

PDK1 Attenuation Fails to Prevent Tumor Formation in PTEN-Deficient Transgenic Mouse Models

Katharine Ellwood-Yen¹, Heike Keilhack¹, Kaiko Kunii¹, Brian Dolinski¹, Yamicia Connor¹, Kun Hu¹, Kumiko Nagashima¹, Erin O'Hare¹, Yusuf Erkul¹, Alessandra Di Bacco¹, Diana Gargano¹, Nirah H. Shomer¹, Minilik Angagaw¹, Erica Leccese¹, Paula Andrade¹, Melissa Hurd¹, Myung K. Shin³, Thomas F. Vogt³, Alan Northrup¹, Ekaterina V. Bobkova¹, Shailaja Kasibhatla¹, Roderick T. Bronson², Martin L. Scott¹, Giulio Draetta¹, Victoria Richon¹, Nancy Kohl¹, Peter Blume-Jensen¹, Jannik N. Andersen¹, and Manfred Kraus¹

Abstract

PDK1 activates AKT suggesting that PDK1 inhibition might suppress tumor development. However, while PDK1 has been investigated intensively as an oncology target, selective inhibitors suitable for *in vivo* studies have remained elusive. In this study we present the results of *in vivo* PDK1 inhibition through a universally applicable RNAi approach for functional drug target validation in oncogenic pathway contexts. This approach, which relies on doxycycline-inducible shRNA expression from the *Rosa26* locus, is ideal for functional studies of genes like PDK1 where constitutive mouse models lead to strong developmental phenotypes or embryonic lethality. We achieved more than 90% PDK1 knockdown *in vivo*, a level sufficient to impact physiological functions resulting in hyperinsulinemia and hyperglycemia. This phenotype was reversible on PDK1 reexpression. Unexpectedly, long-term PDK1 knockdown revealed a lack of potent antitumor efficacy in 3 different mouse models of PTEN-deficient cancer. Thus, despite efficient PDK1 knockdown, inhibition of the PI3K pathway was marginal suggesting that PDK1 was not a rate limiting factor. *Ex vivo* analysis of pharmacological inhibitors revealed that AKT and mTOR inhibitors undergoing clinical development are more effective than PDK1 inhibitors at blocking activated PI3K pathway signaling. Taken together our findings weaken the widely held expectation that PDK1 represents an appealing oncology target. *Cancer Res*; 71(8); 3052–65. ©2011 AACR.

Introduction

Abnormal regulation of the PI3K/AKT signaling pathway is one of the most frequent epidemiological observations in human malignancy. Loss of the tumor suppressor PTEN (1, 2), mutations in receptor tyrosine kinases, as well as activating PI3K mutations all result in elevated levels of PIP₃ that activates the AKT-survival pathway (3). PIP₃ causes PDK1 and AKT to colocalize at the plasma membrane via their pleckstrin homology domains (4, 5). Here, PDK1 phosphorylates the kinase T-loop residue AKT^{T308}, an event that is

absent in PDK1 knockout embryonic stem cells (6) and tissue-specific PDK1 ablation mouse models (7–9). Full activation of AKT also requires phosphorylation of AKT^{S473} by mTORC2 (10). The role of PDK1 in phosphorylating the T-loop of numerous AGC kinases makes it an attractive target for anticancer therapy (11). Several proteins phosphorylated by PDK1, including AKT, S6K, RSK, and certain PKC isoforms, are themselves kinase drug targets (12–15).

Several strategies have been used to evaluate the therapeutic potential of inhibiting PDK1 function in cancer. These include *in vitro* and *in vivo* pharmacological and genetic approaches. Kinase selectivity represents a key challenge when using pharmacological inhibitors to study the involvement of a particular kinase in a cellular process (16, 17). For example, the indolinone-based PDK1 inhibitor BX-795 and its analogues inhibit cell cycle progression, induce apoptosis, and show efficacy in a xenograft model (18). However, BX-795 causes G2/M cell cycle arrest in both PDK1 wild-type (WT) and PDK1 knockout cells suggesting that the effects on cell cycle are unrelated to PDK1 inhibition (19). At present, analysis of small-molecule PDK1 inhibitors, covering a dozen structural classes (11), has not pinpointed any selective compound suitable for *in vivo* efficacy studies.

In contrast to pharmacological agents, genetic approaches are valued for the inherent specificity by which point mutations,

Authors' Affiliations: ¹Merck Research Laboratories; ²Harvard Medical School, Boston, Massachusetts; and ³Merck Research Laboratories, Rahway, New Jersey

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

K. Ellwood-Yen, H. Keilhack, K. Kunii, and B. Dolinski contributed equally to this work.

Corresponding Author: Jannik N. Andersen or Manfred Kraus, Merck, 33 Avenue Louis Pasteur, Boston, MA 02115. Phone: 617-992-2477; Fax: 617-992-2486; E-mail: Jannik_Andersen@dfci.harvard.edu, Manfred_Kraus@merck.com

doi: 10.1158/0008-5472.CAN-10-2282

©2011 American Association for Cancer Research.

gene deletion, or knockdown perturb protein function. In particular, manipulation of the mouse genome has been utilized for modeling of *in vivo* tumorigenesis and for drug target validation (20). For example, PTEN deficiency in mice faithfully models many aspects of human tumor development. While the constitutive PTEN knockout ($PTEN^{-/-}$) is embryonic lethal, heterozygous $PTEN^{+/-}$ mice spontaneously develop a broad spectrum of tumors consistent with epidemiological observations in humans (21–23). In addition, the *Cre-loxP* technology, which enables conditional gene modulation (24), has allowed for the homozygous PTEN deletion in adult mice circumventing embryonic lethality. The loss of both alleles of PTEN results in a more rapid and reproducible onset of tumorigenesis compared to the constitutive $PTEN^{+/-}$ model as exemplified in the $PTEN^{fl/fl}PBcre$ prostate cancer model (25). Importantly, some kinase inhibitors show antitumor activity in mouse models of PI3K/AKT pathway activation, highlighting the utility of engineered mice as a platform to evaluate response to targeted therapies and/or genetic perturbations (20, 26).

It was reported that $PDK1^{-/fl}$ hypomorphic mice, which express low levels of PDK1, when crossed to $PTEN^{+/-}$ mice, suppress PTEN-driven tumorigenesis (27). This observation has put PDK1 to the forefront as an anticancer target in context of PTEN deficiency (11). However, the constitutive knockdown of PDK1 impairs mouse development (27, 28), and therefore does not ascertain whether acute knockdown of PDK1 in adult mice, a situation mimicking more closely therapeutic intervention, will be efficacious.

To address this question, inducible, *in vivo* RNAi-mediated knockdown represents a promising technology for modulation of gene expression in adult mice (29, 30). An inducible gene silencing approach is particularly suited for functional studies of genes like PDK1, where constitutive models lead to strong developmental phenotypes or embryonic lethality (6, 27). Several methods for *in vivo* short hairpin (sh)RNA expression have been described (31–34). Here, we report on an RNAi mouse line where the *Rosa26* locus has been specifically targeted to achieve reversible, doxycycline-dependent expression of a shRNA targeting mouse PDK1 ($Rosa26^{PDK1-RNAi}$). To genetically assess target efficacy the PDK1-RNAi mice were crossed with 3 distinct PTEN-deficient tumor models. We observed potent knockdown of PDK1, but found no prevention of tumor development or efficient pathway modulation. This result is further supported by pharmacological PDK1 inhibitor studies in primary *ex vivo* cultures. In contrast to AKT inhibitor treatment, small-molecule PDK1 inhibitors were not capable of completely reversing elevated phospho-AKT^{T308} levels caused by PTEN deficiency, challenging a pharmacological intervention potential.

Materials and Methods

Mice

$Rosa26^{PDK1-RNAi}$ and $Rosa26^{PDK1-RNAi(2)}$ mice were generated by targeting the *Rosa26* locus (30). Targeted ES cell lines were treated with doxycycline for 2 days (1 g/kg), followed by PDK1-specific reverse transcriptase PCR (RT-PCR) to identify shRNA constructs with efficient knockdown (shPDK1: 5'-GGCTAGA-

GATCTTGTGGAA-3'; shPDK1(2): 5'-GAATTTGCACCAGCAGACA-3'). $PDK1^{-}$ and $PDK1^{fl}$ alleles (mixed genetic background) were obtained from Dario Alessi (University of Dundee), rederived with C57BL/6NTac and then intercrossed to generate hypomorphic $PDK1^{-/fl}$ mice. Out of 103 offspring, 2 hypomorphic $PDK1^{-/fl}$ mice were obtained (2%) versus the expected 25%. $PTEN^{+/-}$ mice were generated by intercrossing $PTEN^{-/fl}$ mice with a ubiquitous *Cre*-deleter strain [C57BL/6NTacGt(ROSA)26Sortm16(cre)Arte]. $PTEN^{fl}$ and *PB-cre* lines were obtained from Hong Wu (25) and $Rosa26^{creERT2}$ mice from Taconic (30). Primary mammary epithelial cells were prepared from virgin female mice as described (35). All studies were approved by the Merck Institutional Animal Care and Use Committee (IACUC).

Metabolic measurements

Blood glucose was assayed with a glucometer (Lifescan). Plasma insulin was determined by ELISA (ALPCO immunoassays). Glucose tolerance tests (GTT) were performed on overnight fasted mice. D-glucose was injected intraperitoneally (2 g/kg). Insulin tolerance tests (ITT) were performed on fasted (4h) animals by intraperitoneal injection of human insulin (0.75 U/kg; Eli Lilly).

Western blot and Fluorescence activated cell-sorting analysis

Tissue and cell homogenates were prepared using radioimmunoprecipitation assay buffer (Pierce) plus protease and phosphatase inhibitor cocktails (Roche). Immunoblots were probed with antibodies against: PDK1, p-PDK1^{S241}, AKT, p-AKT^{T308}, p-AKT^{S473}, S6K1, p-S6K1^{T389}, p-S6K1^{S229}, S6, p-S6^{S235/236}, p-ERK1/2^{T202/204}, and β -actin (Cell Signaling Technologies); p-RSK^{S221} (Invitrogen); and p-70S6K^{T229} (Abcam). The specificity of the PDK1 antibodies is evaluated in Figure S1. Fluorescence activated cell-sorting (FACS) analysis was conducted as described previously (36).

