Pten loss promotes MAPK pathway dependency in HER2/neu breast carcinomas

Saya H. Ebbesen[a,b,c], Maurizio Scaltriti[d,e], Carl U. Bialucha[b,1], Natasha Morse[d], Edward R. Kastenhuber[c,f], Hannah Y. Wen[e], Lukas E. Dow[c,2], José Baselga[d,g,h], and Scott W. Lowe[c,i,3]

[a]Watson School of Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; [b]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; [c]Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065; [d]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065; [e]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY 10065; [f]Louis V. Gerstner Jr. Graduate School of Biomedical Sciences, Memorial Sloan Kettering Cancer Center, New York, NY 10065; [g]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065; [h]Department of Medicine, Weill Cornell Medicine, New York, NY 10065; and [i]Howard Hughes Medical Institute, New York, NY 10065

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Loss of the tumor suppressor gene *PTEN* is implicated in breast cancer progression and resistance to targeted therapies, and is thought to promote tumorigenesis by activating PI3K signaling. In a transgenic model of breast cancer, Pten suppression using a tetracycline-regulatable short hairpin (sh)RNA cooperates with human epidermal growth factor receptor 2 (HER2/neu), leading to aggressive and metastatic disease with elevated signaling through PI3K and, surprisingly, the mitogen-activated protein kinase (MAPK) pathway. Restoring Pten function is sufficient to down-regulate both PI3K and MAPK signaling and triggers dramatic tumor regression. Pharmacologic inhibition of MAPK signaling produces similar effects to Pten restoration, suggesting that the MAPK pathway contributes to the maintenance of advanced breast cancers harboring Pten loss.

breast cancer | mouse models | tumor suppressors | RNAi | targeted therapies

The *PTEN* (phosphatase and tensin homolog) tumor suppressor gene is mutated or silenced in a wide range of tumor types (1). *PTEN* encodes a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that counters the action of the phosphatidylinositol 3-kinases (PI3Ks), which otherwise transmit growth factor signals from receptor tyrosine kinases to downstream mediators such as the AKT family of serine/threonine-specific protein kinases (2). AKT, in turn, activates a series of downstream effectors that promote cellular proliferation and survival. Consequently, PTEN loss leads to hyperactivation of the PI3K pathway, and it is widely believed that this is the primary mechanism whereby PTEN loss drives tumorigenesis (3). Although cross-talk and feedback signaling makes the situation more complex (4), this molecular framework provides a strong rationale to target PI3K pathway components in PTEN-deficient tumors, and indeed, a variety of small-molecule antagonists with such activities are currently in clinical trials (5, 6).

Deregulation of the PI3K pathway is common in breast cancer and most frequently occurs through mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) (7). By contrast, *PTEN* mutation or loss is less frequent at diagnosis but instead is associated with disease progression (8, 9). For example, PTEN inactivation often arises in oncogenic receptor tyrosine kinase human epidermal growth factor receptor 2 (*HER2/neu*) amplified tumors in patients who acquire resistance to the HER2/neu targeting agent trastuzumab (10–14). Similarly, *PTEN* mutations were recently reported in a patient harboring *PIK3CA* mutations that developed resistance to the PI3Kα inhibitor BYL719 (15). Thus, PTEN inactivation occurs in advanced disease in patients with poor prognosis, defining a breast cancer subtype for which there is an unmet clinical need.

Studies using mouse models have confirmed the importance of PI3K signaling in breast cancer (16). Transgenic mice that overexpress mutant PIK3CA in conjunction with HER2/neu recapitulate resistance to anti-HER2/neu therapies (17), and conditionally overexpressed mutant PIK3CA in the mammary gland gives rise to tumors at long latency that regress upon oncogene withdrawal (18). Although these observations contribute to the rationale for targeting PI3K pathway components in breast cancer, they use a model in which mutant PIK3CA is expressed at unphysiological levels and serves as the initiating event. Furthermore, studies using conditional knockout mice indicate that deregulation of the endogenous PI3Ks indirectly through Pten inactivation can promote advanced disease in combination with HER2/neu (19, 20). Still, whether sustained PTEN inactivation is needed for the maintenance of advanced cancers remains unknown. Resolving this issue may reveal cellular dependencies and, as such, instruct the clinical use of molecularly targeted agents attacking the PTEN network. In this study, we explore the impact of genetic and pharmacologic manipulation of the Pten pathway in breast cancer. Unexpectedly, our results reveal that Pten loss is required to maintain advanced disease by enhancing signaling through both the PI3K and mitogen-activated protein kinase (MAPK) cascades.

Significance

***PTEN* mutations are associated with disease progression and therapy resistance in human epidermal growth factor receptor 2 (*HER2/neu*)-amplified breast cancer patients but the role of PTEN loss in breast cancer maintenance is unknown. Here, using a regulatable RNAi mouse model of HER2/neu-driven metastatic breast cancer, we show that *Pten* silencing accelerates disease progression and that restoration of endogenous *Pten* expression triggers marked disease regression. By comparing and contrasting how pharmacologic perturbations of various signaling pathways compare to genetic reactivation of Pten, we identify a requirement for Mek signaling in Pten-suppressed tumors. Our findings imply that even advanced tumors can remain dependent on Pten loss and provide a rationale for exploring the utility of MEK inhibitors in therapy-resistant breast cancer patients acquiring *PTEN* mutations.**

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[1]Present address: Novartis Institutes for Biomedical Research, Cambridge, MA 02139.

