The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation

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The phosphatase and tensin homolog (PTEN) is a tumor suppressor that is inactivated in many human cancers. PTEN loss has been associated with resistance to inhibitors of the epidermal growth factor receptor (EGFR), but the molecular basis of this resistance is unclear. It is believed that unopposed phosphatidylinositol-3-kinase (PI3K) activation through multiple receptor tyrosine kinases (RTKs) can relieve PTEN-deficient cancers from their "dependence" on EGFR or any other single RTK for survival. Here we report a distinct resistance mechanism whereby PTEN inactivation specifically raises EGFR activity by impairing the ligand-induced ubiquitylation and degradation of the activated receptor through destabilization of newly formed ubiquitin ligase Cbl complexes. PTEN-associated resistance to EGFR kinase inhibitors is phenocopied by expression of dominant negative Cbl and can be overcome by more complete EGFR kinase inhibition. PTEN inactivation does not confer resistance to inhibitors of the MET or PDGFRA kinase. Our study identifies a critical role for PTEN in EGFR signal termination and suggests that more potent EGFR inhibition should overcome resistance caused by PI3K pathway activation.

Cbl | ubiquitylation | PTEN | glioma | drug resistance

The phosphatidylinositol-3-kinase (PI3K) pathway has emerged as the most frequently deregulated signaling pathway in human cancer. Activation of this pathway in cancer cells can occur through a variety of mechanisms, including mutations in genes encoding the catalytic (PIK3CA) or regulatory (PIK3R1) subunit of class IA PI3Ks, the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), Akt family members, Ras family members, neurofibromin 1, and/or various growth factor receptors. Inactivation of *PTEN* through missense mutations, deletions, and epigenetic mechanisms represents the most common cause of PI3K pathway activation in human cancer (1).

The PTEN protein exhibits dual protein and lipid phosphatase activity. Most of PTEN's tumor suppressor functions have been attributed to its lipid phosphatase activity which hydrolyzes phosphatidylinositol 3,4,5-trisphosphate [*PtdIns(3,4,5)P₃*] at the D3 position of the inositol ring and directly antagonizes the function of phosphatidylinositol-3-kinase. PTEN loss leads to accumulation of *PtdIns(3,4,5)P₃* which recruits proteins containing pleckstrin homology domains to cellular membranes, including the serine/threonine kinase Akt. In addition to its role in tumor suppression, PTEN has emerged as a determinant of tumor cell response to ATP-site competitive inhibitors of the epidermal growth factor receptor (EGFR) in *EGFR* amplified cancer cell lines (2) and in glioblastoma (GBM) patients whose tumors expressed the oncogenic EGFR variant III (EGFRvIII) mutant receptor (3).

How PTEN's functions as tumor suppressor and drug response modifier relate to each other is currently unclear. One possibility is that PTEN inactivation relieves *EGFR* mutant cancer cells from their dependence on EGFR for survival by allowing sufficient *PtdIns(3,4,5)P₃* accumulation and Akt activation through other growth factor receptors. However, although there is evidence for receptor tyrosine kinase coactivation in cancer (4) and certain growth factor receptors have been shown to mediate resistance to EGFR kinase inhibitors (5–7), it is unknown which kinase(s), if any, might substitute for EGFR in the setting of PTEN inactivation. Furthermore, PTEN inactivation has only been associated with clinical resistance to inhibitors of EGFR and its coreceptor HER2, but not other growth factor receptors. In this study, we sought to determine the molecular mechanism whereby PTEN inactivation confers resistance to EGFR kinase inhibitors.

Results

PTEN Knockdown Confers Resistance to EGFR, but Not MET or PDGFR Kinase Inhibitors. To test whether PTEN inactivation relieves cancer cells from their "dependence" on any single growth factor signal for survival, we selected a panel of cancer cell lines with distinct activated growth factor receptors, infected them with a retroviral PTEN shRNA, derived sublines with stable PTEN knockdown, and then determined how PTEN knockdown influenced the response of these cells to inhibitors of the respective oncogenic kinase. We focused on EGFR, platelet-derived growth factor receptor A (PDGFRA) and MET because mutations in these growth factors are found in multiple human cancer types and because small molecule inhibitors of these kinases are either already in use or in advanced stages of clinical development for the treatment of human cancer.

Treatment of *EGFR*-amplified A431 cells with the irreversible EGFR kinase inhibitor EKB-569 resulted in dose-dependent induction of cell death as determined by the trypan-blue method and cleavage of the Caspase3 substrate PARP. In contrast to parental A431 cells, A431 cells with stable PTEN knockdown (Fig. 1*A*) were markedly resistant to cell death induction by EKB-569

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A

A431 (*EGFR* AMP) — PTEN, p85 (parental, PTEN shRNA)
HCC827 (Mut *EGFR*) — PTEN, p85
HCC4006 (Mut *EGFR*) — PTEN, p85

B

A431 (*EGFR* AMP) — % Cell Death — Parental / PTEN shRNA — 64 nM, 128 nM, 256 nM — EKB-569

C

HCC827 (*EGFRdel746-750*) — % Cell Death — Parental / PTEN shRNA — 2 nM, 4 nM, 8 nM
HCC4006 (*EGFRdel746-749*) — Parental / PTEN shRNA — 65 nM — EKB-569

