed the presence of larger molecular weight forms of the target (Fig. 2C). These larger forms were verified to be ubiquitin conjugates that had accumulated due to proteasome inactivation. To investigate whether $TrkA^{PPFRS2\alpha}$ -induced FRS2 α degradation was dependent on phosphorylation by TrkA, we performed immunoblot analysis of PC12 cells that had been incubated with the phenylalanine-containing $TrkA^{NPFRS2\alpha}$. The null phosphoPROTAC was not able to reduce FRS2 α levels and did not block NGF-triggered Erk1/2 activation (Fig. 2D).

This demonstrates the phosphorylation-dependent activity of the phosphoPROTAC, i.e., the “conditional” degradation of FRS2 α .

Similar to NGF, EGF and IGF1 stimulate Erk1/2 activation in PC12 cells, albeit via their own cognate receptor tyrosine kinases ErbB1 and IGF-1R, respectively. However, their cognate receptor transautophosphorylation sequences differ from that of TrkA such that these other growth factors are unable to cause phosphorylation of the phosphoPROTAC, as underscored by the results in Fig. S3. Therefore, EGF and IGF1 should spare FRS2 α from degradation by $TrkA^{PPFRS2\alpha}$. To test the specificity of activation of $TrkA^{PPFRS2\alpha}$ in intact cells, we treated PC12 cells with $TrkA^{PPFRS2\alpha}$ in the presence of NGF, EGF, or IGF1 and measured their levels of FRS2 α . Due to the presence of a small, ligand-independent (“tonic”) level of TrkA activity in the PC12 cells (23), which could over time partially activate the phosphoPROTAC even in the absence of NGF, it was necessary to reduce this tonic activity of TrkA in non-NGF-treated cells with an oxindole-based, small molecule, selective TrkA inhibitor (24). Using this strategy, we were able to activate selectively either the EGF receptor or the IGF-1R in the absence of tonic TrkA activity and monitor the corresponding effect of $TrkA^{PPFRS2\alpha}$ on FRS2 α levels (Fig. 2E). We found that $TrkA^{PPFRS2\alpha}$ -induced FRS2 α degradation was induced by NGF, but not by other growth factors. Moreover, addition and activation of $TrkA^{PPFRS2\alpha}$ in PC12 cells did not significantly interfere with subsequent EGF- or IGF-triggered downstream signaling to Erk1/2 (Fig. 2F), underscoring the lack of off-target effects of the phosphoPROTAC.

PhosphoPROTAC-Dependent Blockade of Neuronal Differentiation.

We next determined whether incubation of PC12 cells with $TrkA^{PPFRS2\alpha}$ could inhibit the cellular consequences of NGF treatment: biologically meaningful protein knockdown of FRS2 α should reduce the induction of neuronal differentiation by NGF. As shown in Fig. S44, increasing concentrations of $TrkA^{PPFRS2\alpha}$ dose-dependently inhibited NGF-driven neurite extension in PC12 cells. In addition to this phenotype, PC12 cell differentiation can

be detected by measuring the amount of the neuron-associated cytoskeletal protein neurofilament, which is up-regulated by NGF (25). Indeed, treatment of PC12 cells with NGF for 48 h resulted in a large increase in neurofilament expression, which was blocked by cotreatment with $\text{Trk}^{\text{KA}}\text{PP}_{\text{FRS2}\alpha}$ at concentrations that also blocked neurite extension (Fig. S4B). Taken together, the results demonstrate the feasibility of a conditional phosphoPROTAC that is critically dependent on phosphorylation by a distinct RTK to induce posttranslational degradation of a specific target protein.

PhosphoPROTAC Targeting of Phosphatidylinositol-3-Kinase in Response to Neuregulin-ErbB2/ErbB3 Signaling. To test the applicability of our conditional knockdown strategy beyond the NGF-TrkA-FRS2 α pathway, we turned to a different receptor tyrosine kinase pathway, the ErbB2/ErbB3-phosphatidylinositol-3-kinase (PI3K) pathway. Studies have firmly established the importance of this signal transduction pathway in mitogenesis as well as in suppression of apoptosis. Heterodimerization of the EGF receptor family RTKs ErbB2 and ErbB3 occurs upon binding of the latter to its agonist, neuregulin (NRG). Formation of the heterodimer and transphosphorylation to activate signaling can also occur from overexpression of ErbB2, as frequently occurs in breast cancer and ovarian cells (26, 27). ErbB3 phosphorylation results in the recruitment of the lipid kinase PI3K via a SH2 domain of the PI3K p85 regulatory subunit (28). In turn, this results in PI3K activation and culminates in stimulation of the survival-promoting kinase Akt (protein kinase B), which is critically important in tumor cells for inactivation of proapoptotic mechanisms. Much attention has recently focused on inhibiting the ErbB3/PI3K-signaling pathway as an antitumor strategy.