Immunohistochemistry

Tumors were fixed in formalin and embedded in paraffin blocks. Sections were hematoxylin and eosin (H&E) stained (Leica ST5020 multistainer). Ki67-positive cells (clone SP6; 1:2,000; Thermo Scientific) were scored using an Ariol automatic scan system (Applied Imaging). All histopathological analyses were performed at the Rodent Pathology Core, Harvard Medical School. Incidence of hyperplasia in lymph nodes, thyroids, adrenal glands, and uteri were scored on blinded samples (Table 1).

Results

Generation of an inducible PDK1 RNAi-mediated knockdown mouse

To study PDK1 function in adult mice, an inducible approach to modify PDK1 expression was chosen as traditional knockout mice ($PDK1^{-/-}$) are embryonic lethal (6) and $PDK1^{-/fl}$ hypomorphic mice, which display constitutively low levels of PDK1, show strong developmental phenotypes including a 30% to 40% reduction in body size (27). For these

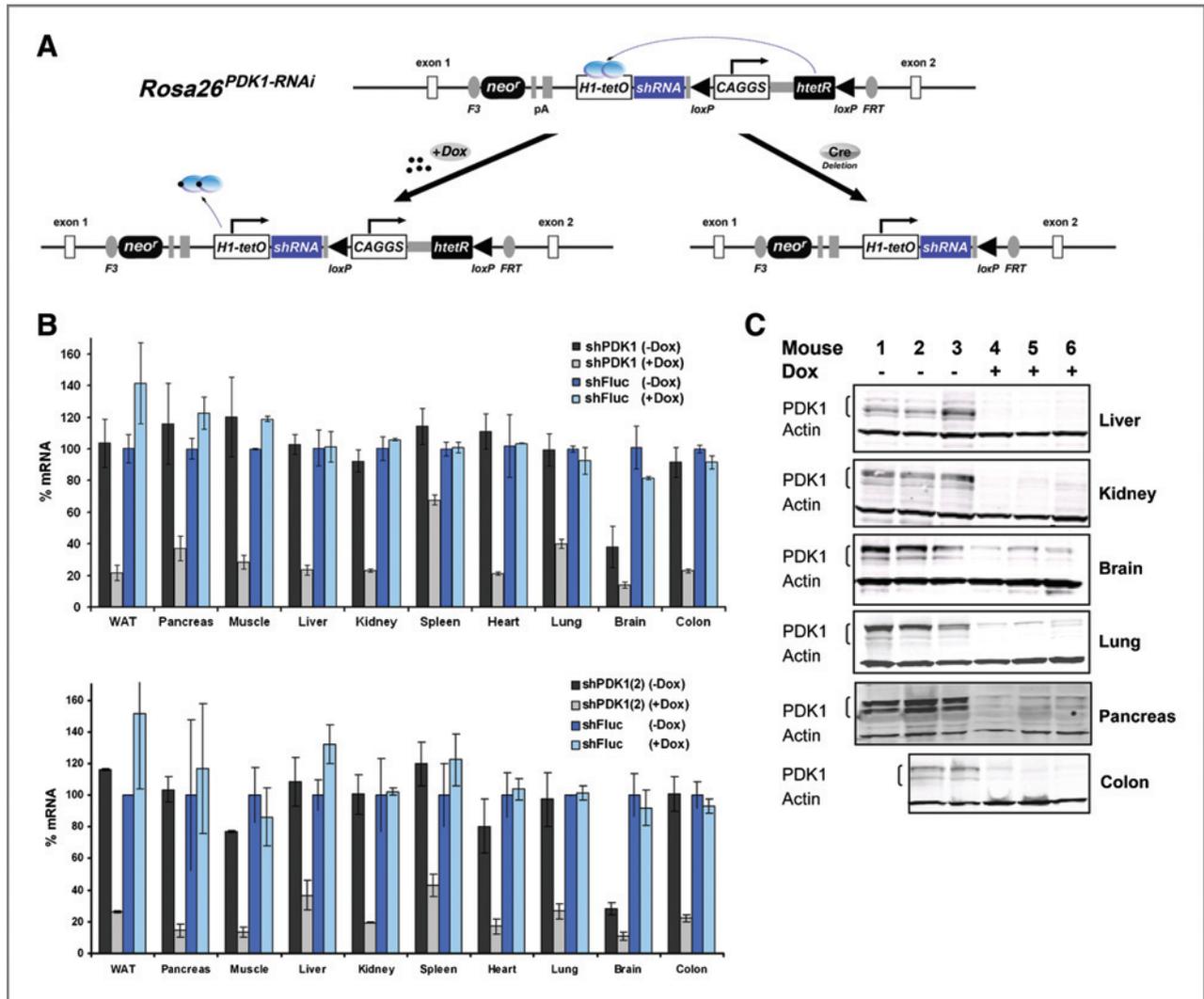


Figure 1. Conditional RNAi-mediated knockdown of PDK1 in mice. **A**, overview of the targeted *Rosa26* locus and tetR-driven repression of shRNA. *H1* promoter driven shRNA expression occurs in the presence of doxycycline (left) or after cre-mediated deletion of the *loxP* flanked tetR (right). **B**, inducible knockdown of PDK1 mRNA *in vivo* determined by qRT-PCR. *Rosa26^{PDK1-RNAi/+}*, *Rosa26^{PDK1-RNAi(2)/+}*, and *Rosa26^{shFluc/+}* mice (*n* = 3 per group) were given doxycycline (2 mg/mL) in the drinking water for 10 days. Knockdown levels were normalized to GAPDH. Standard deviation is shown. **C**, PDK1 protein levels in tissues from *Rosa26^{PDK1-RNAi/+}* mice treated with doxycycline (+) or untreated (-) for 10 days.

reasons, and to more closely mimic a cancer treatment setting, we pursued the approach that affords reversible and temporal control of doxycycline-induced shRNA expression (30). The *Rosa26* locus in mouse ES cells was gene-targeted to encode a single construct containing a PDK1-shRNA-driven by the tet-inducible *H1* promoter and tetR-driven by the *CAGGS* promoter (Fig. 1A). The tetR regulatory cassette is flanked by *loxP* sites, which in addition to doxycycline-regulated RNAi, allows for Cre-recombinase-mediated expression of the shRNA *in vivo* in an irreversible manner.

Two targeted mouse lines (*Rosa26^{PDK-RNAi/+}* and *Rosa26^{PDK-RNAi(2)/+}*) expressing nonoverlapping shRNAs [shPDK1 or shPDK1(2)] were generated with these ES cells and analyzed at 5 weeks of age to evaluate knockdown efficiency following 10 days of doxycycline treatment (Fig. 1B and C). Mice expressing a luciferase targeting shRNA

(shFluc) were used as a control (30). In response to doxycycline, efficient knockdown was observed at both mRNA and protein level. In the absence of doxycycline, 9 out of 10 tissues had normal PDK1 mRNA levels compared to the shFluc controls revealing tight tetR-mediated control of gene silencing. Only brain showed a significant reduction in PDK1 expression in the absence of doxycycline suggesting incomplete tetR-mediated suppression of the shRNA in this organ. In summary, both PDK1-RNAi mouse lines show robust drug-inducible knockdown of PDK1 in all tissues tested.

PDK1 knockdown in *Rosa26^{PDK1-RNAi/+}* mice is reversible and induces a metabolic phenotype

Next, we tested whether the acute knockdown of PDK1 was sufficient to have physiological consequences. Because the PI3K pathway plays an integral part in glucose homeostasis we