[2]Present address: Department of Medicine, Weill Cornell Medicine, New York, NY 10021.

[3]To whom correspondence should be addressed. Email: lowes@mskcc.org.

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Results

A Model for Mammary Gland-Specific *Pten* Silencing. Genetically engineered mouse models (GEMMs) are powerful tools for the study of gene function in disease (21, 22). We previously optimized an efficient pipeline that implements recombinase-mediated cassette exchange to introduce tet-responsive shRNAs into embryonic stem cells at a defined genomic locus, thereby providing a platform to explore the requirement for sustained gene loss in disease progression and maintenance (23, 24). To build a GEMM that enables inducible and reversible knockdown of Pten in the mammary epithelium, we bred mice carrying a whey acidic protein (*WAP*) gene promoter-driven *Cre* transgene (25) and a *CAGs-LoxPStopLoxP-rtTA3-ires-mKate2* (*CAGs-LSL-RIK*) allele (26), which together drive mammary luminal ductal epithelium-restricted expression of a reverse tet-transactivator (rtTA3) and a far-red fluorescent protein (mKate2) in response to lactogenic hormones. The expression of rtTA3 enables doxycycline (dox)-dependent GFP-linked shRNAs (*TRE-GFP-miR30-shRNA* or *TG-shRNA*) to be expressed from a transgene integrated downstream of the *col1a1* locus (Fig. 1 *A*–*C* and Fig. S1*A*).

In this dual-color "Dox-On" system, mKate2 reports Cre recombinase activity and expression of rtTA3, whereas GFP fluorescence identifies cells with shRNA expression and *Pten* silencing (23). Noninvasive, in vivo imaging of fluorescent markers allows for detection of transgene activation and longitudinal surveillance of tumor progression (Fig. S1*B*). We used two transgenic shRNA strains that demonstrate efficient *Pten* silencing to confirm that our phenotypes were due to loss of Pten and not shRNA-specific off-target effects (27). Luminal specific expression of shRNAs targeting *Pten* in post-pregnancy (parous) adult animals conferred robust target protein suppression (Fig. 1*D* and Fig. S1*C*) but did not result in morphological defects of the mammary glands (Fig. S1*D*). Both *WAP-Cre/CAGs-LSL-RIK/TG-shPten.1522* (abbreviated hereafter as *shPten*) and *WAP-Cre/CAGs-LSL-RIK/TG-shPten.2049* (*shPten.2049*) animals that were affected only by *Pten* knockdown did not develop tumors for up to 300 d of dox treatment ($n > 6$ for both shRNAs).

Pten Loss Accelerates HER2/neu-Driven Disease Onset and Progression. To drive breast tumor initiation, we included a mammary-specific HER2/neu oncogene (*MMTV-neu*^{V664E}) (28) (Fig. 1*A*). Continuous postpartum dox treatment of *WAP-Cre/CAGs-LSL-RIK/MMTV-neu*^{V664E}*/TG-shPten.1522* (abbreviated thereafter as *shPten/neu*) animals led to the onset of palpable mammary tumor development in 100% of mice at a median latency of 62 d (Fig. 2*A*). At this time no control animals (*WAP-Cre/CAGs-LSL-RIK/MMTV-neu*^{V664E}*/TG-shRenilla.713* or *shRen/neu*) showed palpable tumors (median