To develop a phosphoPROTAC targeting PI3K in response to activated ErbB2/ErbB3, we synthesized another phosphoPROTAC composed of the same VHL-binding sequence and octa-D-Arg motif at its C terminus but having a different, 24-amino acid stretch at the N terminus (Fig. S5A) taken from a PI3K-binding domain on the intracellular region of ErbB3. Upon phosphorylation of the dual tyrosine residues in this motif by ErbB2, this 24-amino-acid sequence is predicted to bind PI3K and recruit it for ubiquitination and degradation. Accordingly, we designated this phosphoPROTAC as $\text{ErbB2}^{\text{PP}}\text{PI3K}$. We also synthesized a phospho-null variant of this phosphoPROTAC, $\text{ErbB2}^{\text{NP}}\text{PI3K}$ (Fig. S5B). As ErbB3 has more than one predicted PI3K-binding site, we first verified by pull-down assay that our selected 24-amino-acid targeting sequence was sufficient to recruit PI3K. We synthesized biotinylated versions (Fig. S5A and B) of the 24-amino-acid targeting sequences of $\text{ErbB2}^{\text{PP}}\text{PI3K}$ and $\text{ErbB2}^{\text{NP}}\text{PI3K}$:

these pull-down peptides each possess a C-terminal biotin group instead of the VHL-binding/octa-D-Arg moiety and tyrosines that are already phosphorylated. Upon incubating each peptide in whole-cell lysates from human breast cancer cell line MCF-7 followed by streptavidin-agarose pull-down, we detected the p85 subunit of PI3K in the pull-down from the phosphotyrosine-containing peptide, but not in the phenylalanine-substituted peptide (Fig. 3A). This demonstrated that our selected 24-amino-acid sequence from ErbB3 is sufficient to bind PI3K.

To test whether this PI3K-targeting phosphoPROTAC would work as effectively in living cells as the FRS2 α -targeting phosphoPROTAC, we treated MCF-7 cells with increasing concentrations of $\text{ErbB2}^{\text{PP}}\text{PI3K}$ in the presence of neuregulin-1 α (Fig. 3B). Encouragingly, a dose-dependent decrease in p85 PI3K levels was detected by immunoblotting. Similarly to $\text{Trk}^{\text{KA}}\text{PP}_{\text{FRS2}\alpha}$ in PC12 cells, concentrations of $\text{ErbB2}^{\text{PP}}\text{PI3K} \leq 20 \mu\text{M}$ were not effective at knocking down PI3K, and maximum target knock-down was achieved at concentrations above 40 μM . Furthermore, the loss of PI3K resulted in decreased Akt activation, indicating an important biological consequence of the PI3K knockdown by $\text{ErbB2}^{\text{PP}}\text{PI3K}$. Immunoblotting for CD54 (an unrelated membrane-associated protein) and SOCS3 showed no decreases in their abundance upon $\text{Trk}^{\text{KA}}\text{PP}_{\text{FRS2}\alpha}$ treatment, reflecting a degree of target selectivity by the phosphoPROTAC. Moreover, treatment of MCF-7 cells with the cognate phospho-null variant $\text{ErbB2}^{\text{NP}}\text{PI3K}$ (Fig. 3C) reaffirmed the pull-down result: that tyrosine phosphorylation of the peptide was necessary for it to be active, indicating the conditional nature of the knockdown.

Knockdown of PI3K in Cancer Cells Results in Loss of Viability. Because PI3K knockdown occurred in a phospho-dependent manner, we next tested whether $\text{ErbB2}^{\text{PP}}\text{PI3K}$ was able to produce the typical cytotoxic effect in cancer cells following inhibition of PI3K by small molecule inhibitors. For example, treatment of MCF-7 cells with the selective PI3K inhibitor LY294002 will decrease cell viability due to the resultant loss of Akt activation. Similarly, incubation of MCF-7 cells with increasing concentrations of $\text{ErbB2}^{\text{PP}}\text{PI3K}$ caused a loss of viability (Fig. 4). Note that, due to the longer time intervals required to monitor a decrease in cell viability (days vs. hours), concentrations of $\text{ErbB2}^{\text{PP}}\text{PI3K}$ lower than those needed to see a biochemical knockdown in a shorter time frame were able to mediate the toxic response. However, treatment of MCF-7 cells with equivalent concentrations of $\text{ErbB2}^{\text{NP}}\text{PI3K}$ caused no statistically significant changes in 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) conversion relative to control