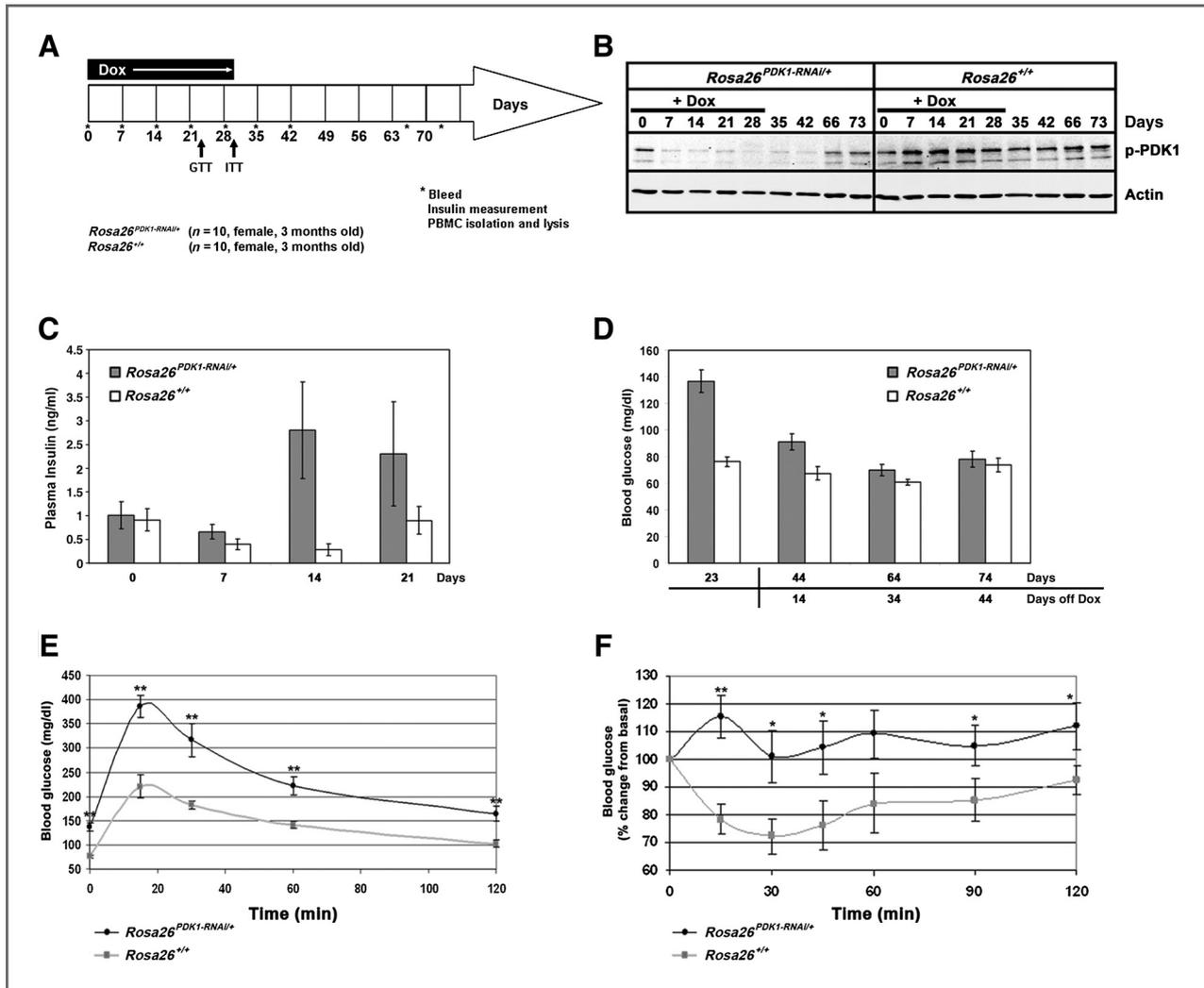


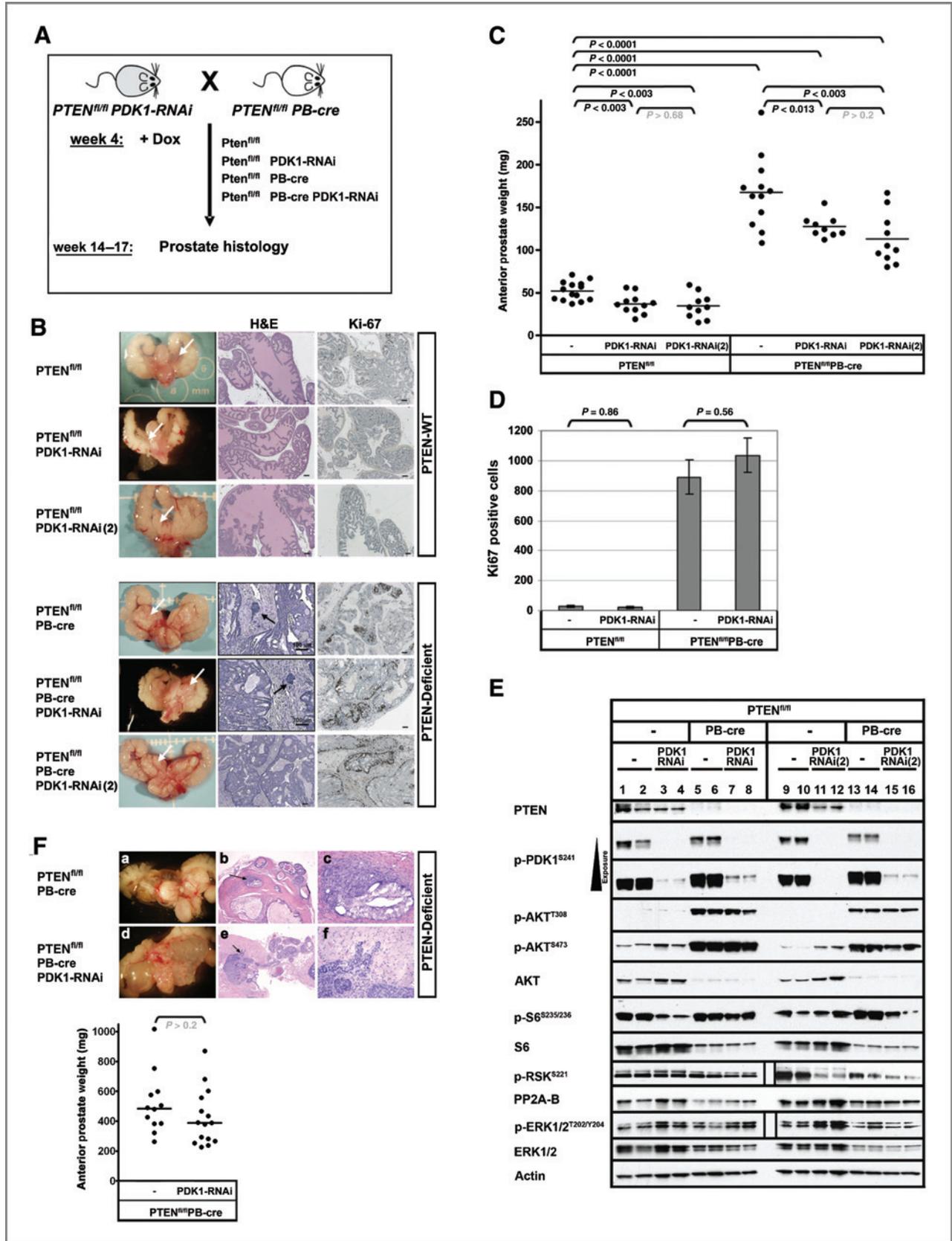
Figure 2. PDK1 knockdown in *Rosa26^{PDK1-RNAi/+}* mice is reversible and induces a metabolic phenotype. **A**, outline of the experiment. Mice were fed doxycycline (2 g/kg of chow) for 30 days and then returned to a regular diet. **B**, PDK1 levels in PBMCs (pooled samples for each group; n = 10). **C**, blood plasma insulin concentration. **D**, blood glucose levels in overnight fasted mice were determined while on doxycycline diet for 23 days and after removal of doxycycline to determine reversibility of elevated glucose levels. **E**, GTT performed at day 23 on doxycycline. **F**, ITT after 30 days on doxycycline. Values depict mean \pm SEM as percentage of initial concentration. Student *t*-test determined: *, $P < 0.05$, **, $P < 0.01$.

characterized the PDK1-RNAi mice for a diabetic phenotype. Female mice carrying the PDK1 knockdown allele (*Rosa26^{PDK1-RNAi/+}*) or WT littermates were administered doxycycline for 30 days and blood glucose and plasma insulin levels were monitored as well as PDK1 knockdown levels in peripheral blood mononuclear cells (PBMC) (Fig. 2A).

Of note, we tested several anti-PDK1 antibodies by Western blotting and observed that they often depicted, in a tissue-dependent manner, nonspecific signals close to the molecular weight of PDK1 complicating evaluation of knockdown efficiency (Fig. S1). By contrast, the anti-phospho-PDK1^{S241} antibody was highly specific and since this phosphorylation event is absolutely required for PDK1 activity (15) it was routinely employed to evaluate knockdown efficiency (Fig. 2B). The use of phospho-PDK1^{S241} to evaluate PDK1 knockdown levels was

further supported by quantitative mass-spectrometric analysis that identified greater than 99% phospho-occupancy at PDK1^{S241} (data not shown).

PDK1 protein levels were rapidly downregulated in PBMCs after feeding doxycycline diet and reversed within 5 weeks after withdrawal of the inducer (Fig. 2B). Similar kinetics for target knockdown have been reported for the insulin receptor RNAi model (30) and is consistent with reports that saturation of a system with doxycycline is an intrinsically faster process than depletion (37, 38). Notably, systemic PDK1 knockdown *in vivo* resulted in hyperinsulinemia as well as hyperglycemia (Fig. 2C and D). Thus, GTT and ITT were performed to further investigate a potential diabetes phenotype. Mice with PDK1 knockdown exhibited considerably higher fasting blood glucose levels



and impaired ability to clear glucose during intraperitoneal GTT (Fig. 2D and E). Moreover, *Rosa26^{PDK1-RNAi/+}* mice had decreased insulin sensitivity compared to WT littermates during ITT (Fig. 2F). The efficiency of PDK1 knockdown in peripheral insulin-responsive tissues (fat, liver, and muscle) was analyzed and found to be greater than 90% in each tissue (Fig. 1C and data not shown). Subsequently, we removed doxycycline from the diet to test whether the diabetic phenotype would be reversible. Similar to the recovery of PDK1 protein levels (Fig. 2B) fasted blood glucose returned to normal levels within 6 to 7 weeks after doxycycline withdrawal (Fig. 2D). Altogether, we conclude that *Rosa26^{PDK1-RNAi/+}* mice enable an efficient and reversible knockdown of PDK1 in adult, mature tissues, sufficient to impact physiological functions.

Prostate tumors develop in PTEN-deficient context despite long-term knockdown of PDK1

To better understand the role PDK1 inhibition could play in preventing PI3K-pathway-dependent tumorigenesis, we crossed both PDK1-RNAi mouse lines with a conditional PTEN-deficient prostate tumor model (Fig. 3A). It is well established that the *PTEN^{f/f}PB-cre* mice develop prostatic intraepithelial neoplasia shortly after probasin cre-mediated PTEN deletion (i.e., 4 weeks of age) and that invasive adenocarcinomas can be seen by 9 to 12 weeks of age (25). To model systemic PDK1 inhibition (analogous to a small-molecule kinase inhibitor) the mice were treated with doxycycline starting at 4 weeks of age. Dissection at 14 to 17 weeks revealed that anterior prostate lobes were visibly enlarged and histological analysis showed massive hyperplasia in the PTEN-deficient prostates irrespective of PDK1 knockdown (Fig. 3B and C). PDK1 deficiency limited the weight increase of the anterior prostate to a small extent, however a similar weight reduction was also found in the nontumorigenic PTEN-WT context (Fig. 3C). Thus, it is unlikely antitumor efficacy but rather due to PDK1's role in cell size regulation (28).