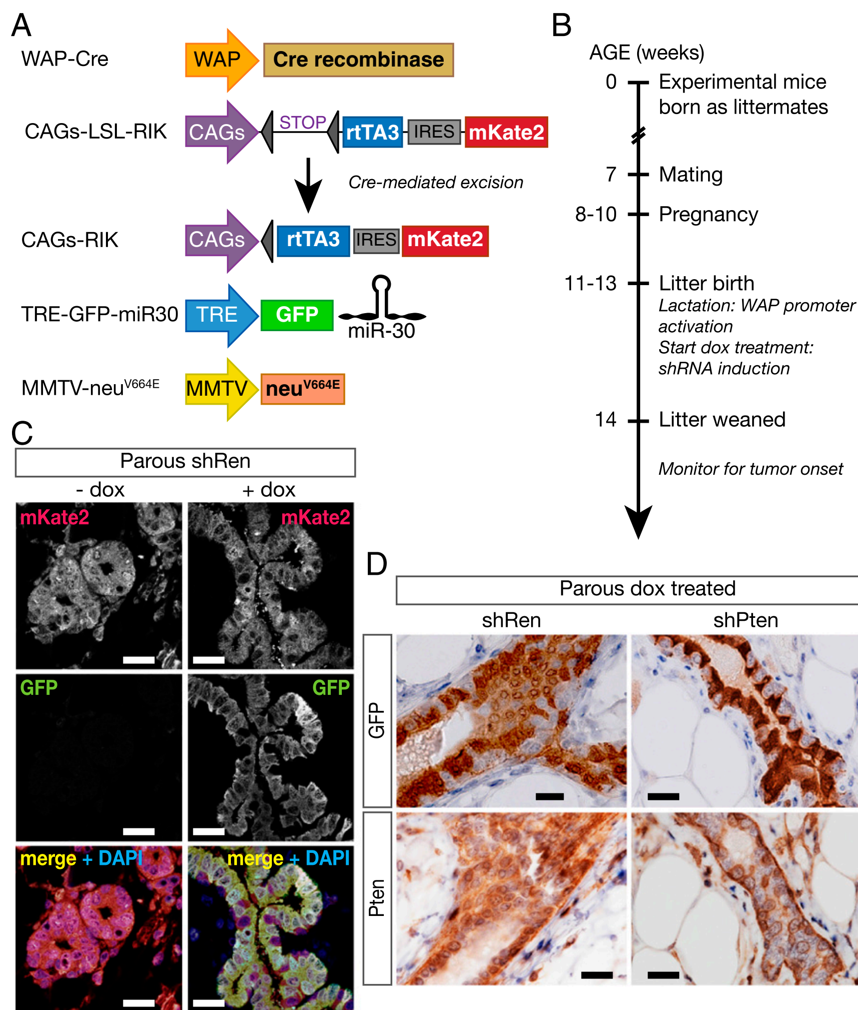

Fig. 1. Mammary gland-specific expression of dox-inducible miR30 shRNAs. (*A*) Schematic description of the five transgenic alleles incorporated into the "Dox-On" multiallelic model, with the *CAGs-LSL-rtTA3-IRES-mKate2* (*CAGs-LSL-RIK*) allele shown in its configuration before and after Cre-mediated recombination upon lactogenic stimulus. This *RIK* transgene contains a CMV early enhancer element and chicken beta actin (*CAGs*) ubiquitous promoter upstream of the *LSL*, followed by a modified reverse tet-transactivator with increased dox sensitivity (*rtTA3*), internal ribosomal entry site (*IRES*), and a monomeric far-red fluorescent protein (*mKate2*). Because the lactation-induced *WAP* promoter provides the tissue specificity, we opted for the use of a ubiquitous promoter upstream of the rtTA element such that its expression levels are not modulated by changes in cellular state over the course of tumorigenic transformation. Following pregnancy, the CAGs promoter drives constitutive expression of rtTA3 and fluorescent reporter mKate2. Once the luminal cells of the mammary ductal epithelium express rtTA3, dox can be used to induce expression of the shRNA transgenic allele *TRE-GFP-miR30-shRNA* (*TG-shRNA*). The tetracycline-responsive element promoter (*TRE*) is active when bound by rtTA3, and GFP has been coupled with the expression of the shRNA such that the strength of the fluorescent signal corresponds inversely to the knockdown level of the target protein. The resulting model provides postadolescent, luminal epithelial-specific knockdown of the target protein of interest. (*B*) Time line of procedural steps for transgene activation including lactation and dox treatment. Experimental animals (carrying either the *Ren.713* control shRNA or *Pten* shRNA) are generated as littermate cohorts to control for the influence of the mixed genetic background. Mice are mated at 7 wk of age after the development of the mammary gland, and pregnancy lasts 21 d. Lactation after litter birth induces the *WAP* promoter, and as such dox treatment is started simultaneously to litter birth to induce shRNA expression. Nursing pups are weaned after 3 wk, and experimental mice are monitored weekly for onset of tumor growth. (*C*) Representative images of immunofluorescence (IF) analysis of mammary glands from parous *WAP-Cre/CAGs-LSL-RIK/TG-shRen.713* (abbreviated as "*shRen*") control animals either with or without dox treatment. Fluorescent reporter mKate2 staining indicates cells expressing rtTA3. In the presence of dox, rtTA3 binds the TRE promoter and drives expression of the GFP reporter and shRNA. (Scale bars: 20 μm.) (*D*) Representative images of IHC analysis of mammary glands from parous, dox-treated *shRen* and *shPten* (abbreviation for *WAP-Cre/CAGs-LSL-RIK/TG-shPten.1522*) animals. GFP staining indicates cells expressing miR30 shRNAs, and Pten protein loss is observed specifically in luminal ductal cells in *shPten* ducts. (Scale bars: 20 μm.)