D

MKN45 (*MET* AMP) — PTEN, p85 (parental, PTEN shRNA)
EBC1 (*MET* AMP) — PTEN, p85
H1703 (*PDGFRA* AMP) — PTEN, p85
TS-543 (*PDGFRA* AMP) — PTEN, p85

MKN45 — % Cell Death — Parental / PTEN shRNA — 100, 200, 400, 800 — SU11274 [nM]
EBC1 — Parental / PTEN shRNA — 100, 200, 400, 800 — SU11274 [nM]
H1703 — Parental / PTEN shRNA — 250, 1000 — Imatinib [nM]
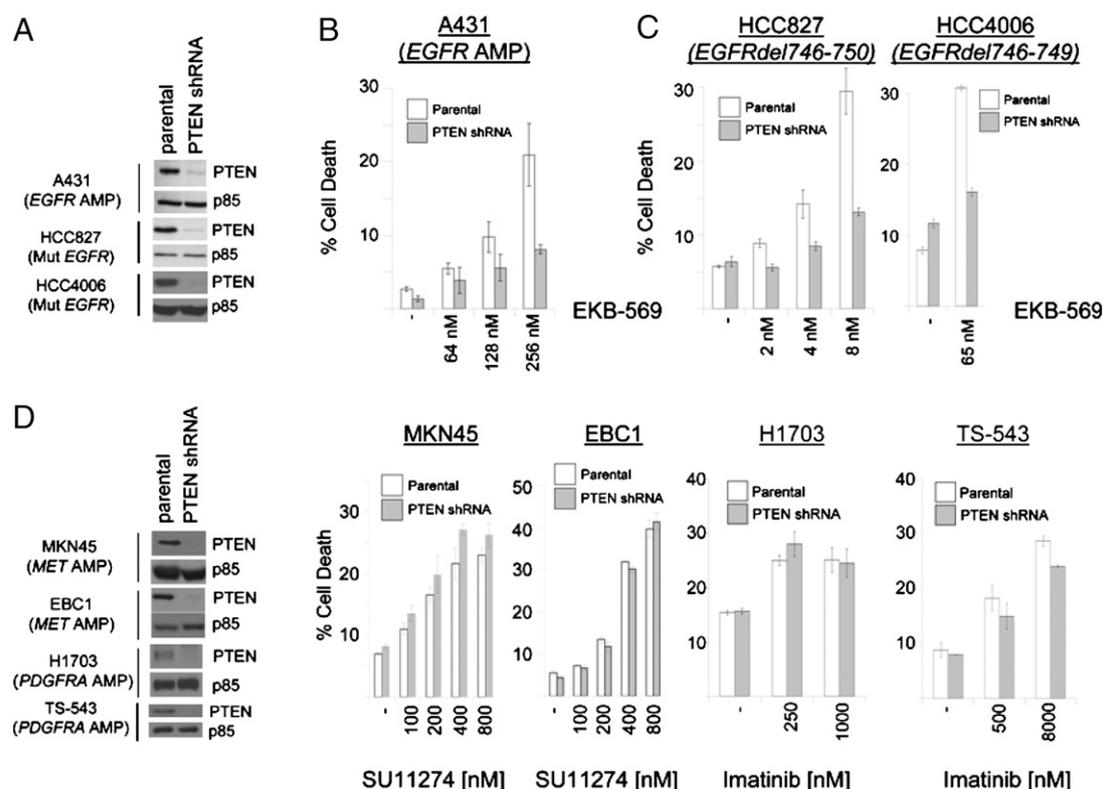TS-543 — Parental / PTEN shRNA — 500, 8000 — Imatinib [nM]

Fig. 1. PTEN silencing confers resistance to EGFR kinase inhibitors, but not to inhibitors of c-Met or PDGFRA. (*A*) Knockdown of PTEN in cancer cell lines harboring amplified wild-type or mutant EGFR. Shown are PTEN and p85 immunoblots of whole cell lysates from A431, HCC827, and HCC4006 cells stably transduced with a PTEN shRNA. (*B* and *C*) PTEN inactivation is sufficient to confer resistance to the EGFR kinase inhibitor EKB-569. A431 cells (*A*), which carry a highly amplified wild-type EGFR gene, or HCC827 and HCC4006 (*C*), which carry mutant EGFR, were treated with the indicated doses of EKB-569. The graph shows cell death (means ± SE) 5 days after treatment. (*D*) PTEN inactivation does not confer resistance to MET or PDGFR inhibitors. Immunoblots show knockdown of PTEN in cancer cell lines with *MET* (MKN45 and EBC1) or *PDGFRA* (H1703 and TS-543) amplification (*Left*). The bar graphs show cell death induction by the MET kinase inhibitor SU11274 or the PDGFR kinase inhibitor imatinib.

(Fig. 1*B* and Fig. S1*A*). To confirm that this resistance was indeed due to PTEN knockdown, rather than potential off-target effects of the PTENshRNA construct, we reconstituted PTEN in A431 PTENshRNA cells using a murine PTEN cDNA (mPTEN) that lacks the targeting sequence of our hairpin. Reintroduction of wild-type PTEN, but not a naturally occurring PTEN mutant that lacks lipid phosphatase activity [mPTEN(G129E)], restored the sensitivity of A431 PTENshRNA cells to EGFR kinase inhibitors (Fig. S1*B*). This result documents the specificity of our PTEN shRNA construct and indicates that the lipid phosphatase activity of PTEN is the critical mediator of PTEN-associated EGFR kinase inhibitor resistance.