Strikingly, both RNAi models (*PTEN^{f/f}PB-creRosa26^{PDK1-RNAi/+}* and *PTEN^{f/f}PB-creRosa26^{PDK1-RNAi(2)/+}*) could not prevent the formation of microinvasive tumors (Fig. 3B). Consistent with the observed hyperplasia in PTEN-deficient tissue, the number of Ki67-positive cells was increased compared to PTEN-WT prostate (Fig. 3B and D).

PDK1 knockdown had no significant effect on tumor cell proliferation based on this marker (Fig. 3B and D).

Next we evaluated whether extended PDK1 inhibition (>10 months) could achieve antitumor efficacy during the final stages of prostate tumor progression. Mice were analyzed at an age of 47 ± 3 weeks for *PTEN^{f/f}PB-cre* and 45 ± 4 weeks for *PTEN^{f/f}PB-crePDK1-RNAi*, respectively. Irrespective of PDK1 knockdown, tumors further progressed resulting in enlarged anterior prostates compared to younger animals (Fig. 3C and F). Histological examination confirmed no difference in tumor stage and invasiveness despite potent and long-term PDK1 knockdown (Fig. 3F).

Both PDK1-RNAi models achieved efficient PDK1 knockdown in the PTEN-WT (*PTEN^{f/f}*) and PTEN-deficient (*PTEN^{f/f}PB-cre*) context (Fig. 3E). Surprisingly, long-term knockdown of PDK1 had no measurable effect on AKT^{T308} phosphorylation in the PTEN-deficient context but impacted modestly phosphorylation of p90RSK^{S221} and S6^{S235/236} (Fig. 3E). Notably, we observe 2 feedback mechanisms. First, p-ERK1/2^{T202/Y204} levels increase in response to PDK1 knockdown (Fig. 3E) possibly due to inhibition of RSK. A similar effect was observed *in vitro* with BI-D1870, a selective pharmacological inhibitor of p90RSK (39). Second, total AKT protein expression is downregulated in response to PTEN deletion suggesting a compensatory mechanism to the observed activation/hyperphosphorylation of AKT (Figs. 3E, 4D, and 5). In conclusion, PDK1 knockdown (>90%) is not able to prevent tumor formation in PTEN-deficient prostate consistent with the lack of strong PI3K/AKT-pathway modulation.

PDK1 knockdown in an inducible PTEN-deficient leukemia model has minimal impact on survival

PTEN deletion occurs with high frequency in many human cancers, including prostate, brain, breast, and leukemia (1). To model the leukemic event, we generated a conditional mouse model with the capacity for inducible PTEN deficiency in hematopoietic stem cells by combining the *PTEN^{f/f}* allele (25) with the tamoxifen controllable *Rosa26^{creER}* allele (ref. 30; Fig. 4A). These mice (*PTEN^{f/f}Rosa26^{creER/+}*) developed typical characteristics of leukemia [Acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML)] after tamoxifen-induced PTEN deletion. At 5 weeks post Cre-induction, mice started to show significant weight

Figure 3. PDK1 knockdown does not prevent invasive prostate tumor formation. A, experimental overview for Fig. 3B to E. Two PDK1-RNAi mouse lines were bred with the PTEN-dependent prostate tumor model. The following cohorts of mice: *PTEN^{f/f}*, *PTEN^{f/f}PDK1-RNAi*, *PTEN^{f/f}PDK1-RNAi(2)*, *PTEN^{f/f}PB-cre*, *PTEN^{f/f}PB-crePDK1-RNAi*, and *PTEN^{f/f}PB-crePDK1-RNAi(2)* ($n = 14, 11, 10, 13, 10, 10$, respectively) were fed with doxycycline (1 g/kg) continuously beginning at 4 weeks of age and prostate tissue harvested at 14 to 17 weeks of age. B, prostate anatomy and histology. Anterior prostate lobes (white arrows) are enlarged in the PTEN-deficient context irrespective of PDK1 knockdown. Representative tissue sections of anterior prostate lobes showing microinvasive tumors (black arrows) and Ki67-positive cells. C, anterior prostate weights. Each dot represents an individual mouse, geometric mean is indicated. *P*-values were calculated by student *t*-test. D, quantification of Ki67 staining. E, analysis of PDK1 knockdown and pathway modulation in anterior prostate lobes. For each genotype, protein lysates from 2 representative mice are shown. F, prostate anatomy and histology in aged animals. *PTEN^{f/f}PB-cre* ($n = 12$; age 47 ± 3 weeks) *PTEN^{f/f}PB-crePDK1-RNAi* ($n = 15$; age 45 ± 4 weeks). Anterior prostate lobes are strongly enlarged in the PTEN-deficient context irrespective of PDK1 knockdown (a, d, and graph). b, H&E of prostate showing normal glands (top left), mucin-filled cysts (bottom), and left of center a hyperplastic gland with hyperchromatic cells extending to the left (arrow). c, higher magnification of (b) showing the hyperplastic gland at bottom right and a proliferation of hyperchromatic, pleiomorphic cells extending away from the hyperplastic gland and invading surrounding stroma. e, hyperplastic prostate glands to right and adjacent skeletal muscle to left. In the center of the muscle and extending upwards into the muscle are projections of tumor cells. f, higher magnification of (e) showing tumor cells invading into skeletal muscle.

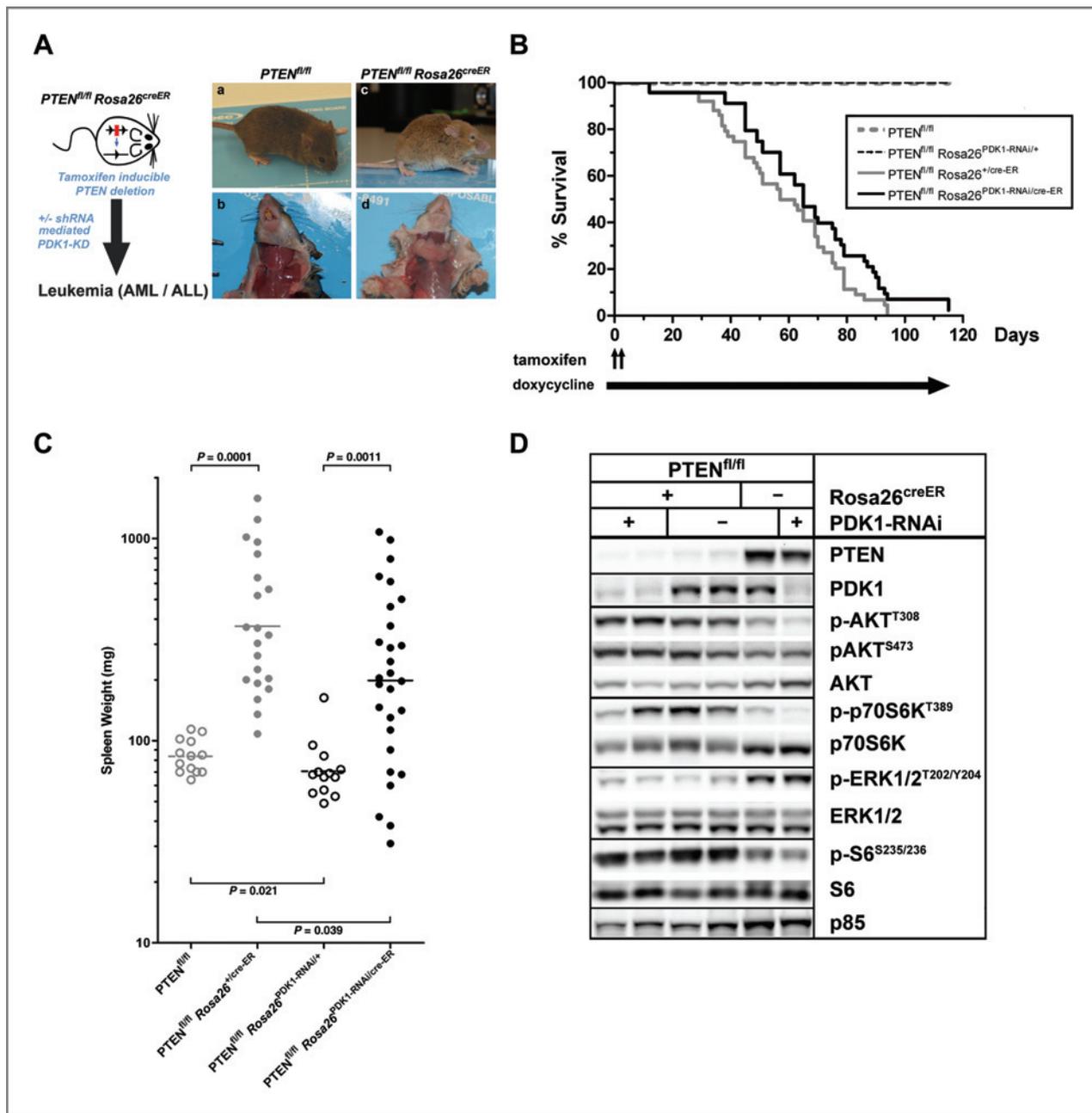


Figure 4. Impact of PDK1 knockdown on leukemia development in a PTEN-deficient context. **A**, experimental overview. Mature PTEN^{fl/fl}Rosa26^{+cre-ER} and PTEN^{fl/fl}Rosa26^{PDK1-RNAi/cre-ER} mice (12–14 weeks old) were placed on doxycycline diet (2 g/kg) and 4 days later tamoxifen was administered (day 0 and day 2) to induce PTEN deletion. Leukemia (AML/ALL) developed in these mice which caused a hunched posture (c) and enlarged lymph nodes (d) compared to PTEN^{fl/fl} (a, b) controls. **B**, impact of PDK1 knockdown on survival. PTEN^{fl/fl} mice without the Rosa26^{cre-ER} allele mice did not develop disease and survival was not affected by PDK1 knockdown (n = 31, dashed black) in comparison to controls with normal PDK1 levels (n = 23, dashed gray). Presence of Rosa26^{cre-ER} conferred PTEN deletion and leukemia development. PTEN^{fl/fl}Rosa26^{PDK1-RNAi/cre-ER} mice (solid black, n = 48) had a longer median survival (65 vs. 57 days) than their PTEN^{fl/fl}Rosa26^{+cre-ER} (solid gray, n = 49) littermates (P = 0.0564, log-rank test). **C**, spleen weights in PTEN^{fl/fl}Rosa26^{+cre-ER} and PTEN^{fl/fl}Rosa26^{PDK1-RNAi/cre-ER} mice were determined when mice were sacrificed because of advanced disease state. Nondiseased PTEN^{fl/fl} and PTEN^{fl/fl}Rosa26^{PDK1-RNAi/+} mice were kept on doxycycline for more than 5 weeks. Horizontal lines indicate geometric means (P-values are based on a nonparametric t-test). **D**, PI3K pathway activity in splenocytes 5.5 weeks after tamoxifen treatment. Each lane represents an individual animal. All mice are homozygous PTEN^{fl/fl}. "+" denotes the presence of the Rosa26^{creER} and PDK1-RNAi alleles, respectively.