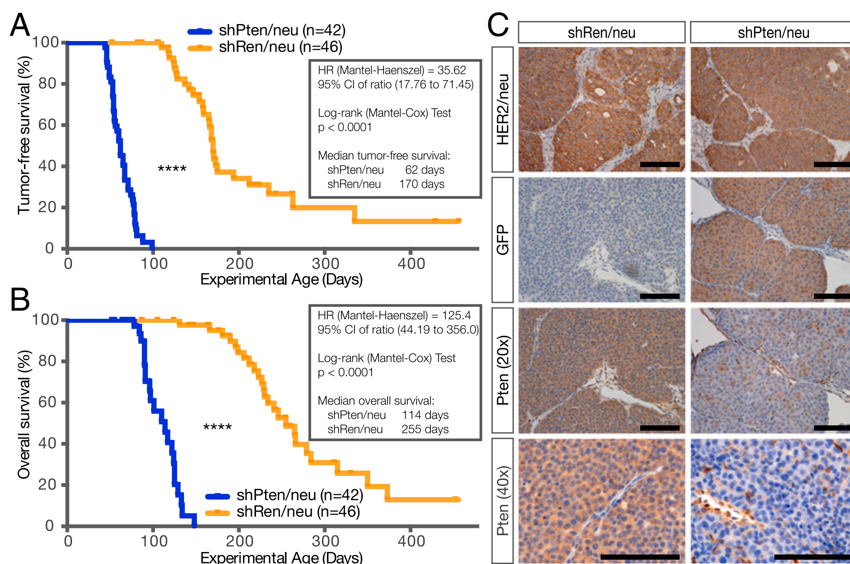

Fig. 2. Pten loss accelerates HER2/neu-driven disease onset and progression. Kaplan–Meier (KM) curves for cohorts of parous, dox-treated quadruple transgenic mice (*WAP-Cre/CAGs-LSL-RIK/MMTV-neu*^{V664E}*/TG-shRNA* abbreviated as "*shRNA/neu*" with corresponding shRNA target gene name) monitored weekly through palpation showing (*A*) tumor-free and (*B*) overall survival for *shPten/neu* (n = 42) and *shRen/neu* (n = 46). Age has been normalized to date of litter birth, which was also the start of dox treatment in this experiment. (*C*) Representative images of IHC analysis of primary tumor tissue of parous, dox-treated *shRen/neu* and *shPten/neu* mice at end-stage disease showing robust HER2/neu overexpression in both cohorts and strong Pten protein knockdown specifically in *shPten/neu* tumors. (Scale bars: 100 μm.)

latency 170 d), demonstrating a strong synergistic effect of HER2/neu overexpression and Pten depletion (Fig. 2*A*). As *WAP-Cre* induction is linked to lactation, we confirmed that disease burden was not related to the litter size of parous mice (Fig. S2*A*).

shPten/neu mice showed a dramatic decrease in overall survival compared with *shRen/neu* control animals, which eventually succumb to the oncogenic effect of the *HER2/neu* transgene (Fig. 2*B* and Fig. S2*B*). Although every tumor harvested from *shPten/neu* mice expressed the GFP and mKate2 fluorescent reporters, in *shRen/neu* mice only one quarter (26%) of observed tumors were GFP/mKate2-positive, indicating that *Pten* silencing promoted accelerated tumor growth in a *WAP-Cre* allele-expressing subpopulation of epithelial cells that does not overlap completely with the population of cells that express the *MMTV-neu*^{V664E} allele (Fig. S2*C*). A significant increase in disease burden per mouse at end-stage disease was also seen in *shPten/neu* mice (Fig. S2 *D* and *E*). These results recapitulate previous studies using a conditional Cre-LoxP model of *Pten* loss (19, 20) and highlight the effectiveness of the shRNA approach.

Pten protein depletion was consistently robust in *shPten/neu* tumor tissue, which also expressed GFP (Fig. 2*C*). These areas also displayed strong staining for HER2/neu at the cell membrane and a high Ki-67 index, a marker for cellular proliferation (Fig. 2*C* and Fig. S3*A*). Pten protein was detected in all tumors analyzed from *shRen/neu* mice (n = 18/18; Fig. 2*C*), indicating that HER2/neu-driven tumor development did not select for stochastic Pten loss. Histopathology of primary mammary tumors revealed large nodular nests with central necrosis, similar in morphology to the parental *MMTV-neu*^{V664E} strain (Fig. S3*B*). Both *shRen/neu* and *shPten/neu* tumors at end-stage disease showed characteristics of human invasive ductal carcinomas, with marked nuclear pleomorphism, high mitotic count, and lack of tubule formation. Thus, the *shPten/neu* mice develop tumors that reflect the most common malignant breast tumor type in humans and show pathological characteristics (high grade, poorly differentiated) that carry a poor clinical prognosis. Immunohistochemical analysis revealed estrogen receptor alpha (ERα) and CK19 expression in both control and Pten knockdown tumors (Fig. S3*A*). In contrast, there was a decrease in HER3/ErbB3 protein levels detected only in *shPten/neu* tumor tissue (Fig. S3*A*), which, in line with previous observations (29, 30), is suggestive of elevated PI3K/Akt activity and increased pathway feedback. Collectively, these results demonstrate strong cooperation between Pten loss and activated HER2/neu in a histopathologically accurate model of luminal epithelial breast cancer.

We also noted increased metastatic burden in *shPten/neu* animals (n = 19/21) compared with *shRen/neu* controls (n = 9/13) at end-stage disease (Fig. S4 *A* and *B*). Histologically, lung metastases resembled the primary tumors (Fig. S4*C*), showing strong HER2/neu overexpression and Pten knockdown (Fig. S4*D*). Importantly, key phenotypes (accelerated tumor initiation and progression, lung metastasis, and histopathology) were validated with two independent shRNAs targeting *Pten* (Fig. S5). Together, our results demonstrate that shRNA-mediated *Pten* silencing has a dramatic impact on tumor initiation and progression in HER2/neu-mediated mammary tumorigenesis.