The EGFR mutant lung cancer cell lines HCC827 (*EGFRdel746-750*) and HCC4006 (*EGFRdel746-749*) underwent cell death at lower EKB-569 concentrations than A431 cells, consistent with the reported enhanced sensitivity of these EGFR mutants to EGFR kinase inhibitors. This effect was again markedly blunted by PTEN knockdown (Fig. 1 *A* and *C*). In contrast to our findings with *EGFR* mutant cancer cell lines, PTEN knockdown did not protect cancer cell lines harboring amplification of the *MET* growth factor receptor kinase (MKN45 and EBC1 cells) from cell death in response to the MET kinase inhibitor SU11274. PTEN knockdown also did not protect cell lines with amplification of the *PDGFRA* gene (H-1703 and TS543 cells) from cell death induction by the PDGFR inhibitor imatinib (Fig. 1*D*). These results were confirmed with other inhibitors of the MET and PDGFR kinase.

Increased EGFR Activity in PTEN Deficient Cells. Our finding that PTEN inactivation did not affect the response of *MET* or *PDGFRA*

amplified cancer cells to inhibitors of the MET and PDGFR kinase, respectively, pointed toward a more intricate relationship between PTEN and EGFR. We therefore examined the biochemical effects of PTEN on the EGFR protein. Immunoblotting of A431 whole cell lysates with phosphosite-specific antibodies against EGFR demonstrated dose-dependent EGFR inhibition by EKB-569 in both parental and PTEN knockdown cells. However, PTEN knockdown cells consistently showed greater EGFR phosphorylation than their matched controls in the absence of drug and at equimolar concentrations of EKB-569 (Fig. 2*A Upper*). PTEN knockdown did not increase phosphorylation of the MET receptors under steady-state conditions and also did not right-shift the biochemical response of these receptors to SU11274 (Fig. 2*A Lower*), respectively.

We also examined the relationship between PTEN, EGFR phosphorylation, and EGFR kinase inhibitor response in pretreatment tumor samples from GBM patients who either responded to ($n = 5$) or failed ($n = 5$) EGFR kinase inhibitor therapy in the context of our previously reported clinical trial (3). The group of PTEN-deficient tumors that failed treatment showed a statistically significant increase in EGFR phosphorylation compared to PTEN-expressing tumors from clinical responders ($P = 0.021$, Fisher's exact test) (Fig. 2*B*). Loss of PTEN protein expression was similarly associated with increased EGFR phosphorylation in glioblastomas, which did not express the EGFRvIII mutant (Fig. S2).

Because EGFR phosphorylation provides only a limited readout for EGFR kinase activity, we next examined the effects of PTEN on a broader panel of EGFR substrates. These experiments took advantage of a microfluidic-based phosphopeptide array, which measures phosphorylation rates of 144 matrix-spotted
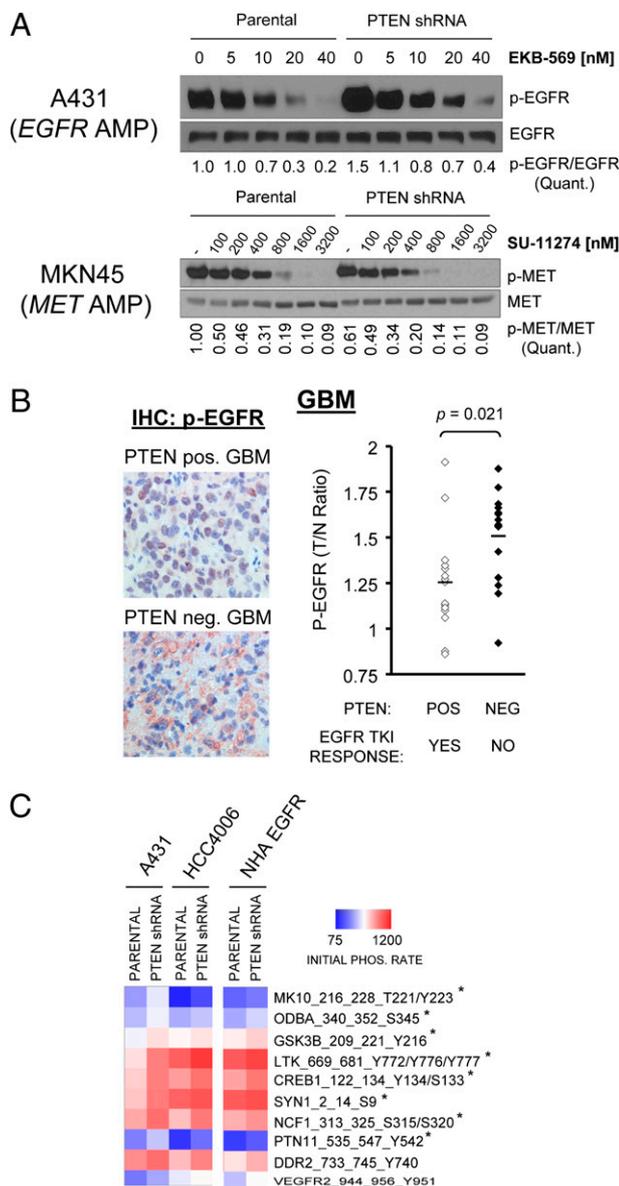
Fig. 2. PTEN-deficient cancers show increased EGFR activation. (*A*) PTEN inactivation raises EGFR phosphorylation in A431 cells and enhances residual EGFR phosphorylation during treatment with EKB-569. Cells were treated with the indicated concentrations of EKB-569 for 6 h and whole cell lysates immunoblotted with the indicated antibodies. (*B*) PTEN deficient glioblastomas from patients that fail EGFR kinase inhibitor therapy (n = 5) show greater EGFR phosphorylation than PTEN expressing tumors from patients that respond (n = 5). Tissue sections were stained with an antibody against activated EGFR and staining intensity was quantified as tumor/normal ratio in three areas per tumor. The *Left* panels show examples of p-EGFR IHC staining from a PTEN positive tumor (GBM no. 4) and PTEN-negative tumor (GBM no. 20). The solid line on the *Right* graph indicates the median pEGFR staining intensity per group. (*C*) PTEN inactivation enhances phosphorylation of 10 EGFR-regulated peptides. Whole cell lysates from four different cell lines and their matching PTEN knockdown sublines were incubated on Pamgene phosphopeptide arrays (*Materials and Methods* and Fig. S3). Peptides that are significantly (P < 0.05) up-regulated in all three EGFR overexpressing cell line pairs (A431, HCC4006, and NHA-EGFR,) are marked with an asterisk. The heat map shows initial phosphorylation velocities for each peptide.