loss, became hunched, and developed a scruffy coat in comparison to PTEN^{fl/fl} controls (Fig. 4A). These phenotypes are similar to a previously described interferon-driven indu-

cible PTEN deficiency model (40, 41). PTEN^{fl/fl}Rosa26^{creER/+} mice displayed consistent changes in the composition of white blood cell fractions detectable as early as 2 weeks post

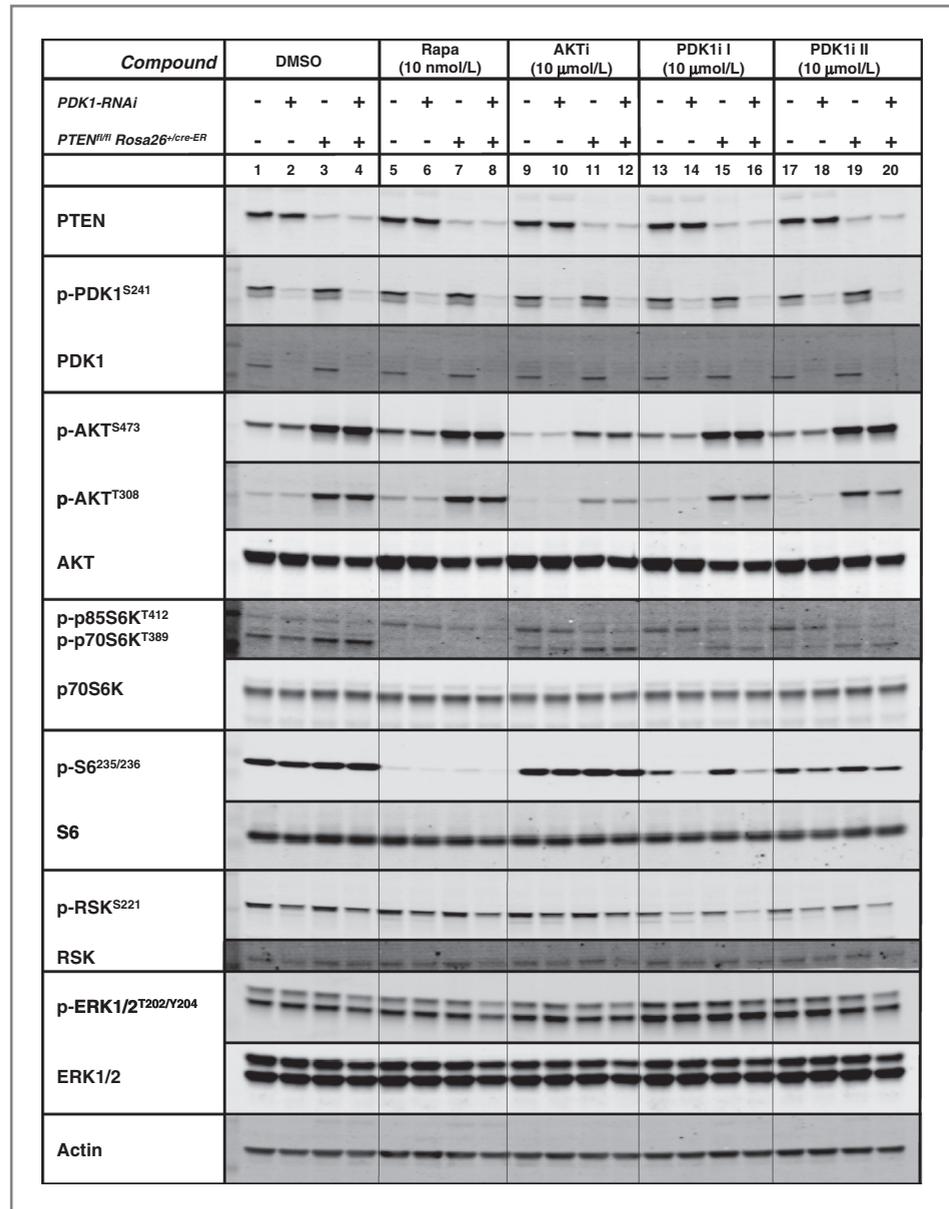


Figure 5. Combined pharmacological and genetic inactivation of PDK1 does not suppress AKT activation in a PTEN-deficient setting. *PTEN^{fl/fl}* and *PTEN^{fl/fl}Rosa26^{cre-ER}* mice with or without the *Rosa26^{PDK1-RNAi/+}* allele were placed on doxycycline diet (2 g/kg), treated with tamoxifen, and mammary epithelial cells were isolated 2 weeks later. Cells were cultured in medium containing 2 μg/mL doxycycline, 5% FBS, 5 ng/mL EGF, and 10 μg/mL insulin for 3 days. Inhibitors were added for 4 hours prior to cell lysis.

PTEN deletion. The B220⁺ B cell fraction was reduced while the fraction of CD11b⁺ cells was strongly increased (data not shown). The extent of these changes however did not correlate with disease progression or length of survival in the study. Thus, we conclude that these are systemic changes linked to PTEN deficiency, not reflecting a leukemic disease.

Disease progression was fast and overall body condition deteriorated such that more than 90% of animals needed to be sacrificed at 5 to 12 weeks post PTEN deletion (Fig. 4B). At sacrifice, most *PTEN^{fl/fl}Rosa26^{creER/+}* animals presented with enlarged lymph nodes and spleens (Fig. 4A and C). Leukemic disease was heterogeneous: some mice had a transplantable T-ALL with an outgrowth of CD4⁺CD8⁺ or CD4⁺ T cells in the periphery, while others had an out-

growth of CD11b⁺ cells in lymphoid organs and bone marrow (Fig. S2).

Next, we investigated whether a strong reduction of PDK1 expression in the PTEN-deficient leukemia model could effectively modulate activity of the PI3K pathway and prevent or slow disease onset. *PTEN^{fl/fl}Rosa26^{PDK1-RNAi/cre-ER}* mice were fed doxycycline diet 4 days prior to the induced PTEN deletion to ensure effective PDK1 knockdown before heightened PI3K pathway activity would occur. Interestingly, these mice all developed disease with comparable characteristics as their littermates without the PDK-RNAi allele (Fig. 4C and data not shown). At sacrifice, greater than 90% PDK1 knockdown was observed in the *PTEN^{fl/fl}Rosa26^{PDK1-RNAi/creER}* diseased animals (Fig. 4D). PDK1 knockdown had a small effect on spleen

weights irrespective of PTEN status (Fig. 4C). However, despite PDK1 knockdown most $PTEN^{fl/fl}Rosa26^{PDK1-RNAi/creER}$ mice still displayed significantly enlarged spleens when compared to control mice (Fig. 4C). Disease onset and progression was not significantly delayed by PDK1 knockdown (Fig. 4B). Median survival was 65 days for $PTEN^{fl/fl}Rosa26^{PDK1-RNAi/creER}$ mice versus 57 days in $PTEN^{fl/fl}Rosa26^{+/creER}$ littermates.

Following PTEN deletion, PI3K-pathway activation was detectable in both peripheral white blood cells (Fig. S3) and the spleen from $PTEN^{fl/fl}Rosa26^{creER/+}$ mice (Fig. 4D). However, while knockdown of PDK1 in PTEN-WT context reduced phosphorylation of AKT^{T308}, p70S6K^{T389}, and S6^{S235/236}, PDK1 knockdown in PTEN-deficient context ($PTEN^{fl/fl}Rosa26^{PDK1-RNAi/creER}$), did not lower p-AKT^{T308} or p-S6^{S235/236} levels, which remained significantly above the basal levels seen in PTEN-WT controls (Fig. 4D).

We conclude that similar to the prostate tumor formation, inducible PDK1 knockdown (>90%) in adult mice is not sufficient to prevent development of leukemia following PTEN deletion.

Combined pharmacologic and genetic inactivation of PDK1 cannot reverse phospho-AKT^{T308} elevation resulting from PTEN deficiency

One possible explanation for the missing p-AKT^{T308} down-regulation despite more than 90% PDK1 knockdown is that the remaining PDK1 protein is still sufficient to perform the catalysis. To test this hypothesis, we isolated mammary epithelial cells from PTEN-WT ($PTEN^{fl/fl}$) and PTEN-deficient ($PTEN^{fl/fl}Rosa26^{+/cre-ER}$) mice with or without the $Rosa26^{PDK1-RNAi/+}$ allele and treated them *ex vivo* with 2 structurally diverse and cell potent PDK1 inhibitors (Fig. S4). Both inhibitors display single digit nanomolar potency (EC₅₀) against purified PDK1 enzyme. To maximize inhibition of the residual PDK1 activity, we used high compound concentrations (10 μmol/L) only achievable in an *in vitro* setting. As controls we used an allosteric and highly selective AKT inhibitor (42) and the mTOR inhibitor rapamycin.