Restoration of Pten Causes Tumor Regression. To assess whether sustained Pten loss is required for tumor maintenance, we took advantage of the unique ability of regulated RNAi to reverse gene silencing and restore endogenous Pten protein in established tumors. For this, we identified a cohort of *shPten/neu* animals with palpable tumors in at least one quadrant region (n = 19, 52–97 d dox treated), withdrew dox to restore Pten expression, and monitored tumor growth with caliper measurements and small animal magnetic resonance imaging (MRI). Restoration of Pten protein and loss of shRNA-linked GFP was confirmed in tumor tissue 2 wk after dox withdrawal (Fig. 3*A*).

Most tumors arising in *shPten/neu* mice showed dramatic, and often complete, regression during the first 3 wk following dox withdrawal (n = 15/17), whereas those in *shPten/neu* animals maintained on dox exhibited exponential tumor growth (Fig. 3 *B* and *C*). Tumor regression was indeed due to Pten protein restoration, as tumors in the second shRNA strain, *shPten.2049/neu*, displayed similar regression patterns whereas those arising in *shRen/neu* control mice at long latency remained unaffected by dox treatment withdrawal (Fig. S6). Neither total tumor burden at the time of dox withdrawal nor duration of dox treatment before dox withdrawal correlated with the maximal response in tumor regression documented in each animal (Fig. S7). In most cases, tumor regression was sustained for at least 50 d after dox withdrawal (Fig. S8), leading to a significant increase in overall survival (Fig. 3*D*). Nonetheless, multifocal disease relapse driven by HER2/neu overexpression eventually occurred in almost all *shPten/neu* mice maintained off dox (n = 18/19) (Fig. 3*D*). Still, this dramatic regression of exponentially growing HER2/neu-driven mammary tumors upon Pten restoration suggests that Pten knockdown not only accelerates tumor initiation but also is required for tumor maintenance.

Pten Loss Drives PI3K/Akt and MAPK Signaling Pathways in HER2/neu Mammary Tumors. We postulated that restoration of Pten protein in Pten knockdown tumor tissue disrupts the signaling pathways governing cellular proliferation and/or survival, leading to tumor