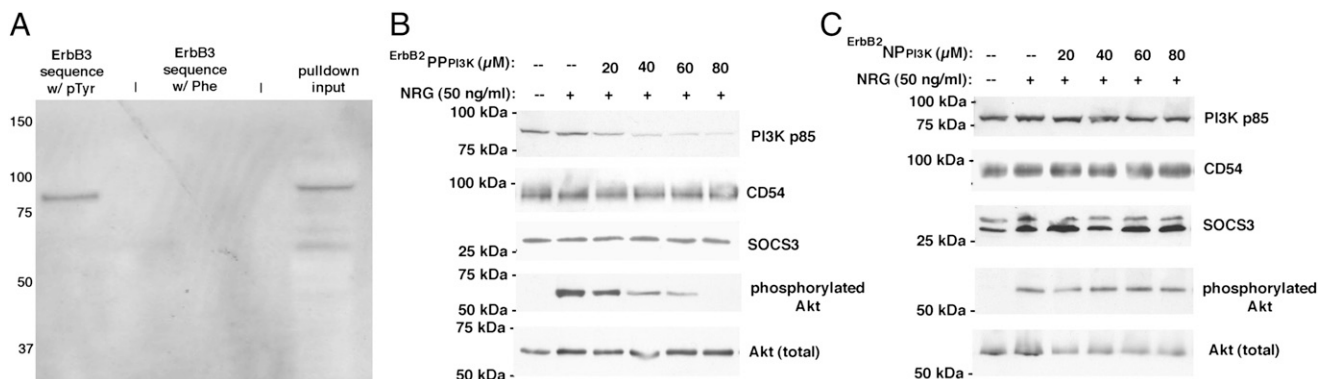


Fig. 3. $\text{ErbB2}^{\text{PP}}\text{PI3K}$, a phosphoPROTAC that conditionally targets PI3K for degradation upon ErbB2 activation. (A) Streptavidin pull-downs from MCF-7 cells using biotinylated peptides demonstrate efficacy and phospho-dependence of a selected dual phosphotyrosine-containing ErbB3 sequence to bind PI3K p85. Pull-downs electrophoresed and immunoblotted with anti-p85 antibody as described in *Materials and Methods*. (B) $\text{ErbB2}^{\text{PP}}\text{PI3K}$ -mediated degradation of PI3K is target specific and blocks NRG-dependent Akt activation in MCF-7 cells. (C) Null-phosphoPROTAC $\text{ErbB2}^{\text{NP}}\text{PI3K}$ cannot cause degradation of PI3K p85 even in the presence of neuregulin-activated ErbB2/ErbB3 signaling.

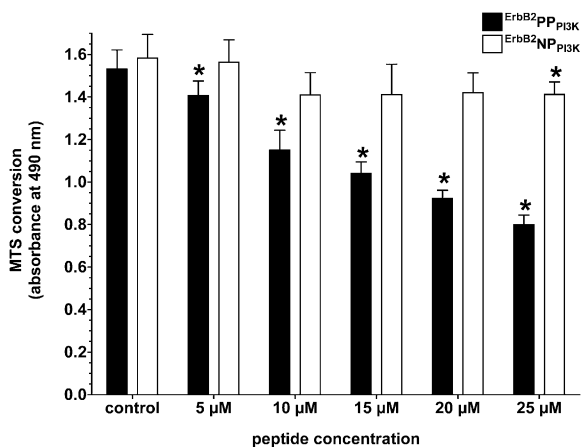


Fig. 4. Inhibition of PI3K signaling due to targeted knockdown by $\text{ErbB2PP}_{\text{PI3K}}$ is phospho-dependent and reduces cell viability. MCF-7 cells were incubated with varying concentrations of $\text{ErbB2PP}_{\text{PI3K}}$ or $\text{ErbB2NP}_{\text{PI3K}}$ for 48 h, following which viability was assessed using MTS conversion. Bars represent the mean \pm SD of three replicate experiments. Asterisk denotes significant difference in MTS conversion compared with control (untreated) cells determined by Student *t* test ($P < 0.05$).

cells (except at 25 μM), indicating a strongly attenuated toxic response to this null variant. The toxic effect seen with either peptide at 25 μM must not be due to a phosphorylation event, as $\text{ErbB2NP}_{\text{PI3K}}$ cannot be phosphorylated.

Consistent with the prediction that $\text{ErbB2PP}_{\text{PI3K}}$ reduces cell viability through inhibition of PI3K signaling, combined treatment of MCF-7 cells with both $\text{ErbB2PP}_{\text{PI3K}}$ and LY294002 led to a more pronounced reduction in MTS conversion than the sum of either treatment done separately (Fig. S64). For example, 2.5 μM LY294002 by itself causes a small 5% decrease in MTS conversion after 2 d, and 5 μM $\text{ErbB2PP}_{\text{PI3K}}$ alone also causes a small decrease of 5%; however, when added as a combined treatment to MCF-7 cells, the decrease in viability is close to 36%, rather than the sum of the individual treatments (10%). This synergy of inhibition supports the notion that both molecules are negatively affecting the same target, albeit in different modes: $\text{ErbB2PP}_{\text{PI3K}}$ causes stoichiometric knockdown of the PI3K regulatory subunit (p85), whereas LY294002 binds to and inhibits the catalytic subunit p110.

To confirm the phospho-dependent cytotoxicity in another cell line, we similarly tested both $\text{ErbB2PP}_{\text{PI3K}}$ and $\text{ErbB2NP}_{\text{PI3K}}$ in MDA-MB-175 cells. Like MCF-7 cells, MDA-MB-175 cells are breast cancer derived; unlike MCF-7 cells, MDA-MB-175 cells secrete their own neuregulin as part of an autocrine loop. MDA-MB-175 cells were even more sensitive (Fig. S6B) to the cytotoxic effect of $\text{ErbB2PP}_{\text{PI3K}}$ than were the MCF-7 cells. In agreement with this, $\text{ErbB2PP}_{\text{PI3K}}$ more potently knocked down PI3K levels in MDA-MB-175 cells than in MCF-7 cells (Fig. S7A). In addition to MDA-MB-175 and MDA-MB-231 (Fig. S7B) breast cancer cells, $\text{ErbB2PP}_{\text{PI3K}}$ was also able to knock down PI3K in two independent ovarian cancer cell lines, OVCAR8 and SK-OV-3 (Fig. S7B). The small differences in $\text{ErbB2PP}_{\text{PI3K}}$ -induced PI3K knockdown among various cell lines may reflect variations in signaling component levels or perhaps the relative degree to which the cells tested are dependent upon PI3K signaling for survival.

PhosphoPROTAC-Mediated Inhibition of Tumor Growth in Vivo. Given the activity of phosphoPROTACs to elicit biological responses in vitro, we tested whether $\text{ErbB2PP}_{\text{PI3K}}$ could inhibit tumor growth in vivo. We elected to use the OVCAR8 cells for our model tumor line due to their demonstrated in vitro sensitivity to $\text{ErbB2PP}_{\text{PI3K}}$, the lack of need for estrogen administration to the mice (a necessary adjuvant for MCF-7 cell tumorigenesis), and OVCAR8

dependence on activated ErbB2/ErbB3-to-Akt signaling for tumorigenicity. Athymic mice were s.c. implanted dorsally with OVCAR8 cells following which $\text{ErbB2PP}_{\text{PI3K}}$ was administered daily by i.p. injection at 10 mg/kg, which we determined to be the maximum tolerated dose as evidenced by lack of toxicity symptoms and steady weight gain by the mice over the course of the experiment (Table S1). For comparison, other mice were injected daily with either an equal dose of $\text{ErbB2NP}_{\text{PI3K}}$ or an equal volume of PBS vehicle (“control”). After a total of 47 d of daily peptide injection, mice were killed, and the s.c. tumors (for control, $n = 16$; for $\text{ErbB2PP}_{\text{PI3K}}$, $n = 16$; and for $\text{ErbB2NP}_{\text{PI3K}}$, $n = 18$) were surgically removed and weighed (Fig. S8). Mice that were treated daily with $\text{ErbB2PP}_{\text{PI3K}}$ showed an average tumor weight that was $\sim 40\%$ less than that in control mice. Conversely, mice that had received daily i.p. $\text{ErbB2NP}_{\text{PI3K}}$ injections developed tumors that were on average 10% smaller than in control mice. There was a statistically significant difference between the groups as determined by one-way ANOVA [$F(2,46) = 3.802$, $P = 0.030$]. Newman-Keuls post hoc analysis further specified where these differences exist: tumor growth in $\text{ErbB2PP}_{\text{PI3K}}$ -receiving mice was significantly different from that in control mice ($P < 0.05$) and in $\text{ErbB2NP}_{\text{PI3K}}$ -receiving mice as well ($P < 0.05$). However, there was no significant difference between the control and the $\text{ErbB2NP}_{\text{PI3K}}$ -receiving groups ($P > 0.05$). These data show that $\text{ErbB2PP}_{\text{PI3K}}$ retains its anticancer activity in live animals and further strongly suggest that in vivo activity of phosphoPROTACs is still dependent on phosphorylation of the peptide.

Discussion

Delineating the importance of various tyrosine kinase pathways in cell biology is an enormous challenge given overlapping downstream effectors and the limited number of kinase-specific small molecule inhibitors. In this report, we describe an approach to inhibit tyrosine kinase pathways that may take advantage of the intrinsic selectivity inherent in each signaling pathway. The first level of specificity exploited by this phosphoPROTAC approach arises from the specificity that individual tyrosine kinases possess for their respective substrates. By incorporating peptide sequences known to be phosphorylated by particular kinases, we take advantage of the natural specificity of individual signaling pathways. This was demonstrated by the lack of FRS2 α degradation by IGF-1R and ErbB1 (Fig. 2E), despite the ability of both kinases (shared with TrkA) to activate downstream MAPK. This methodology also inhibits particular signaling cascades not by targeting the kinases themselves but by inducing the degradation of particular downstream-signaling components such as FRS2 α and PI3K. Again, by using the natural affinity inherent in phosphotyrosine-containing peptides that serve as ligands for these SH2/PTB domain-containing proteins, we can effectively couple their degradation to the activation state of their cognate signaling cascade within the cell. Thus, these two levels of selectivity, i.e., at the kinase/substrate level and the effector protein/phosphoPROTAC binding level, co-opt nature’s ability to delineate particular signaling pathways from one another, alleviating the need to artificially engineer specificity into these cellular probes.

In addition to its ability to distinguish the activation state of various signaling pathways within a cell, this methodology may offer the ability to distinguish normal tissue from diseased tissue. Because dysregulation of various tyrosine kinase pathways leads to uncontrolled cell proliferation, the ability of a phosphoPROTAC to couple protein degradation to a particular growth factor-signaling pathway offers a possible cell-type selective anticancer strategy. Use of nucleic acid-based approaches for target protein knockdown in a mature, whole organism are being developed; conversely, use of peptide therapeutics, although not without caveats regarding their delivery and biological half-life, is well established. Furthermore, the conditional nature of the activation makes phosphoPROTACs suitable for the selective treatment of

malignant cells. For example, one could envision that ubiquitous PROTAC-mediated knockdown of a protein involved in cell growth or survival could have systemic deleterious effects (e.g., inhibition of PI3K by the small molecule inhibitor LY294002 has been found to be toxic in animal studies). In this study, we were able to administer to mice a dose of phosphoPROTAC that was able to blunt tumor growth with no apparent ill effects on animal health (as evidenced by the maintenance of body mass and lack of toxicity symptoms over the course of the experiment). The signaling context-dependent phosphoPROTAC knockdown of PI3K in only those cells with high levels of ErbB2/ErbB3 signaling could preferentially inhibit breast cancer or ovarian or pancreatic cancer cell proliferation (29).

Another strength of this approach is that it is unlikely to elicit drug-resistant mutations. Current antitumor tyrosine kinase inhibitors such as Tarceva and Iressa halt cell proliferation via inhibition of receptor tyrosine kinase activity, providing a strong selective pressure for target mutations that mitigate inhibitor binding and preserve kinase activity. In contrast, conditional phosphoPROTACs depend on the misregulated kinase activity found in many tumor types to stop cellular signaling and growth. Thus, mutation of kinase structure to prevent phosphoPROTAC activation would likely result in a loss of kinase signaling as well.

Finally, a key advantage of this methodology is its accessibility to the cell biology community. The generation of kinase-specific small molecule inhibitors currently requires the concerted efforts of chemists, pharmacologists, and biochemists. However, as shown here, these signaling pathway-specific cellular probes can be readily synthesized using an automated peptide synthesizer. Moreover, the “portability” of this approach is clear: because many of the important tyrosine kinase phosphotyrosine residue: SH2- or PTB-domain-containing protein pairings are already known, it is conceivable that additional phosphoPROTACs may prove useful in the exploration of the cell biology of different signaling pathways, especially in instances where no specific small molecule inhibitor currently exists.

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