At the time of cell harvest, mammary glands had not developed tumors in the PTEN-deficient context but appeared hyperplastic. As shown in Figure 5, the PI3K pathway was strongly activated by PTEN loss (indicated by p-AKT and p-p70S6K) whereas the phosphorylation of the mitogen activated protein kinase (MAPK) pathway components p90RSK and extracellular signal regulated kinase (ERK) was unchanged (lanes 1 vs. 3). AKT inhibitor and rapamycin treatment resulted in potent downregulation of p-AKT (lanes 1 vs. 9 and 3 vs. 11) and p-p70S6K levels (lanes 1 vs. 5 and 3 vs. 7), respectively. Similar to our *in vivo* findings, knockdown of PDK1 by more than 90% was not able to counteract the elevated phosphorylation of AKT^{T308} caused by PTEN-deletion (lanes 3 vs. 4). In contrast, PDK1-knockdown decreased phosphorylation of p90RSK^{S221} about 2-fold across all treatment groups.

For each of the 4 genotypes, addition of pharmacological PDK1 inhibitors reduced the phosphorylation levels of the PDK1 substrates p70S6K, p90RSK, and AKT (Fig. 5, lanes 13–16 and 17–20) compared to dimethyl sulfoxide (DMSO)

controls (Fig. 5, lanes 1–4). Moreover, consistent with a direct pharmacological inhibition of PDK1 kinase activity, only p-AKT^{T308} was reduced whereas p-AKT^{S473} was not altered in the primary mammary gland cultures. PDK1 inhibitor treatment did not alter p-PDK1^{S241} or total PDK1 protein levels. In contrast to pharmacological PDK1 inhibition, rapamycin and the AKT inhibitor potently inhibited the phosphorylation of S6 and AKT phosphosites, respectively (Fig. 5). Although a modest inhibition of p-AKT^{T308} was observed *ex vivo* by the 2 PDK1 inhibitors, p-AKT^{T308} was still elevated when compared to PTEN-WT controls (lane 15 vs. 1 and 19 vs. 1). Combined PDK1 knockdown and pharmacological inhibition (lanes 14, 16, 18, 20) showed a further inhibitory effect on p-AKT^{T308} phosphorylation, however, the effects remained small compared to direct AKT inhibition (lanes 9–12). An additive effect of combined knockdown and pharmacological inhibition was also observed for other pathway nodes (p90RSK^{S221} and pS6^{S235/236} phosphorylation).

In summary, compared to direct AKT inhibition using an AKT1/2 specific inhibitor, combined genetic and pharmacological inhibition of PDK1 still remains inferior as a means to inhibit AKT activity.

Tumor incidence in heterozygous $PTEN^{+/-}$ mice

Thus far, in 2 models of inducible homozygous PTEN loss, efficient PDK1 knockdown did not prevent tumor formation. By contrast, $PDK1^{fl/-}$ hypomorphic mice, which display a constitutive reduction of PDK1 enzymatic activity by about 80% to 90% in all tissues, have been reported to prevent tumorigenesis in a heterozygous $PTEN^{+/-}$ context (27).

First, to ensure that this discrepancy was not caused by significant differences in the remaining PDK1 protein levels between RNAi and hypomorphic knockdown models, we compared tissues from these models for PDK1 expression by Western analysis (Fig. 6A). Mice with a single hypomorphic allele ($PDK1^{fl/+}$) showed around 45% reduction in PDK1 expression as previously reported (27). Stronger reduction of PDK1 levels (>80%) was observed in the $PDK1^{fl/fl}$ and $PDK1^{fl/-}$ hypomorphic mice and these low levels were comparable to the RNAi-mediated PDK1 knockdown observed in prostate, T cells, mammary epithelial organoids, and liver (Fig. 6A). Thus, we conclude that the reduction in PDK1 protein is comparable between the 2 model systems.

Next, to test whether the difference in efficacy outcome could be explained by homozygous versus heterozygous PTEN deficiency, we combined the inducible $Rosa26^{PDK1-RNAi}$ allele with heterozygous $PTEN^{+/-}$ mice. Similar to the approach of Bayascas and colleagues, we tested whether PDK1 knockdown would have efficacy on tumor burden in multiple organs (Fig. 6B). Taking advantage of the inducible knockdown system, adult mice were continuously fed either doxycycline or control diet beginning at 6 weeks of age, which circumvents potential developmental issues of constitutive knockdown models. All animals were sacrificed within 1 week, at an age of 6 to 7 months. This was the stage when visible lymphomas became apparent in the $PTEN^{+/-}$ background.

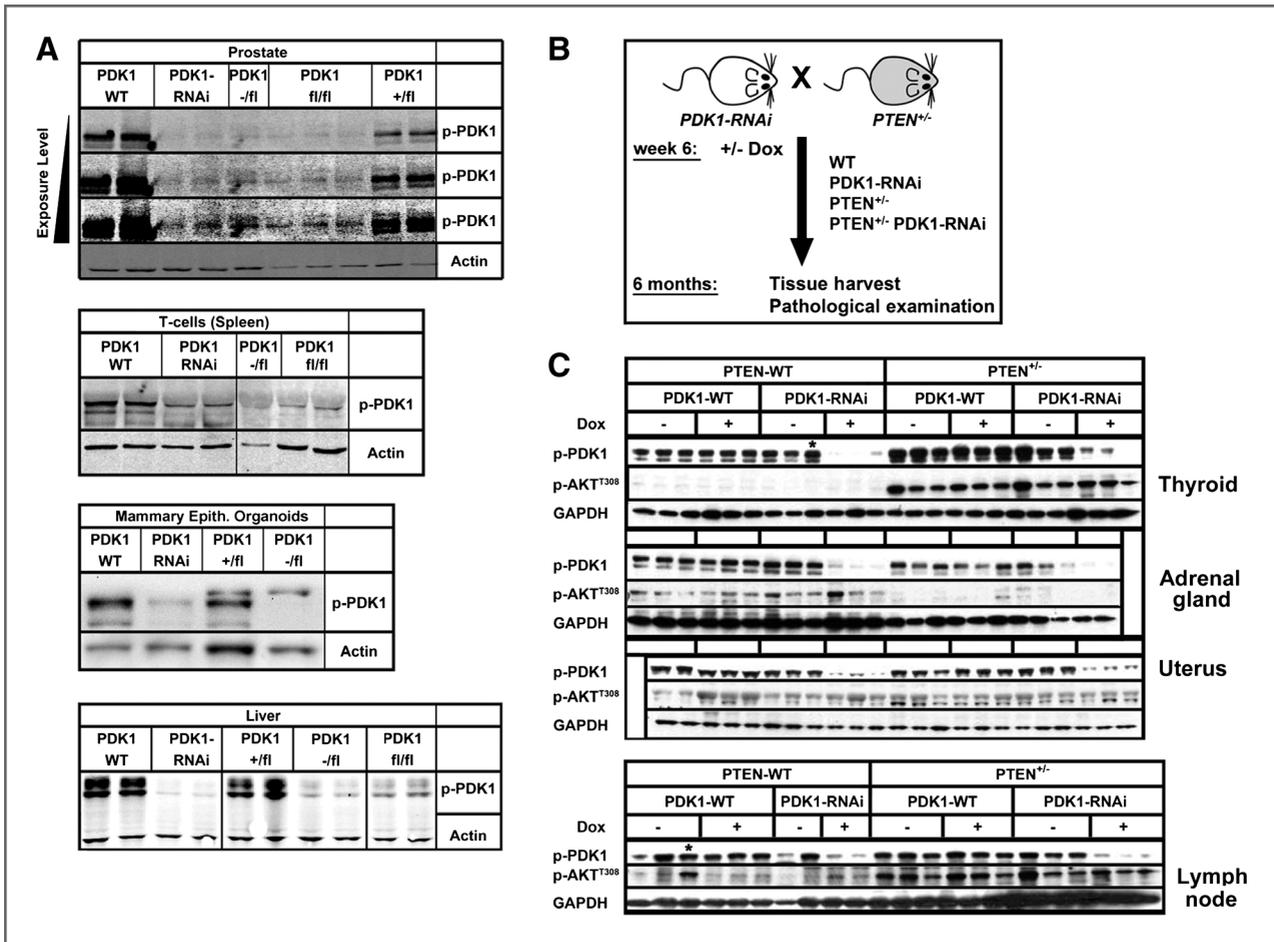


Figure 6. Assessment of knockdown efficiency across models and impact of PDK1 knockdown in heterozygous *PTEN*^{+/-} mice. **A**, comparison of PDK1 knockdown levels in tissues from *Rosa26*^{*PDK1-RNAi/+*} mice, hypomorphic *PDK1*^{-fl} and *PDK1*^{fl/fl} mice. Cell lysates from prostate (top), splenic T cells (MACS-purified CD90.2⁺), mammary fat pads, and liver were immunoblotted with anti-phospho-PDK1 (S241). RNAi-mediated knockdown of PDK1 was induced 10 weeks prior to tissue harvest. **B**, experimental overview. Adult mice were fed with doxycycline (2 g/kg) starting at 6 weeks of age until sacrifice at 6 months. Thyroid, lymph node, adrenal gland, and uterus were harvested from each mouse for histologic and Western blot analysis. **C**, Western analysis of p-PDK1^{S241} and p-Akt^{T308} in the *PTEN*^{+/-} tumor model. Long-term knockdown of PDK1 was achieved in *Rosa26*^{*PDK1-RNAi/+*} and *PTEN*^{+/-} *Rosa26*^{*PDK1-RNAi/+*} animals in response to doxycycline-induced RNAi. In a pathologic examination, all *PTEN*^{+/-} tissue samples showed hyperplasia/tumor whereas all *PTEN*-WT tissues presented with normal histology except one thyroid and lymph node sample (indicated by *).

Thyroid, uterus, lymph nodes, and adrenal glands were harvested and examined in a blinded comparative histopathologic analysis (Table 1). Western blot analysis confirmed that efficient PDK1 knockdown (>80%) was achieved in all tissues analyzed (Fig. 6C).