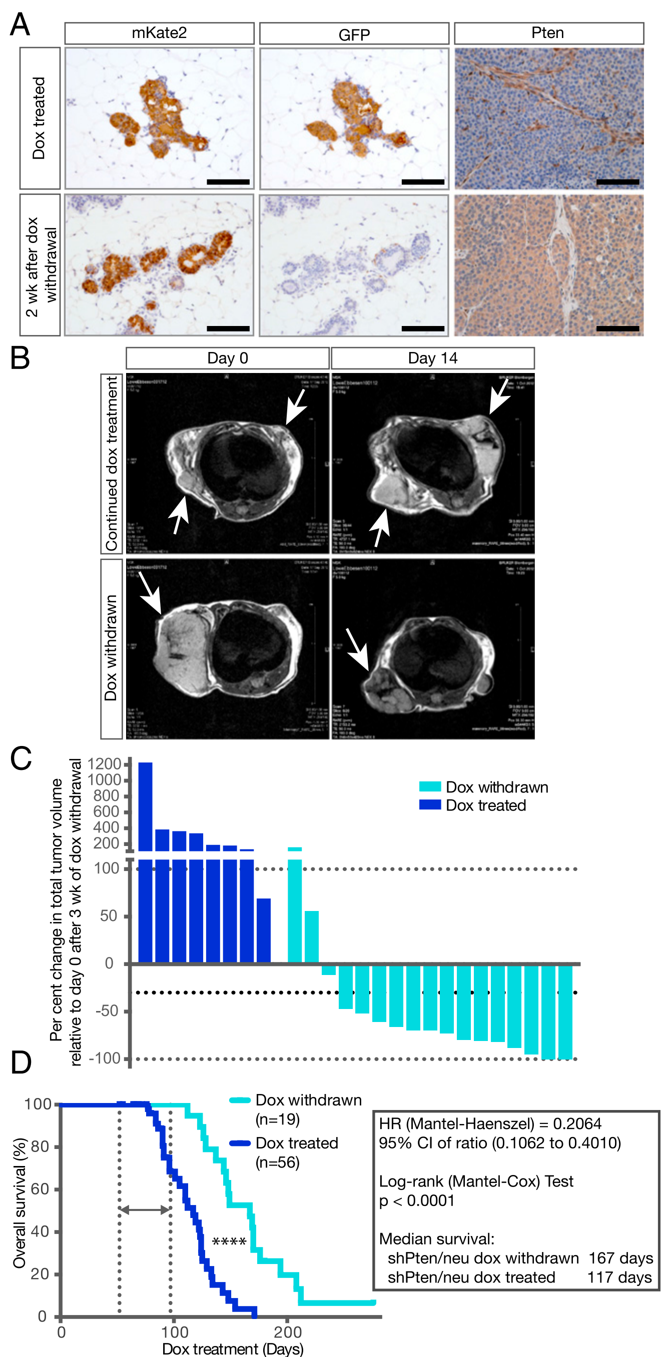

Fig. 3. Pten loss is required for tumor maintenance. (*A*) Representative images of IHC analysis of hyperplastic ductal epithelium of the mammary gland or mammary tumor of parous *shPten/neu* mice either continuously dox treated or 2 wk after dox withdrawal. (Scale bars: 100 μm.) (*B*) Representative axial plane MRI images of continuously dox-treated (n = 2) and dox-withdrawn (n = 3) cohorts of *shPten/neu* mice analyzed by MRI at days 0 and 14. (*C*) Waterfall plot displaying percentage of change in total tumor burden volume per mouse at the time point of 3 wk after dox withdrawal for a cohort of *shPten/neu* mice (n = 17) compared with a representative cohort of continuously dox-treated animals (n = 8). Dotted lines indicate +100 and −30% thresholds. (*D*) KM curve depicting the overall survival benefit of animals after dox withdrawal (n = 19) compared with dox-treated animals (n = 56; data partially presented in Fig. 2). Double arrow indicates range of dox withdrawal start date.

shrinkage. To explore this in more detail, we performed immunohistochemical (IHC) and immunoblot (IB) analyses of *shRen/neu* and *shPten/neu* primary mammary tumors (Fig. 4 and Fig. S9*A*). In regressing *shPten/neu* tumors examined 3 wk after dox withdrawal, tumors showed reduced proliferation (as measured by BrdU incorporation and Ki-67 staining) but no detectable increase in apoptosis (as assessed by cleaved caspase-3 staining) (Fig. S9*B*). Although these observations suggest that tumor shrinkage was independent of apoptosis, we cannot rule out the possibility that transient increases in signal were missed at the examined time point. Consistent with the ability of Pten to suppress PI3K signaling, Pten knockdown tumors showed an increase in Akt phosphorylation that was not apparent in tumors arising in *shRen/neu* mice (Fig. 4*B*). Akt levels were suppressed upon Pten restoration, and notably, relapsed tumors maintained re-expression of Pten (Fig. 4).

Unexpectedly, we also noted a consistent elevation in phosphorylated Erk (p-Erk) levels in primary tumors and pulmonary metastases arising in dox-treated *shPten/neu* mice compared with *shRen/neu* mice, suggesting that Pten suppression triggered hyperactivation of the MAPK pathway (Fig. 4 and Fig. S9*C*). Indeed, high p-Erk expression was not merely a secondary event in tumors generated by Pten knockdown, as restoration of endogenous Pten expression in *shPten/neu* tumors led to a reproducible suppression of p-Erk levels (Fig. 4). Consistent with the delay seen in the *shPten/neu* cohort tumor regression response, Pten levels remained low at 7 d after dox withdrawal (Fig. S9*D*) but returned to baseline by 14 d (Fig. 4*B*). High p-Akt and p-Erk levels were sustained at 7 d post-dox withdrawal but were suppressed at 2 wk. Thus, despite constitutive overexpression of an overactive HER2/neu oncogene, restoration of endogenous Pten induced a striking reduction in both PI3K/Akt and Mek/Erk signaling that correlated with disease regression.

Confounding further in-depth analysis of the relapsed tumors is the multifocal nature of the disease that develops in this GEMM. Despite our capacity to track mKate2-expressing cells in the absence of dox, given the large number of tumor-initiating events documented per mouse, it was difficult to determine whether the tumors that progressed off dox were arising from relapses of an originally responsive malignancy or rather from "background" tumors expected to arise eventually from HER2/neu-expressing cells that never expressed the Pten shRNA. Indeed, it seems most likely that they represented a mixture of both classes, as some expressed high levels of the lineage tracing mKate2 reporter linked to the rtTA3 (indicating they can express the Pten shRNA when dox treated) whereas others did not. Regardless, all of the tumors that progressed showed abundant Pten expression and did not contain elevated p-Akt or p-Erk levels, indicating that secondary mechanisms capable of recapitulating Pten loss were not acquired.

Mek Signaling Is Required to Maintain Tumors with Suppressed Pten. The marked reduction in PI3K/Akt and Mek/Erk signaling following Pten restoration prompted us to ask whether the activity of each pathway was critical for disease progression and, thus, whether pharmacological inhibition could also drive tumor regression. To provide a controlled experimental setting for comparing different treatment arms, we generated cohorts of matched secondary transplants from primary tumor fragments harvested from *shPten/neu* mice. These transplants showed consistent tumor growth (on dox) and response to Pten restoration (dox withdrawn), mirroring the primary disease in transgenic mice (Fig. 5). In all, we assessed four treatment arms using agents targeting oncogenic HER2/neu (Lapatinib), PI3K/Akt signaling (α-Akt: MK-2206; α-pan-PI3K: NVP-BKM120), or Mek/Erk activity (α-Mek: GSK1120212) at doses routinely used in preclinical studies (15, 29, 31, 32). Drug efficacy against the mouse protein target was verified by assessing the impact on signaling in treated tumor protein extracts (Fig. S10).