peptides following the addition of whole cell lysates, adenosine-5′-triphosphate, and a fluorescently labeled antiphosphotyrosine antibody (Fig. S3*A*). We previously identified 10 of these peptides as EGFR-regulated peptides (See *Materials and Methods*) and confirmed in preliminary experiments that most of these peptides

indeed showed increased phosphorylation when we compared lysates from normal human astrocytes (NHA) engineered to express "high EGFR" levels with a matching "low EGFR" expressing subline (Fig. S3*B*). We then compared whole cell lysates from three different PTEN knockdown lines (A431, HCC4006, and NHA-EGFR) with lysates from their matching parental cell lines and found that 8/10 EGFR-related peptides showed significantly increased phosphorylation in all three PTEN knockdown lines (Fig. 2*C*). The other two peptides (DDR2_733_745_Y740 and VEGFR2_944_956_Y951) showed increased phosphorylation in 2/3 PTEN knockdown lines (Table S1). None of the other detectable peptides showed a similarly consistent change in phosphorylation rates in response to PTEN knockdown (P = 0.0173).

PTEN Accelerates Down-Regulation of Activated EGF Receptors. To determine how PTEN might regulate EGFR activity, we first surveyed RNA transcript levels for EGFR, its coreceptors, and its ligands in the A431 and HCC827 isogenic pairs, but did not find any statistically significant differences between PTEN-intact and
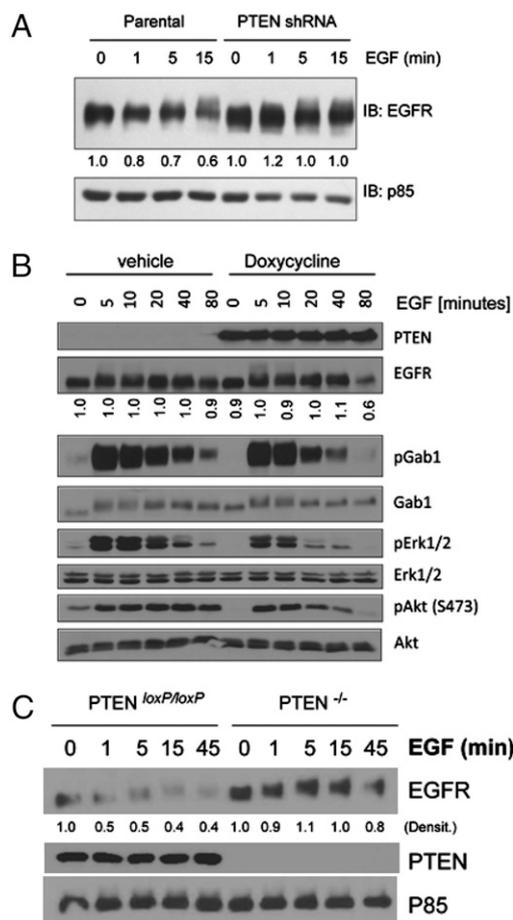

Fig. 3. PTEN inactivation blunts EGFR signal termination. (*A*) PTEN modulates ligand-induced EGFR down-regulation. Inactivation of PTEN through RNAi (*A*) or gene targeting (*C*) impairs ligand-induced down-regulation of EGFR. A431 and A431-PTENshRNA cells, or PTEN *loxP/loxP* and PTEN⁻/⁻ MEFs were serum starved and challenged with EGF for the indicated times. Lysates were generated and probed with EGFR, p85, and PTEN antibodies as indicated. Shown underneath are densitometric measurements of EGFR levels. (*B*) Acute PTEN restoration in PTEN-deficient cells corrects their defect in EGFR signal termination. PC3 prostate cancer cells expressing a tetracycline-inducible allele of PTEN were serum starved in the presence or absence of doxycycline and challenged with EGF as indicated. Lysates were immunoblotted with various pathway-related antibodies as shown.

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PTEN-deficient sublines (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession no. GDS2958).

We next examined the effect of PTEN on ligand-induced EGFR activation and subsequent signal termination. Down-regulation of the EGFR protein in response to EGF was blunted in the A431 subline with PTEN knockdown when compared to parental A431 cells (Fig. 3A, compare lanes 8 and 4). We conducted similar experiments in PC3 cells, which are naturally PTEN deficient but in which PTEN expression could be restored acutely using a doxycycline-inducible PTEN allele. Pretreatment of these cells with doxycyline for 36 h resulted in robust PTEN induction and inhibition of pAkt (Fig. 3B). Treatment with EGF resulted in down-regulation of EGFR protein levels only after PTEN expression had been restored by pretreatment with doxycycline (Fig. 3B, lane 12 versus lane 6). Consistent with the accelerated down-regulation of EGFR, phosphorylation of the EGFR substrate Gab1 and the EGFR effector molecule ERK1/2 in response to EGF was markedly shortened in the presence of PTEN (Fig. 3B).

To explore whether PTEN also regulates EGFR signal termination in nonmalignant cells, we used embryonic fibroblasts derived from mice with a "floxed" PTEN gene [PTEN$^{loxP/loxP}$ mouse embryonic fibroblasts (MEFs)]. Due to the low amount of endogenous EGFR in these cells, we first retrovirally introduced human wild-type EGFR into these cells, derived stable subclones through antibiotic selection, and subsequently inactivated PTEN in a second round of infection with Cre recombinase. Consistent with our observations in the PC3 system, PTEN inactivation markedly blunted down-regulation of EGFR protein levels in response to EGF (Fig. 3C, lanes 6–10 vs. 1–5).

PTEN Promotes Ubiquitylation of Activated EGF Receptors. Down-regulation of activated EGF receptors involves EGFR internalization from the cell surface and subsequent shuttling of the receptors through various intracellular compartments to the lysosome (8). We found no defect in EGFR internalization in PTEN-deficient cells using immunofluorescence and by measuring the shuttling of biotinylated EGFR from the cell surface to the cytoplasm (Fig. S4).

We next examined the effect of PTEN on the ligand-induced ubiquitylation of EGFR, a process that triages the endocytosed receptor to the degradative, lysosomal pathway (i.e., rather than receptor recycling to the cell surface) (9). EGF stimulation of PTEN$^{loxP/loxP}$ MEFs for 5 minutes resulted in EGFR ubiquitylation, seen as high-molecular weight bands on ubiquitin immunoblots of EGFR immunoprecipitates. EGFR ubiquitylation was markedly reduced in PTEN$^{-/-}$ MEFs (Fig. 4A). We observed a similar effect of PTEN on EGFR ubiquitylation in all examined cancer cell lines. In A431 cells, EGF stimulation for 5 minutes resulted in EGFR ubiquitylation, seen again as upward smearing on ubiquitin immunoblots of EGFR immunoprecipitates and also, less dramatically, by the upward smearing on EGFR immunoblots. EGF-induced EGFR ubiquitylation in A431 cells was markedly reduced in the PTEN knockdown line (Fig. 4B). Conversely, acute PTEN restoration in PTEN-deficient PC3 cells (Fig. S5A) or in PTEN-deficient U87 glioblastoma cells (Fig. S5B) augmented EGF-induced ubiquitylation of EGFR (Fig. S5A). The endogenous del746-750 EGFR mutant expressed in HCC827 cells showed less ubiquitylation than wild-type EGFR in response to EGF, as previously reported (10), but this ubiquitylation defect was further enhanced by PTEN knockdown (Fig. S5C), suggesting that PTEN inactivation and EGFR mutation may affect receptor ubiquitylation through distinct molecular mechanisms. Of note, we did not observe any impairment in HGF-induced ubiquitylation of the Cbl client MET in our PTEN-isogenic cell lines, suggesting that the effect of PTEN on Cbl-mediated EGFR ubiquitylation was not due to a general impairment in Cbl function.

To determine whether the effect of PTEN on EGF-induced ubiquitylation of EGFR was mediated by the lipid phosphatase
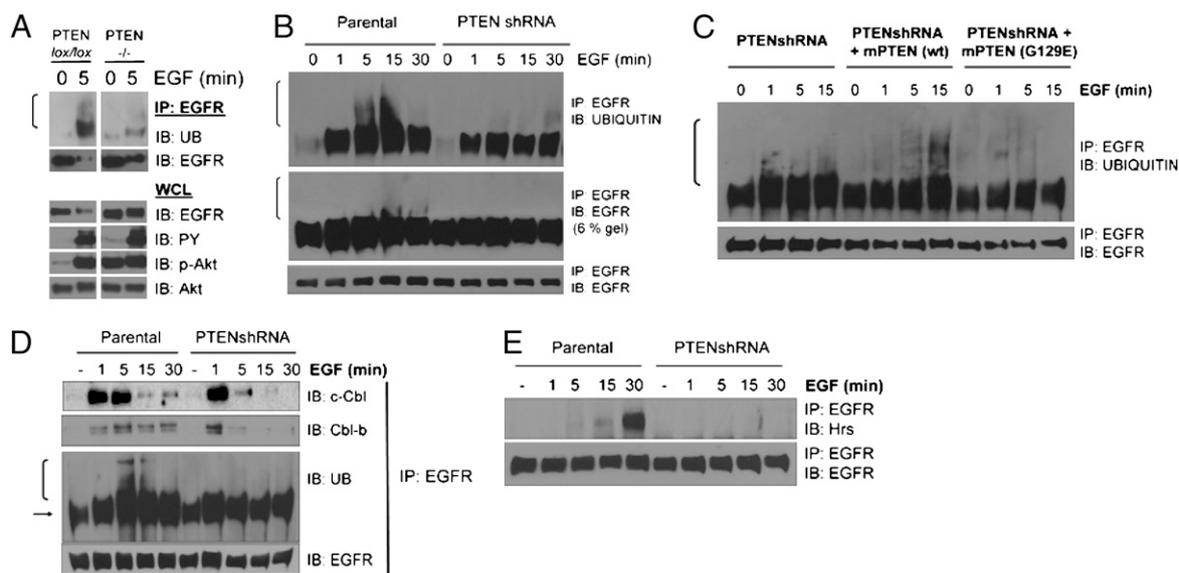

Fig. 4. PTEN inactivation impairs EGFR ubiquitylation through destabilization of the EGFR–Cbl complex. PTEN inactivation inhibits EGFR ubiquitylation in MEFs (A) and A431 cells (B, C, and D). (A) PTEN$^{loxP/loxP}$ and PTEN$^{-/-}$ MEFs were serum starved and incubated for 5 min with EGF. EGFR immunoprecipitates (Upper) and whole cell lysates (WCL, Lower) were probed with the indicated antibodies. The Left brace indicates more extensively ubiquitylated EGFR. (B) A431 and A431-PTEN-shRNA cells were stimulated with 100 ng/mL EGF for the indicated times and cell lysates were analyzed as indicated. The Left bracket indicates more extensively ubiquitylated EGFR. (C) Replacement of endogenous PTEN with heterologous wild type, but not lipid-phosphatase-dead PTEN can preserve ligand-induced EGFR ubiquitylation. A431 cells were sequentially and stably transduced with shRNA-resistant forms of either wild-type or a lipid-phosphatase-dead (G129E) PTEN cDNA, followed by a PTEN shRNA that targets endogenous PTEN. The resulting cells were subjected to ubiquitylation analysis as in B. The Left bracket indicates more extensively ubiquitylated EGFR. (D) PTEN knockdown destabilizes the EGFR–Cbl complex. A431 and A431-PTENshRNA cells were serum starved, challenged with 100 ng/mL EGF for the indicated times, and lysed under nondenaturing conditions. Shown are EGFR immunoprecipitates probed with antibodies against EGFR, c-Cbl, and Cbl-b. The bracket indicates more extensively ubiquitylated EGFR, whereas the arrow points toward less extensively ubiquitylated receptors. (E) PTEN inactivation impairs the ligand-induced recruitment of Hrs to EGFR. A431 and A431-PTEN-shRNA cells were treated and lysed as in D. EGFR immunoprecipitates were analyzed by Western blot using the indicated antibodies.

activity of PTEN, we reexamined ligand-induced EGFR ubiquitylation in A431 PTEN shRNA cells with reconstitution of either wild-type or lipid-phosphatase-dead PTEN. Reconstitution of wild-type *PTEN*, but not the G129E *PTEN* mutant, was sufficient to restore EGFR ubiquitylation in response to EGF (Fig. 4C). Overexpression of a constitutively active mutant of Akt1 found in sporadic human cancers (*Akt1 E17K*) blunted the EGF-induced ubiquitylation of EGFR (Fig. S6A) and conferred resistance to EKB-569 (Fig. S6B). Together, these data suggest that PTEN regulates EGFR ubiquitylation through $PtdIns(3,4,5)P_3$ and Akt-dependent mechanisms.

PTEN Stabilizes the EGFR–CBL Complex. EGFR ubiquitylation is mediated by the Cbl family of RING domain E3 ubiquitin ligases, which bind to the activated receptor and assemble a multiprotein complex including hepatocyte growth factor-regulated substrate (Hrs) and other members of the endosomal sorting complex required for transport (ESCRT) (9). Because both c-Cbl and Cbl-b are recruited to the activated EGFR and serve redundant functions in receptor ubiquitylation, we examined the effect of PTEN on EGFR association with these Cbl family members. In parental A431 cells, immunoprecipitation of EGFR under nondenaturing conditions showed rapid recruitment of both Cbl family members to EGFR (Fig. 4D) with a more sustained association between EGFR and Cbl-b as previously reported (11). A431 cells with PTEN knockdown showed similarly rapid recruitment of both Cbl family members to EGFR, but the durability of the EGFR–Cbl complex was substantially shortened with barely detectable EGFR–CBL association beyond 5 min following EGF induction (Fig. 4D).

Consistent with the early disassembly of the EGFR–Cbl complex and incomplete EGFR ubiquitylation in the PTEN knockdown cells, we were unable to detect recruitment of ubiquitin binding protein Hrs to the EGFR complex in these cells (Fig. 4E).

Residual EGFR Activity Determines Cell Death in Response to EGFR Kinase Inhibition. Our finding that PTEN regulates EGFR signal termination raised the question of whether this function of PTEN might contribute to EGFR kinase inhibitor resistance in cells that require chronic EGFR signaling for survival ("EGFR-dependent cells"). If PTEN inactivation indeed conferred drug resistance through its effects on EGFR activity, i.e., rather than EGFR-independent mechanisms, we would expect that: (*i*) genetic inactivation of Cbl, the critical mediator of the PTEN–EGFR crosstalk, should be sufficient to confer EGFR kinase inhibitor resistance, and (*ii*) more complete EGFR kinase inhibition should overcome EGFR kinase inhibitor resistance associated with PTEN inactivation.

We first examined whether ectopic expression of 70Z-Cbl (12), a naturally occurring c-Cbl mutant, which lacks RING domain activity and functions as a dominant negative allele, rendered EGFR mutant cells resistant to cell death induction by EKB-569. Overexpression of 70Z-Cbl was sufficient to confer resistance to EKB-569 (Fig. 5A and Fig. S7).

We next examined whether PTEN-associated resistance to EGFR kinase inhibitors could be overcome by more complete EGFR kinase inhibition. Higher concentrations of EKB-569 were indeed able to completely overcome the protective effects of PTEN inactivation on cell death induction by this drug (Fig. 5B Left). To ascertain that cell death at these increased EKB-569 concentrations was due to inhibition of the EGFR/HER2 signaling unit rather than EKB-569 effects on other kinases, we engineered a HER2 mutant, which constitutively active due to an insertion in the HER2 kinase domain (*YVMA HER2*) and was also resistant to EKB-569 due to a second mutation in a drug-interaction residue (Cys805Ser). Stable expression of this double mutant (*HER2 YVMA-CS*) in HCC827-PTENshRNA cells restored phosphorylation of EGFR and HER2 in the presence of EKB-569 (Fig. 5B Right) and rescued cell death induction by EKB-569, even at increased EKB-569 concentrations (Fig. 5B Middle).
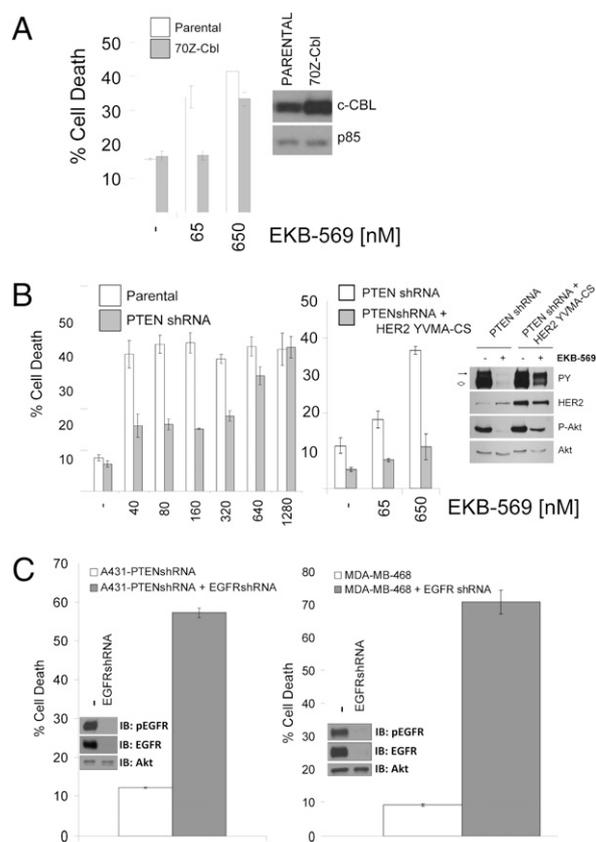

Fig. 5. EGFR signal strength determines apoptotic response to EGFR kinase inhibitors. (*A*) Dominant-negative Cbl confers EGFR kinase inhibitor resistance. HCC4006 cells were stably transduced with the naturally occurring 70Z-Cbl allele and cultured for 5 days in the presence of the indicated concentrations of EKB-569. Cell death was assessed by the trypan blue exclusion (means ± SE). Expression of the 70Z-Cbl transgene was confirmed by immunoblotting (*Inset*). (*B*) More complete EGFR inhibition overcomes PTEN-associated resistance. (*Left*) HCC827 and HCC827-PTENshRNA cells were treated with the indicated concentrations of EKB-569. Cell death was assessed 5 days after treatment (*Left*) (means ± SE). (*Center* and *Right*) HCC827-PTEN shRNA cells were stably transduced with a constitutively active and EKB-569-resistant allele of HER2 (HER2 YVMA-CS) and cultured with the indicated doses of EKB-569. Cell death was assessed as elsewhere (means ± SE). The expression of the mutant HER2 allele and its resistance to EKB-569 were documented by immunoblot as shown on the *Right*. (*C*) Efficient EGFR knockdown promotes massive cell death in PTEN-deficient EGFR-amplified cells. A431-PTENsh RNA (*Left*) and MDA-MB-468 (*Right*) cells were acutely infected with the indicated retroviruses and cell death was assessed by trypan blue exclusion. EGFR knockdown was confirmed by immunoblot (*Insets*). Akt was used as a loading control.

Finally, we acutely knocked down EGFR in A431-PTENshRNA cells and in MDA-MB-468 cells which are naturally PTEN deficient and harbor *EGFR* amplification. In both cell lines, acute EGFR knockdown triggered massive cell death (Fig. 5C).

Discussion

Our study describes a previously unrecognized role of the PTEN tumor suppressor in EGFR signal termination. Several mutant growth factor receptors found in human cancer have been shown to derive their oncogenic properties by escaping Cbl-mediated receptor down-regulation (13). It is intriguing to speculate that the strong genetic association between *EGFR* amplification and *PTEN* inactivation in glioblastoma (Fig. S8) and perhaps other human cancers (14) is, at least in part, due to the effects of PTEN on EGFR signal output.

Several EGFR kinase domain mutants found in lung cancer and the EGFRvIII mutant (Fig. S9) are substrates of the Cbl E3 ligase,

albeit with reduced affinity relative to wild-type EGFR. In contrast to mutations in *EGFR* which impair Cbl recruitment to the mutant receptor (15), PTEN inactivation impairs EGFR signal termination at a later step during receptor down-regulation, namely the continued association between EGFR and Cbl family members (Fig. 4*D*). Maintenance of the EGFR–Cbl complex is critical to counteract the activity of deubiquitylating enzymes in the early endosomal compartments and allow efficient interaction between fully ubiquitylated EGF receptors with members of the ESCRT complex (16). Whereas our results with mutant alleles of PTEN and Akt1 suggest that PTEN regulates EGFR signal termination, through its lipid phosphatase activity, we cannot formally exclude the possibility that $PtdIns(3,4,5)P_3$-dependent and Akt-independent effects further contribute to the effects of PTEN inactivation on EGFR. Further work is required to dissect whether changes in the phosphoinositide pool caused by other oncogenic perturbations of the PI3K pathway may also affect the stability of the EGFR–CBL interaction.

Our results have several important implications for therapeutic targeting of growth factor receptors in cancer. Our finding that PTEN inactivation does not impair the cell death response of *MET*- or *PDGFRA*-amplified cancer cells to MET and PDGFR kinase inhibitors, respectively, is encouraging for future therapeutic attempts to target these growth factor receptors in cancer types with coexistent mutations in PTEN or PI3K. In terms of EGFR, we show that activation of the PI3K pathway does not relieve EGFR-driven cancer cells from their dependence on EGFR for survival but, instead, shifts the EGFR kinase inhibitor response curve toward drug concentrations, which may be difficult to reach in tumor tissue, in particular in the central nervous system (17). Our finding that the modest boost in EGFR activity conferred by PTEN inactivation is sufficient to confer resistance to EGFR kinase inhibitors is reminiscent of the observation that the *T790M-L858R EGFR* mutant, responsible for about 50% of cases with acquired EGFR kinase inhibitor resistance in lung cancer (18), confers resistance even when present at an allelic frequency of only ~3% (19). Collectively, these results indicate that maximal EGFR kinase inhibition, perhaps only achievable by pulsatile kinase inhibitor therapy (20) or multipronged attacks on the EGFR life cycle, remains a therapeutic goal for the treatment of EGFR mutant cancer harboring activating lesions in the PI3K pathway.

Materials and Methods

Generation of Stably Transduced Cell Lines. To generate EGFR-expressing Pten$^{lox/lox}$ and Pten$^{-/-}$ MEFs, EGFR retrovirus was made by transfecting pLXSN-Neo-EGFR and pCL-ampho packaging plasmid into 293T cells. Virus was collected and Pten$^{lox/lox}$ MEFs were infected and selected with 800 μg/mL G418. To knockout PTEN, G418-resistant cell populations were infected with retrovirus generated in 293T cells with pMSCV-puro-Cre and pCL-ampho and selected with 5 μg/mL puromycin. A431-WT-PTEN-PTEN-shRNA and A431-PTEN-G129E-PTEN-shRNA cells were generated by sequential stable infections. First, A431 cells were infected with either pLNCX2-mPTEN or pLNCX2-mPTEN-G129E and selected with 800 μg/mL G418. Subsequently, G418-resistant cells were infected with pSIREN-RetroQ-PTEN and selected with 5 μg/mL puromycin. All other stable lines were generated similarly.

Western Blot and Immunoprecipitations. Cells were harvested directly on tissue culture plates. When only Western blots were carried out on cell lysates, 1% triton lysis buffer (Cell Signaling Technologies) supplemented with protease and phosphatase inhibitors was used. Lysates were sonicated, cleared by centrifugation, and normalized to equal amounts of total protein using the DC protein assay (Biorad). Immunoprecipitations to determine EGFR ubiquitylation were done under denaturing conditions. Cells were serum starved and treated with 10 μM lactacystine 30 min before ligand stimulation. Cells were then washed with cold PBS containing 10 μM N-ethylmaleimide and harvested in lysis buffer supplemented with 1% SDS, protease inhibitors, phosphatasae inhibitors, and 10 μM N-ethylmaleimide. Lysates were sonicated and cleared by centrifugation. Cleared lysates were then subjected to immunoprecipitation. After an overnight incubation, 80 μL of proteinA/proteinG beads was added and pull-down was carried out for an additional 2 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer and eluted with 3× SDS loading buffer. EGFR–Cbl coimmunoprecipitations were done similarly with the following modification: the lysis buffer contained 50 mM hepes (pH 7.4), 150 nM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% glycerol, protease, and phosphatase inhibitors. After addition of lysis buffer, lysates were incubated on ice for 30 min without sonication before they were cleared by centrifugation. Cleared lysates were subjected to immunoprecpitation as described.

Phosphopeptide Array. We examined phosphorylation of 144 peptides spotted on a three-dimensional porous matrix produced and commercialized by Pamgene (www.pamgene.com). Phosphorylation on these arrays is measured kinetically, using a microfluidic device with an integrated CCD camera as described elsewhere (21).

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