Tissue analysis of our *PTEN*^{+/-} mice showed phenotypes similar to previously reported *PTEN*^{+/-} heterozygous models, where animals develop a variety of tumors in multiple organs including the lymph nodes, mammary gland, uterus, prostate, adrenal glands, and thyroid (21–23). Strikingly, histopathologic examinations revealed that neoplastic lesions were present at high incidence in both *PTEN*^{+/-} and in *PTEN*^{+/-} *Rosa26*^{*PDK1-RNAi/+*} animals, irrespective of PDK1 knockdown status (Table 1). For example, in the presence of doxycycline, 82% of the *PTEN*^{+/-} mice showed thyroid adenomas comparable to the 89% in

PTEN^{+/-} *Rosa26*^{*PDK1-RNAi/+*} animals. Neoplastic lesions in the adrenal gland were less frequent and we observed high variation among control groups (20%–53%). Thus the apparent lowered incidence (4 vs. 2 animals) in the doxycycline treated group may have occurred by chance. Alternatively, we noted that loss of *PTEN* in the adrenal gland had little effect on AKT upregulation, which could provide a setting for PDK1 knockdown to be efficacious (Fig. 6C).

In summary, substantial knockdown of PDK1 (>80%) is not able to prevent tumor development in *PTEN*^{+/-} tissues. This difference in phenotype from the PDK1 hypomorphic mice crossed to *PTEN*^{+/-} mice may be attributed to knockdown in adult versus embryonic tissues as the latter results in a small cell phenotype with significantly altered body size and nutrients uptake defects (27, 28).

Table 1. Incidence of hyperplasia in $PTEN^{+/-}$ mice following PDK1 knockdown

Genotype	Diet	Thyroid hyperplasia		Adrenal pheochromocytoma		Lymph node hyperplasia		Uterus hyperplasia	
		Incidence	%	Incidence	%	Incidence	%	Incidence	%
$PTEN^{+/-}$ PDK1-RNAi	- Dox	8/20	40	4/20	20	7/20	35	5/10	50
	+ Dox	24/27	89	2/26	8	14/31	45	9/21	43
$PTEN^{+/-}$	- Dox	16/21	76	5/22	23	10/22	45	3/9	33
	+ Dox	14/17	82	9/17	53	11/20	55	8/13	61
PDK1-RNAi	- Dox	1/28	4	0/27	0	0/23	0	0/17	0
	+ Dox	0/17	0	0/21	0	0/19	0	0/13	0
WT	- Dox	0/23	0	0/26	0	1/20	5	1/14	7
	+ Dox	0/27	0	0/28	0	0/28	0	0/11	0

NOTE: Inducible long-term knockdown of PDK1 does not reverse hyperplastic and tumor phenotypes in $PTEN^{+/-}$ $Rosa26^{PDK1-RNAi/+}$ Mice. Tabulated results of histological readouts (blinded samples scored) show lack of efficacy in lymph nodes, thyroids and uteri of $PTEN^{+/-}$ and $PTEN^{+/-}$ $Rosa26^{PDK1-RNAi/+}$ animals fed doxycycline. Animal numbers in each group: $PTEN^{+/-}$ $Rosa26^{PDK1-RNAi/+}$ (-Dox, $N = 20$, 10 female; +Dox, $N = 31$, 21 female), $PTEN^{+/-}$ (-Dox, $N = 22$, 9 female; +Dox, $N = 20$, 13 female), $Rosa26^{PDK1-RNAi/+}$ (-Dox, $N = 28$, 17 female; +Dox, $N = 21$, 13 female), WT (-Dox, $N = 26$, 14 female; +Dox, $N = 28$, 11 female). Not all tissues were available for histological analysis due to sample processing.

Discussion

PDK1 is thought to be a key modulator of PI3K-pathway signaling based on elegant studies using $PDK1^{-/-}$ mouse ES cells and knockin mutant mice (15). It is an activator of numerous AGC kinases and more than a dozen companies have disclosed efforts on developing PDK1 inhibitors (11). However, to date, no inhibitors have emerged with suitable kinase selectivity and physiochemical properties to enable meaningful *in vivo* studies (11). In this light, genetic approaches to study PDK1 function become highly valuable. Such approaches directly complement preclinical drug discovery efforts as a means to identify pharmacodynamic biomarkers and explore patient responder hypotheses.

Here, to provide a preclinical assessment of PDK1 target efficacy and safety, we generated mouse lines with efficient doxycycline-induced knockdown of PDK1. Compared with conventional gene-targeted knockout strategies, this approach has several advantages including the reversible and temporal control of genetic-alteration. While recombinase-based knockouts or mutational approaches (exemplified by the Cre/loxP system) enable temporal control, the genetic modification is irreversible. For modeling of many human diseases, such as PTEN deficiency-dependent tumorigenesis, conditional Cre/loxP systems are very powerful as illustrated by the advanced PTEN-null leukemia and prostate cancer models (25, 40, 41). However, as a genetic approach to mimic pharmaceutical intervention, gene knockout models are limited by the fact that they completely deplete the cell of the targeted protein. Pharmacologically, for an enzyme this would equal a 100% chronic inhibition of catalytic activity, which is an unrealistic benchmark for most drug targets. RNAi-mediated inactivation, however, typically lowers the amount of catalytically active drug target, which in this respect is more

similar to a pharmacological intervention. However, inherent differences still remain between RNAi and small-molecule inhibitors. Frequently, small-molecule drugs specifically target enzymatic activity while knockdown impacts all protein functions (i.e., scaffolding and adaptor functions). In some cases, this can result in distinct biological phenotypes (43, 36). However, given the lack of suitable *in vivo* PDK1 tool compounds (11), the present RNAi-based approach is highly warranted.

Important for our inducible gene silencing approach is that substantial PDK1 knockdown is achievable and that shRNA expression is effectively suppressed prior to administration of the inducer (doxycycline). We report tight control of PDK1-shRNA expression and efficient knockdown, consistent with the initial report using this targeting strategy (30). Out of 10 tissues, brain is the only organ with a significant reduction of PDK1 transcripts in the absence of doxycycline. However, treatment with doxycycline resulted in further knockdown and lowered protein levels. The apparent leakiness of shRNA expression in the brain is likely an inherent property of the H1-tetR system, since we also observed it for other genes evaluated by the same RNAi approach (unpublished data).

For PDK1 we have demonstrated that efficient (90%) knockdown is neither sufficient to block PI3K pathway activation in various tissues nor prevent tumor formation and progression resulting from loss of PTEN. This genetically invalidates PDK1 as an attractive mono-therapy drug target in a PTEN-deficient tumor context. Our results clearly indicate that the residual PDK1 was sufficient to support high levels of p-AKT^{T308} and maintain flux through the PI3K-pathway. Alternatively, other factors exist that can compensate for the low PDK1 levels. The latter scenario may be aided by adaptive cellular mechanisms on long-term PDK1 knockdown (i.e., feedback mechanism,

subcellular compartmentalization, changes in phosphatase activities). Taken together, we conclude that PDK1 is not rate limiting for AKT phosphorylation in PTEN-deficient tissues.

While PDK1 is not the rate limiting factor for the pathway, it is still absolutely required for phosphorylation of AKT^{T308} as recently shown in using conditional PDK1 knockouts. The total loss of PDK1 in PTEN-deficient context completely abolishes p-AKT^{T308} and prevents formation of T-Cell leukemia (8, 9).

Our findings are in contrast to what was seen in PDK1 hypomorphic mice, where the neomycin selection cassette within the PDK1 locus reduced PDK1 activity and protein levels by 80% to 90% (27). When combined with *PTEN*^{+/-} mice, tumor development was completely absent until 8 months of age. In older mice (14 to 15 months), the authors also reported a severe suppression of tumor incidence with only 22% of hypomorphic PDK1^{fl/-}PTEN^{+/-} mice having detectable tumors compared to 72% in PDK1^{+/-}PTEN^{+/-} mice (27). This observation, and the fact that PDK1^{-/-} ES cells are unable to activate numerous AGC kinases (6), including AKT, was originally taken to validate PDK1 as a prime drug target for PI3K pathway-addicted tumors.

Notably, our constitutive PTEN^{+/-} animals display shorter tumor latency than the 8 months reported by Bayascas and colleagues (27) and a tumor spectrum that is more similar to that reported by other groups (21–23). Across different tissues, we observed tumor formation at 6 months of age or younger and report no significant antitumor correlation with PDK1 deficiency.

To investigate this discrepancy, we have directly compared PDK1 protein levels between the PDK1-RNAi and hypomorphic models and found comparable lowered protein levels as judged by Western blotting. We cannot formally exclude that a subtly better knockdown in the hypomorphic animals exists, which could lower PDK1 protein levels below a threshold that is required to sustain elevated PI3K signaling in tumor cells. Such a threshold, although in a developmental context, could explain why we observe a less than Mendelian ratio of PDK1^{fl/-} offspring when generating hypomorphic mice (Experimental Procedures). Apparently, a functional threshold for PDK1 hypomorphism exists that depends on the genetic background as backcrossing of the PDK1^{fl/-} mice onto a C57BL/6 background severely reduced viability. Another significant difference between the 2 models, which may contribute to the discrepancy in antitumor efficacy, is the strong developmental impact of PDK1 hypomorphism on body size (30%–40% reduction; ref. 28).

In the current study, we evaluated the impact on glucose metabolism primarily to assess whether PDK1 knockdown levels were sufficient to have immediate physiological consequences. We found that PDK1 knockdown had an acute metabolic effect that coincided with the kinetics of protein knockdown. Consistent with this, tissue specific knockout of PDK1 in liver and pancreatic beta cells has been reported to result in glucose intolerance and diabetes, respectively (7, 44). Also, mutation of the PDK1 PH-domain leads to insulin resistance in mice due to impaired phosphorylation

of AKT^{T308} (45). A general concern of targeting the PI3K-pathway has been the potential impact on glucose homeostasis in terms of potentially limiting the therapeutic window. Importantly, long-term PDK1 knockdown was well tolerated and elevation of blood glucose levels was reversible on removal of doxycycline. PDK1 knockdown animals appeared overall healthy and maintained normal weight. This safety profile is in contrast to the liver specific PDK1 knockout mice, which die from liver malfunction after 4 to 6 weeks (7).