Consistent with the known role for PI3K pathway activation in promoting resistance to HER2/neu inhibition (33, 34), Lapatinib had only a mild effect in slowing tumor growth (Fig. 5*A*). By contrast, pan-PI3K and Akt inhibitors, as predicted, were effective in suppressing tumor growth, although not nearly as efficiently as Pten restoration. Surprisingly, Mek inhibition induced the most robust tumor response, effectively blocking tumor growth over 4 wk of treatment and phenocopying the effect of Pten restoration in these transplants (Fig. 5*A*). Although counterintuitive to the canonical

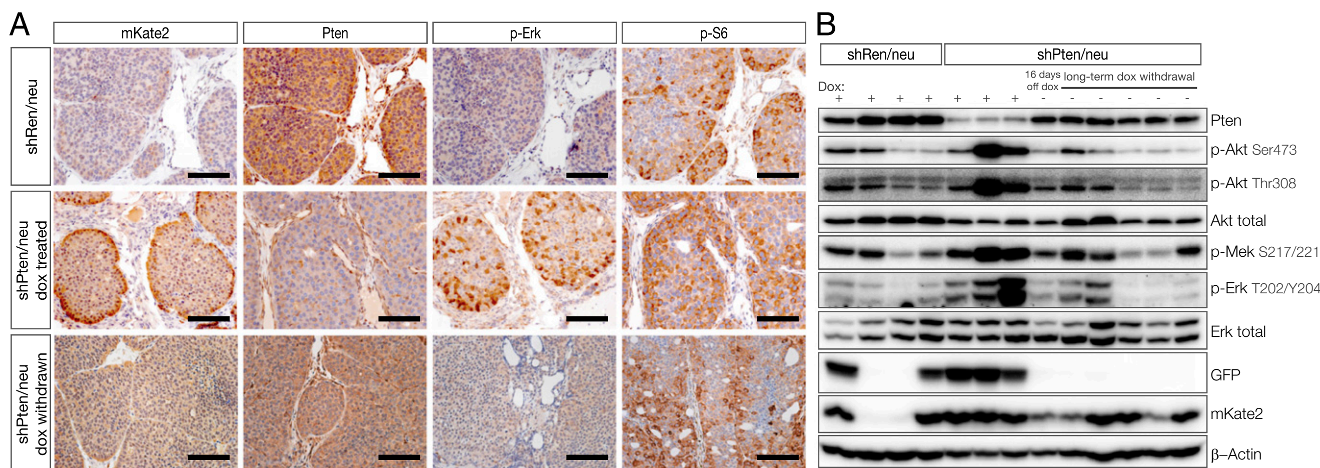

Fig. 4. Heightened PI3K/Akt and MAPK pathway signaling in Pten knockdown tumors. IHC (*A*) and IB (*B*) analysis of end-stage disease mammary tumors of parous *shRen/neu* and *shPten/neu* mice either continuously dox treated or dox withdrawn. (Scale bars: 100 μm.) For *B*, unsorted whole tumor protein lysate was analyzed.

view of Pten as a regulator of PI3K signaling, our results suggest that Pten loss, in addition to up-regulating the PI3K/Akt pathway, causes a functional "cross-activation" of the MAPK pathway that goes beyond the canonical HER2/neu-dependent signaling through Erk and contributes significantly to aggressive tumor growth. Still, it remains a possibility that pan-PI3K and Akt inhibition was less effective than Mek inhibition in our model due to differences in pharmacokinetics, reduced activity against the mouse protein(s), or PI3K-independent functions of Pten (35).

Discussion

Our findings are consistent with previous work in which withdrawal of a conditional *PIK3CA* transgene used to initiate tumorigenesis also produced marked disease regression (18). In our study, Pten inactivation served as a cooperating event to promote tumorigenesis together with the strong initiating driver HER2/neu. Even so, Pten reactivation blunted PI3K and MAPK signaling, leading to a potent antitumor response. Although these studies suggest that pharmacological strategies to achieve similar ends would be therapeutically beneficial in advanced forms of breast cancer, the relative contribution of Pten loss to tumor maintenance may depend on tissue and/or genetic context. Accordingly, we recently showed that the antitumor effects of Pten reactivation in a mouse model of aggressive leukemia were largely limited to leukemic cells at disseminated sites (27).

Additional studies will be required to fully elucidate the complex cross-talk between the PI3K and MAPK pathways (4). Physiologically, PTEN-dependent early endodermal morphogenesis seems to require the Erk, but not the Akt, pathway (36). Furthermore, in Ras-induced oncogenic transformation, PTEN apoptotic function is suppressed via the Raf-Mek-Erk pathway (37). Recent publications using human breast cancer cell lines support the notion that PI3K inhibition can involve an ERK-dependent component (38, 39). In one study, this effect was apparently mediated by the phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 (P-Rex1), which activates Rac1, leading to MAPK pathway activation. In another, enforced expression of PTEN caused a decrease in p-ERK levels. Both confirm that combined inhibition of MEK in conjunction with PI3K or AKT improved antitumor efficacy in breast cancer xenografts. These data are in agreement with earlier reports showing that expression of functionally active PTEN inhibits ERK activation in glioblastoma models (40). Moreover, ectopic expression of PTEN in MCF7 cells results in both AKT and ERK suppression, but only specific inhibition of MEK can abolish the negative effects of PTEN on insulin-mediated cell growth (41).

Our finding that *shPten/neu* primary tumor regression upon restoration of Pten protein levels is dependent on both PI3K/Akt and MAPK pathways reiterates the presence of a complex and interconnected signaling cross-talk network that regulates these tumors' sustained survival and proliferative advantage in a relevant physiological context. Collectively, these data provide a strong rationale for combining agents that target the MAPK

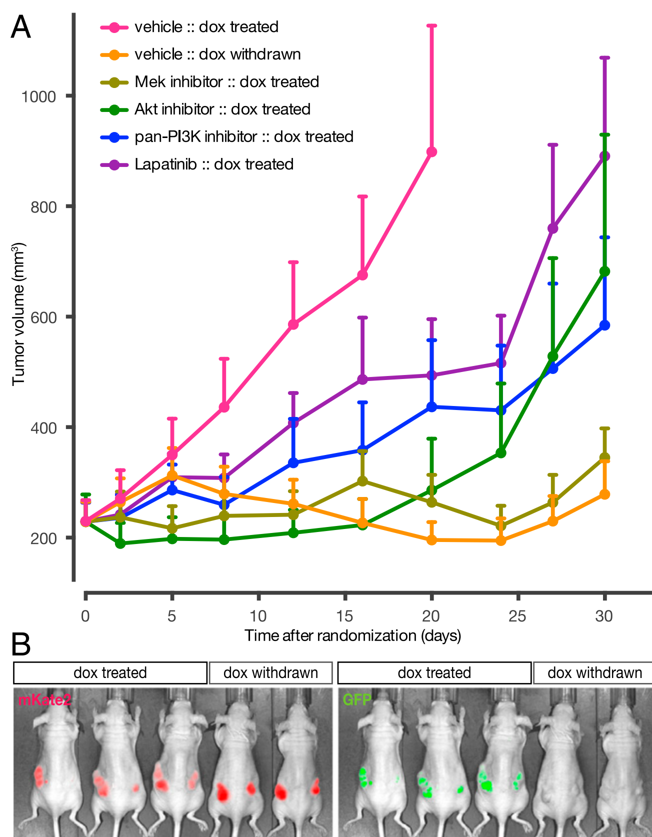

Fig. 5. Pharmacologic inhibition of MAPK pathway recapitulates Pten restoration. (*A*) Dox-treated *shPten/neu* animal-derived primary tumor fragment serial transplantation assay in nude mice. Mice were treated with or without dox with a drug or vehicle (n = 12–14 tumors). Drug dosages: GSK1120212 (3 mg/kg daily), MK-2206 (360 mg/kg daily and then twice weekly after day 14), NVP-BKM120 (31.35 mg/kg daily), and Lapatinib (100 mg/kg daily). Data represent mean ± SEM. (*B*) Representative mKate2 and GFP fluorescence images from in vivo Xenogen IVIS Spectrum imaging of dox-treated and dox-withdrawn (day 10) nude mice from the tumor fragment transplantation assay.

and PI3K pathways, especially in the absence of PTEN (42). As PTEN loss is linked to resistance against anti-HER2/neu agents or single PI3K inhibitors, such combinatorial approaches would address an unmet clinical need.

Materials and Methods

Mouse Strains, Animal Husbandry, and Tumor Cohorts. All mouse strains have been previously described. *MMTV-neu^{V664E}* [strain name: FVB-Tg(MMTV-Erbb2)NK1Mul/J] (28) and *WAP-Cre* [strain name: B6.Cg-Tg(Wap-cre)11738Mam/JKnwJ] (25) were purchased from Jackson Laboratory. In the *MMTV-neu^{V664E}* allele, an activating point mutation (V664E) in the transmembrane domain of *neu* results in increased receptor homodimerization and constitutive activation of the kinase domain (43). The strains *CAGs-LSL-RIK* (26), *TG-shRenilla-Luciferase.713* (23), *TG-shPten.1522* (27), and *TG-shPten.2049* (27) were made by the S.W.L. laboratory. Transgenic strains were not backcrossed onto the same strain background before mating; thus all experimental animals were generated on a mixed strain background. The mating strategy was conducted such that the experimental shRNA and the control shRNA animals were born as littermates. Mice were housed in vented cages with a 12-h light cycle and food and water ad libitum. Experimental female animals, after PCR verification for the correct genotypes, were bred at 7 wk, and doxycycline feed was administered from the date of litter birth. Litters were nursed for 3 wk to induce WAP promoter activity. Parous mice underwent only one pregnancy cycle. Parous mice were monitored weekly, blinded from genotypic information, for tumor formation by physical palpation. Tumor and organ tissue samples were excised from humanely euthanized mice and either fixed and/or snap-frozen. Caliper measurements during tumor regression experiments were used to estimate tumor volume with the formula $V = (4/3) * (pi) * (L/2) * (W/2)^2$, where Length > Width. Total tumor burden volume per mouse was calculated as the sum of all tumor volumes per mouse at each particular time point.

Study Approval. All mice were maintained and experiments were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial Sloan Kettering Cancer Center (MSKCC) under protocol nos. 11–06-015 and 12–10-019.

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