The side-by-side comparison of single digit nanomolar inhibitors targeting either AKT or PDK1 revealed a different ability to impact p-AKT^{T308} levels. Even when combined with efficient PDK1 knockdown, treatment of primary PTEN-deficient cells with high concentrations of PDK1 inhibitor (1,000-fold more than enzymatic EC₅₀) was not capable of normalizing elevated p-AKT^{T308}. In contrast, treatment with the allosteric AKT inhibitor efficiently blocked AKT phosphorylation in this system. Of note, we used primary cells with exclusive deletion of PTEN to study the effect of PI3K-pathway inhibitors. In contrast to transformed cell lines, which have adapted to growth on tissue culture plastic and have acquired additional mutations, the advantage of the *ex vivo* primary cultures is that we can study a defined PTEN deficiency context without direct interference from other activated or suppressed pathways.

In summary, our mouse models predict that human cancer patients, who have elevated p-AKT^{T308} as a biomarker, are at best likely to benefit only marginally, if at all, from PDK1 inhibition as a single agent. However, it is possible that PDK1 knockdown, in combinations with other targeted agents may show a synergistic antitumor effect. For example, the inhibition of p-p90RSK^{S221} in *ex vivo* primary mammary epithelial cultures suggests inhibitory effects on MAPK-pathway signaling. p-p90RSK^{S221} inhibition was unique to PDK1 inhibition and not observed with mTOR and AKT inhibitors. Paradoxically, RSK inhibition appears to be associated with potential compensatory ERK activation (Fig. 5) as described by others (39). In the leukemia model, we also noted that PTEN loss was inversely correlated with p-ERK status, highlighting PI3K/MAPK cross-pathway regulation (Fig. 4D). Moreover, we identified an adaptation or feedback mechanism that lowers AKT protein expression in response to PTEN deficiency (Figs. 3E, 4D, and 5) presumably to limit the activity of AKT.

Since we have observed physiological changes in response to PDK1 knockdown (i.e., reversible diabetes phenotype, effects on spleen size and trend toward prolonged survival in the leukemia model), the presented RNAi model is well positioned for continued evaluation of PDK1 as a potential drug target in other tumor and disease contexts. In this study, we have tested PDK1 target efficacy under a single agent treatment paradigm. Thus, it is possible that PDK1 inhibition may have significant beneficial impact on tumor inhibition either in a combination therapy or in tumors that have less strong activation of the PI3K pathway.

Finally, it remains unknown whether small-molecule PDK1 inhibitors would mimic the results from our genetic efficacy

studies. However, based on our genetic efficacy studies in 3 PTEN-deficient tumor models, we conclude that in cancer patients where blocking elevated AKT activity is highly desirable (i.e., PTEN-deficient tumors), inhibiting AKT directly using an AKT specific inhibitor will be far superior than targeting the upstream phosphorylating kinase PDK1.

Disclosure of Potential Conflicts of Interest

There are no conflicts of interest to disclose.

References

- Li J, Yen C, Liaw D, Podyspanina K, Bose S, Wang SI, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997;275:1943–7.
- Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* 2006;441:424–30.
- Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.
- Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr Biol* 1997;7:261–9.
- Calleja V, Alcor D, Laguerre M, Park J, Vojnovic B, Hemmings BA, et al. Intramolecular and intermolecular interactions of protein kinase B define its activation *in vivo*. *PLoS Biol* 2007;5:e95.
- Williams MR, Arthur JS, Balendran A, Van Der KJ, Poli V, Cohen P, et al. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol* 2000;10:439–48.
- Mora A, Lipina C, Tronche F, Sutherland C, Alessi DR. Deficiency of PDK1 in liver results in glucose intolerance, impairment of insulin-regulated gene expression and liver failure. *Biochem J* 2005;385:639–48.
- Chalhoub N, Zhu G, Zhu X, Baker SJ. Cell type specificity of PI3K signaling in Pdk1- and Pten-deficient brains. *Genes Dev* 2009;23:1619–24.
- Finlay DK, Sinclair LV, Feijoo C, Waugh CM, Hagenbeek TJ, Spits H, et al. Phosphoinositide-dependent kinase 1 controls migration and malignant transformation but not cell growth and proliferation in PTEN-null lymphocytes. *J Exp Med* 2009;206:2441–54.
- Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Dev Cell* 2006;11:859–71.
- Peifer C, Alessi DR. Small-molecule inhibitors of PDK1. *ChemMedChem* 2008;3:1810–38.
- Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene* 2008;27:5511–26.
- Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? *Nat Rev Cancer* 2007;7:554–62.
- Nguyen TL. Targeting RSK: an overview of small molecule inhibitors. *Anticancer Agents Med Chem* 2008;8:710–6.
- Mora A, Komander D, van Aalten DM, Alessi DR. PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* 2004;15:161–70.
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, et al. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007;408:297–315.
- Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 2009;9:28–39.
- Feldman RI, Wu JM, Polokoff MA, Kochanny MJ, Dinter H, Zhu D, et al. Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. *J Biol Chem* 2005;280:19867–74.
- Tamguney T, Zhang C, Fiedler D, Shokat K, Stokoe D. Analysis of 3-phosphoinositide-dependent kinase-1 signaling and function in ES cells. *Exp Cell Res* 2008;314:2299–312.
- Carver BS, Pandolfi PP. Mouse modeling in oncologic preclinical and translational research. *Clin Cancer Res* 2006;12:5305–11.
- Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del BB I, et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol* 1998;8:1169–78.
- Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. *Nat Genet* 1998;19:348–55.
- Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, et al. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* 1999;96:1563–8.
- Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995;269:1427–9.
- Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 2003;4:209–21.
- Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med* 2004;10:594–601.
- Bayascas JR, Leslie NR, Parsons R, Fleming S, Alessi DR. Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/-) mice. *Curr Biol* 2005;15:1839–46.
- Lawlor MA, Mora A, Ashby PR, Williams MR, Murray-Tait V, Malone L, et al. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J* 2002;21:3728–38.
- Kuhn R, Streif S, Wurst W. RNA interference in mice. *Handb Exp Pharmacol* 2007;149–76.
- Seibler J, Kleinridders A, Kuter-Luks B, Niehaves S, Bruning JC, Schwenk F. Reversible gene knockdown in mice using a tight, inducible shRNA expression system. *Nucleic Acids Res* 2007;35:e54.
- Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, et al. Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A* 2004;101:10380–5.
- Oberdoerffer P, Kanellopoulou C, Heissmeyer V, Paeper C, Borowski C, Aifantis I, et al. Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. *Mol Cell Biol* 2005;25:3896–905.
- Dickins RA, McJunkin K, Hernando E, Premrsiruk PK, Krizhanovsky V, Burgess DJ, et al. Tissue-specific and reversible RNA interference in transgenic mice. *Nat Genet* 2007;39:914–21.
- Stern P, Astrof S, Erkeland SJ, Schustak J, Sharp PA, Hynes RO. A system for Cre-regulated RNA interference *in vivo*. *Proc Natl Acad Sci U S A* 2008;105:13895–900.
- Pullan SE, Streuli CH. *Epithelial Cell Culture*. Cambridge, UK: Cambridge University Press; 1996. p. 97–121.
- Kraus M, Pao LI, Reichlin A, Hu Y, Canono B, Cambier JC, et al. Interference with immunoglobulin (Ig)alpha immunoreceptor tyrosine-

Acknowledgments

The authors thank Dario Alessi and the Division of Signal Transduction Therapy, University of Dundee, for generous sharing of reagents; Michele Cleary for shRNA design; Jason Laskey, Roseanne Wexler, Cara Lerner, Clay Efferson for technical support; and Gabriele Staples, Pradip Majumder, Thomas Rosahl for assistance with animal model generation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 23, 2010; revised February 16, 2011; accepted February 26, 2011; published online April 14, 2011.

- based activation motif (ITAM) phosphorylation modulates or blocks B cell development, depending on the availability of an Igbeta cytoplasmic tail. *J Exp Med* 2001;194:455–69.
37. Bocker R, Warnke L, Estler CJ. Blood and organ concentrations of tetracycline and doxycycline in female mice. Comparison to males. *Arzneimittelforschung* 1984;34:446–8.
 38. Baron U, Bujard H. Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. *Meth Enzymol* 2000;327:401–21.
 39. Sapkota GP, Cummings L, Newell FS, Armstrong C, Bain J, Frodin M, et al. BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms *in vitro* and *in vivo*. *Biochem J* 2007;401:29–38.
 40. Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 2006;441:475–82.
 41. Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* 2006;441:518–22.
 42. Bilodeau MT, Balitza AE, Hoffman JM, Manley PJ, Barnett SF, Defeo-Jones D, et al. Allosteric inhibitors of Akt1 and Akt2: a naphthyridinone with efficacy in an A2780 tumor xenograft model. *Bioorg Med Chem Lett* 2008;18:3178–82.
 43. Kung C, Shokat KM. Small-molecule kinase-inhibitor target assessment. *ChemBiochem* 2005;6:523–6.
 44. Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, et al. Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. *Nat Genet* 2006;38:589–93.
 45. Bayascas JR, Wullschleger S, Sakamoto K, Garcia-Martinez JM, Clacher C, Komander D, et al. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Mol Cell Biol* 2008;28:3258–72.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

PDK1 Attenuation Fails to Prevent Tumor Formation in PTEN-Deficient Transgenic Mouse Models

Katharine Ellwood-Yen, Heike Keilhack, Kaiko Kunii, et al.

Cancer Res 2011;71:3052-3065.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/71/8/3052
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/05/13/71.8.3052.DC1

Cited articles	This article cites 43 articles, 17 of which you can access for free at: http://cancerres.aacrjournals.org/content/71/8/3052.full.html#ref-list-1
Citing articles	This article has been cited by 7 HighWire-hosted articles. Access the articles at: /content/71/8/3052.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .