PI3K isoform dependence of PTEN-deficient tumors can be altered by the genetic context

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There has been increasing interest in the use of isoform-selective inhibitors of phosphatidylinositide-3-kinase (PI3K) in cancer therapy. Using conditional deletion of the p110 catalytic isoforms of PI3K to predict sensitivity of cancer types to such inhibitors, we and others have demonstrated that tumors deficient of the phosphatase and tensin homolog (PTEN) are often dependent on the p110β isoform of PI3K. Because human cancers usually arise due to multiple genetic events, determining whether other genetic alterations might alter the p110 isoform requirements of PTEN-null tumors becomes a critical question. To investigate further the roles of p110 isoforms in PTEN-deficient tumors, we used a mouse model of ovarian endometrioid adenocarcinoma driven by concomitant activation of the rat sarcoma protein Kras, which is known to activate p110α, and loss of PTEN. In this model, ablation of p110β had no effect on tumor growth, whereas p110α ablation blocked tumor formation. Because ablation of PTEN alone is often p110β dependent, we wondered if the same held true in the ovary. Because PTEN loss alone in the ovary did not result in tumor formation, we tested PI3K isoform dependence in ovarian surface epithelium (OSE) cells deficient in both PTEN and p53. These cells were indeed p110β dependent, whereas OSEs expressing activated Kras with or without PTEN loss were p110α dependent. Furthermore, isoform-selective inhibitors showed a similar pattern of the isoform dependence in established $Kras^{G12D}$/PTEN-deficient tumors. Taken together, our data suggest that, whereas in some tissues PTEN-null tumors appear to inherently depend on p110β, the p110 isoform reliance of PTEN-deficient tumors may be altered by concurrent mutations that activate p110α.

ovarian cancer | PI3K inhibitors | genetically engineered mouse model

The phosphatidylinositide-3-kinases (PI3Ks) constitute a family of lipid kinases that are subdivided into three subclasses based on their mechanisms of activation, substrate preference, and subunit composition (1–3). Of these, the class-IA PI3Ks has been intensively studied, as this class of enzymes has been demonstrated to be involved in human cancer. This class of PI3Ks is heterodimeric proteins composed of a catalytic p110 and a regulatory p85 subunit, both of which exist in several isoforms. There are three class IA p110 isoforms in mammalian cells, of which p110δ is mostly restricted to the immune system, whereas p110α and p110β are ubiquitously expressed (1, 2). The class IA PI3Ks has long been found to promote oncogenic growth of cells in vitro (4–6) and PI3K signaling is known to be tightly controlled by the tumor suppressor phosphatase and tensin homolog (PTEN) (7). More recently, interest in this protein family has increased due to the identification of cancer specific mutations in the p110α isoform (8). Notably, in the initial study and in all subsequent reports, mutations were seen only in p110α (8–10). To date, activating mutations of p110α have been found in a significant fraction of commonly occurring human cancers, whereas no somatic mutations have been identified in the other class IA isoforms p110β and p110δ (11, 12). On the other hand, there are several cancer types that have been reported to have elevated levels and/or genomic amplifications of these other isoforms, indicating that they too may contribute to cancer (13, 14). In addition, activation of receptor tyrosine kinases such as VEGF receptor, EGF receptor, PDGF receptor, or human epidermal growth factor receptor 2 (HER2) leading to the activation of the PI3K pathway usually requires p110α for signaling and tumorigenesis (15–17). Rat sarcoma (Ras) proteins, which signal in part through PI3K pathway, are also frequently mutated in human cancer (18, 19). For example, Kras- and Hras-dependent lung and skin tumors have been shown to rely on p110α (18, 19), and this isoform has been reported to directly bind Kras. In contrast, p110β does not directly bind Ras proteins (20). In addition, loss-of-function mutations in the tumor suppressor PTEN are common in many human tumors and result in PI3K pathway activation (7). Whereas some mutations of the PI3K pathway are mutually exclusive, mutations in Kras and PTEN coexist in endometrial ovarian tumors (10, 21).

Recently, we found that ablation of p110β can block tumor formation in a mouse prostate tumor model driven by *Pten*-loss (22). Notably, ablation of p110α alone had no effect on tumor formation in this system. It has also been reported that p110β-selective inhibitors reduce AKT phosphorylation in PTEN-null human breast cancer cell lines (23). Other groups found that specific knockdown of p110β, but not p110α, resulted in down-regulation of PI3K pathway signaling and inhibition of growth in both cell-based and in vivo studies of breast and prostate human

Significance

Aberrant activation of the PI3K pathway is a frequent event in human cancer, making PI3K an attractive target in cancer therapy. Early generation inhibitors have poor efficacy and intolerable side effects; new PI3K isoform-selective inhibitors are emerging in the clinic. Much work is ongoing to determine the isoform dependence of different cancers. Of the ubiquitously expressed isoforms, p110α is critical for activated receptor tyrosine kinases or oncogenes, whereas p110β seems essential in many tumors deficient of the phosphatase and tensin homolog (PTEN). We show for the first time, to our knowledge, that PTEN-null ovarian tumors requiring p110β can become dependent on p110α through concurrent activation of the rat sarcoma protein $Kras^{G12D}$. Our results provide critical insights into patient selection and stratification in current and future clinical trial designs with PI3K inhibitors.

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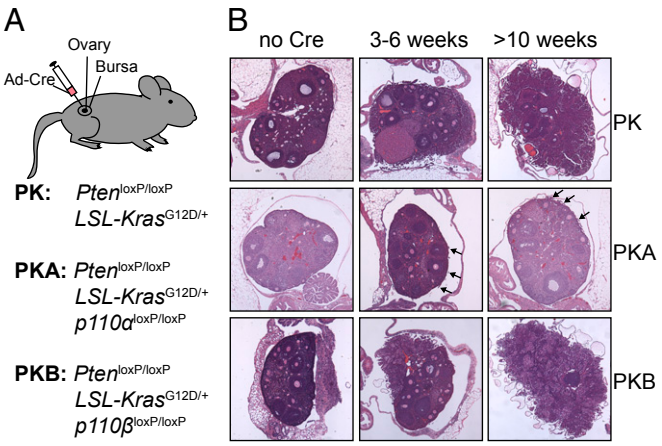
tumor cell lines featuring PTEN mutations (24, 25). All these studies suggested that p110β plays an important role in the tumorigenesis driven by loss of PTEN. Although considerable evidence links PTEN loss with p110β action, there are clearly human tumor cell lines featuring PTEN loss that are not dependent on p110β (23, 25). Additionally, recent work has indicated that loss of PTEN in certain tissues may lead to p110α-dependent tumors in mice (26). Reflecting the complexity of the matter, other reports show that some models may be equally dependent on both p110α and p110β isoforms (27, 28).

In this study we explore the p110 isoform requirements in ovarian tumorigenesis. Little is known about the molecular characteristics and the specific pathways that contribute to ovarian cancer. Consequently, very limited targeted therapies are available to treat this disease. VEGF and poly (ADP-ribose) polymerase (PARP) inhibitors show promising results, but due to the severity and late progression of most ovarian cancers at the time of diagnosis, there is an urgent need for the development of more targeted therapies (29). Here, we use a mouse model of ovarian endometrioid adenocarcinoma that depends upon concomitant activation of oncogenic *Kras*, and deletion of *Pten* (30). Because the former is a known activator of p110α and the latter relies on p110β in most settings, this allows us to explore the critical question of isoform dependence in a model that is genetically more complex and more closely recapitulates the situation in human tumors, which are believed to arise from defects in multiple genes.

Results

p110α but Not p110β Is Required for Tumor Formation in an Ovarian Tumor Model Driven by Concurrent *Pten* Loss and *Kras*^{G12D} Expression. To test the p110 isoform dependence in ovarian tumors arising from multiple genetic lesions, we used a well-documented mouse model in which tumorigenesis is driven by the activation of *Kras* in combination with loss of *Pten* (30). This model uses injection of a replication-deficient adenovirus expressing Cre recombinase (Ad-Cre) through a capillary tube into the ovarian bursa of female mice harboring both *LSL-Kras*$^{G12D/+}$ and *Pten*$^{loxP/loxP}$ alleles (hereafter referred to as PK) to simultaneously activate the oncogenic *Kras*^{G12D} allele and delete the *Pten* gene specifically in ovarian surface epithelial (OSE) cells (Fig. 1*A*). We found that tumor progression in these PK mice recapitulated published findings (30). As early as 3–5 wk after Ad-Cre injection, ovarian endometrioid-like epithelial lesions are observed, which proliferate and penetrate into the ovary at around 6 wk (Fig. 1*B*, *Top*). Visible tumors are grossly apparent at 8–10 wk after Ad-Cre injection and develop into invasive endometrioid adenocarcinomas with 100% penetrance. At 10–16 wk post Ad-Cre administration, roughly 50% of the mice develop hemorrhagic ascites, similar to that seen in human ovarian carcinomas. Notably, either the *Kras* or *Pten* lesions alone are insufficient to drive efficient tumor formation.

To analyze which p110 isoform is required for tumor formation in PK mice, we crossed PK mice with the previously generated conditional knockout mice for p110α and p110β (17, 22). This resulted in mouse lines that we refer to as PKA (harboring *Pten*$^{loxP/loxP}$; *LSL-Kras*$^{G12D/+}$; *p110α*$^{loxP/loxP}$ alleles) and PKB (*Pten*$^{loxP/loxP}$; *LSL-Kras*$^{G12D/+}$; *p110β*$^{loxP/loxP}$), respectively. After injection of Ad-Cre into the ovarian bursa of female PK, PKA, or PKB mice, tumor formation in these mice was followed for up to 6 mo. Interestingly, loss of *p110β* did not have a significant effect in this model, resulting in tumor formation and progression comparable to that of the PK mice as described above (Fig. 1*B*, *Bottom*). The genetic deletion of *p110β* in these PK tumors was confirmed by genomic PCR analysis (Fig. S1). In contrast, loss of *p110α* in PKA mice resulted in a dramatically reduced incidence of tumor formation in the ovaries (Fig. 1*B*, *Middle*).



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	Hyperplasia	Adenocarcinoma
PK	22% (5/23)	78% (18/23)
PKA	69% (9/13)	0% (0/13)
PKB	9% (1/11)	91% (10/11)

Fig. 1. p110α but not p110β is required for tumor formation in an ovarian tumor model driven by concurrent *Pten* loss and *Kras*^{G12D} expression. (*A*) Adenovirus expressing Cre recombinase (Ad-Cre) was injected into the ovarian bursa of female mice carrying the indicated genotypes, inducing expression of oncogenic *Kras* and deletion of *Pten* (PK), alone or with *p110α* (PKA) or *p110β* (PKB) ablation. (*B*) Ovaries were isolated at different time points after intrabursal injection of Ad-Cre and sent for pathology evaluation. Depicted are typical examples of H&E stainings of complete mouse ovaries. Arrows show hyperplastic lesions in the surface epithelium of the ovary. (*C*) Histopathological evaluation of ovaries with the indicated genotypes.

About 30% (4 of 13) of these PKA mice retained normal ovarian structure without a single tumor lesion (Fig. 1 *B* and *C*). The tumor lesions found in 9 of 13 PKA mice only showed a few small hyperplastic lesions in the surface epithelium of the ovary (Fig. 1*B*, arrows). Notably, we found similar hyperplastic lesions in ovaries of PKA mice at 4, 10, and up to 24 wk after Ad-Cre injection (Fig. 1*B*, arrows), indicating that these small lesions never progressed into more blatant tumors during the time frame of observation (up to 24 wk).

We next characterized the ovarian tumor samples. Immunohistochemical analyses revealed that, at early stages after Ad-Cre injection in PK mice, PTEN expression is detectable inside the ovaries and in the epithelium retaining a single layer of cells (Fig. S2*A*, *Center* and *Right* with arrows). However, in the proliferating parts of the epithelium as well as in tumorigenic regions, PTEN expression is lost (Fig. S2*A*, *Left* and *Right* with arrowheads). Importantly, the regions that have lost PTEN expression stain strongly for the proliferation marker Ki67 and the epithelial marker cytokeratin 8, suggesting that tumorigenesis originates in the ovarian surface epithelium in this model (Fig. S2*B*). Western blot analysis of whole ovaries confirmed reduced abundance of PTEN in Ad-Cre–injected PK and PKB ovaries [left (L) side] compared with the mock-injected ovaries [right (R) side] from the same animal (Fig. S3). Because PKA mice fail to develop bulky tumors and only a small portion of the cells in a whole ovarian preparation represent the surface epithelial cells targeted in this model, PTEN expression, although lost in the epithelium, is unchanged in total PKA ovarian preparations (Fig. S3). Because it is well known that loss of PTEN leads to derepression of the PI3K signaling pathway, we also analyzed its downstream signaling in the ovarian tumor samples. We found that Akt phosphorylation was elevated in ovaries with reduced PTEN expression. Phosphorylation of the S6 ribosomal protein

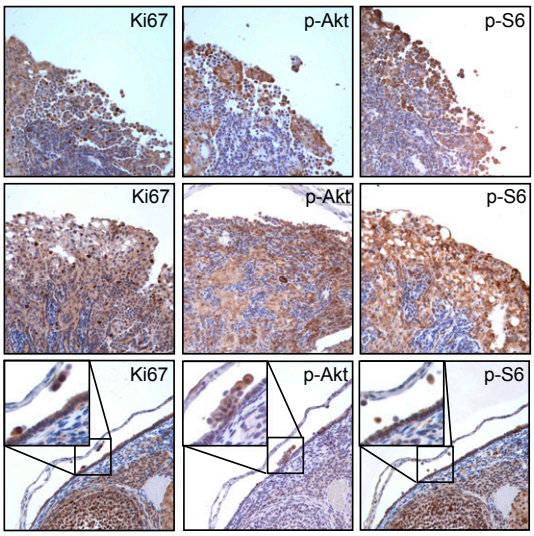

Fig. 2. Immunohistochemical analysis of ovaries after Ad-Cre injection. Immunohistochemical staining of PK, PKA, and PKB ovaries at 10–14 wk after Cre injection with the indicated antibodies. Expression of oncogenic *Kras* and deletion of *Pten* (PK), additional deletion of *p110α* (PKA), and additional deletion of *p110β* (PKB).

also correlated well with tumor formation and Akt activation. In contrast, Akt phosphorylation in ovaries from PKA mice remained at basal levels, consistent with unchanged PTEN levels in whole ovaries (Fig. S3). Immunohistochemistry showed that Akt and S6 protein were phosphorylated in the tumor regions that also showed Ki67 staining in both PK and PKB tissues (Fig. 2, *Top* and *Middle*). In PKA mice, the single layer of surface epithelial cells is largely negative for nuclear staining of Ki67 and p-Akt (Fig. 2, *Bottom*), except for some small hyperplastic lesions on the ovarian surface showing Akt phosphorylation (Fig. 2, *Bottom Inset*). Together, these data suggest that the p110α isoform of PI3K is critical for tumorigenesis driven by concurrent *Pten* loss and *Kras* activation.

The Presence of *Kras*^{G12D} Shifts the PI3K Isoform Dependence of *Pten*-Null Induced Tumorigenesis from p110β to p110α. Whereas tumorigenesis in the *Pten*-null prostate is dependent on p110β (22), the data presented here show that p110α is the more important isoform in an ovarian endometrioid model driven by concurrent *Pten* loss and *Kras*^{G12D} expression. Although it is tempting to speculate that the presence of activated Kras explains this discrepancy, it is also possible that PTEN-null ovarian tumors cells (and perhaps all ovarian tumor cells featuring PI3K signaling) depend on p110α for more tissue-intrinsic reasons. Ideally we would perform the same set of experiments described above using mice with *Pten* loss, but lacking activation of *Kras*. However, tumor formation in these mice was too slow to make these experiments feasible. Hence, we decided instead to examine the p110 isoform dependence of an OSE cell transplantation model.

To this end, we isolated OSE cells from the following genetic mouse lines: *Pten*$^{loxP/loxP}$ (P), *Pten*$^{loxP/loxP}$; *p110α*$^{loxP/loxP}$ (PA), *Pten*$^{loxP/loxP}$; *p110β*$^{loxP/loxP}$ (PB), *LSL-Kras*$^{G12D/+}$ (K), *LSL-Kras*$^{G12D/+}$; *p110α*$^{loxP/loxP}$ (KA), *LSL-Kras*$^{G12D/+}$; *p110β*$^{loxP/loxP}$ (KB), as well as from the PK, PKA, and PKB lines. Isolated primary OSE cells appear as clusters of cobblestone like cells that can grow for 7–10 d before changing in morphology and entering senescence. We immortalized these cells at day 1 post-isolation using retroviral vectors to introduce a combination of a dominant negative form of p53 (p53-DD) and an activated CDK4 allele, R24C, to inactivate both the p53 and pRb pathways. The immortalized OSE cells express cytokeratin, proof of their

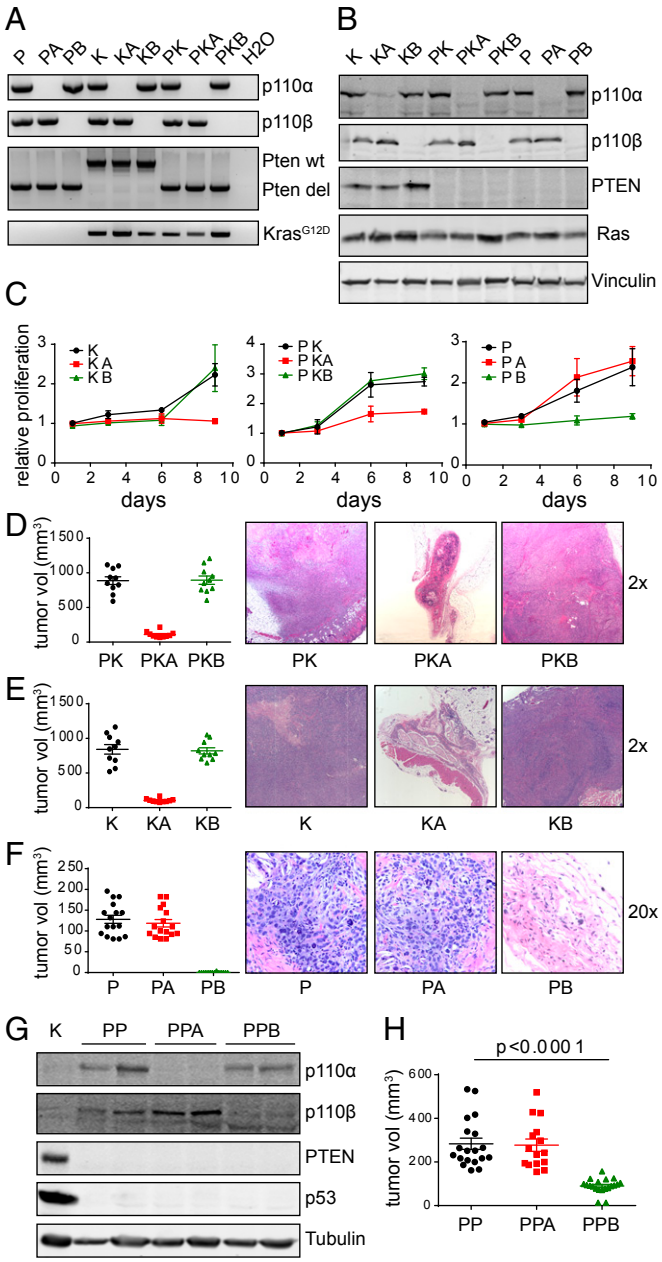

Fig. 3. The presence of *Kras*^{G12D} shifts the PI3K isoform dependence of Pten-null induced tumorigenesis from p110β to p110α. (*A*) Genotyping and (*B*) Western blot (WB) analysis of ovarian surface epithelial (OSE) cells with the following genotypes: Expression of oncogenic *Kras* (K), deletion of *Pten* (P), combination of both lesions (PK), additional deletion of *p110α* (PKA, KA, and PA), additional deletion of *p110β* (PKB, KB, and PB). (*C*) OSE cells with the indicated genotypes were seeded at the same densities, grown for the indicated times in medium without FBS or growth factors, and stained with crystal violet. Shown are averages and SDs from two independent experiments performed in triplicates. (*D–F*) OSE cells with the indicated genotypes were injected s.c. into the flank of NCrNu recipient mice and their tumor sizes measured with a caliper. Shown are single tumor sizes with mean values and SEMs (*Left*) and typical H&E stainings of sections from tumors (*Right*). (*G*) WB analysis of ovarian surface epithelial (OSE) cells with the following genotypes: deletion of *Pten* and *trp53* (PP), additional deletion of *p110α* (PPA), and additional deletion of *p110β* (PPB). (*H*) OSE cells with the indicated genotypes were injected s.c. into the flank of NCrNu recipient mice and their tumor sizes measured with a caliper. Shown are single tumor sizes with mean values and SEMs.

epithelial origin, as well as progesterone receptor (PR) and estrogen receptor (ER) (Fig. S4). These cells were then treated with Ad-Cre to either activate $Kras^{G12D}$, delete *Pten*, or both, in combination with deletion of either *p110α* or *p110β* (Fig. 3 *A* and *B*). After Ad-Cre treatment, we could show complete loss of p110α, p110β, and PTEN on a protein level (Fig. 3*B*). Notably, the protein levels of p110α and p110β remain largely unchanged in these various OSEs in the presence or absence of Pten or mutant Kras (Fig. 3*B*). The presence of the $Kras^{G12D}$ allele was apparent by genotyping (Fig. 3*A*) but did not increase protein levels of Ras (Fig. 3*B*), consistent with the fact that this is a knock-in allele of mutant Kras (31, 32). We also analyzed the PI3K signaling in the OSE-PK cells. After Cre treatment leading to the activation of Kras and loss of Pten, OSE-PK cells showed increased phosphorylation of Akt (Fig. S5). Whereas additional loss of p110β did not change the signaling, additional loss of p110α prevented the increase of p-Akt, consistent with our immunohistochemistry results in the in vivo model (Fig. S5 and Fig. 2). The resulting OSE cells proliferated normally in full growth medium containing FBS and growth factors (Fig. S6), with no evidence of a growth defect arising from loss of any of the floxed genes. However, in serum-starved conditions without FBS or growth factors, the PKA, KA, and PB cell lines grew slower than the other cell lines (Fig. 3*C*), suggesting that some factor(s) in the fully supplemented medium might compensate for the loss of p110α or p110β on proliferation in vitro.

We next tested the ability of these engineered immortalized OSE cells to form tumors in vivo by s.c. injection into the flank of nude mice. We found that OSE cells isolated from PK mice (OSE-PK), when injected at 2×10^6 cells per site, were able to grow into tumors efficiently (Fig. 3*D*). Consistent with our previous experiments, the deletion of *p110β* had no inhibitory effect on this tumor growth, whereas knockout of *p110α* blocked tumor growth (Fig. 3*D*). Anticipating a slower growth rate for cells with wild-type *Pten*, we then injected 5×10^6 OSE-K, OSE-KA, and OSE-KB cells s.c. in nude mice as described before. We found that, whereas OSE-KA cells failed to grow in vivo, both OSE-K and OSE-KB cells were able to form tumors in mice with a similar growth rate (Fig. 3*E*), suggesting that *p110α* is important for tumorigenesis in OSE cells induced by oncogenic Kras.

To gain insight into whether the reliance of OSE-PK and OSE-K cells on p110α for tumor growth is due to a tissue-specific effect or rather caused by the presence of Kras, we conducted parallel experiments using a set of OSE cells derived from P, PA, and PB mice to generate OSE-P, OSE-PA, and OSE-PB cells, respectively. We found that these cells have a much lower tumorigenic potential than OSE-PK cells. Therefore, we increased the number of cells injected to 5×10^6 cells, similar to OSE-K cells. Using this increased cell number, OSE-P and OSE-PA cells formed small tumors (Fig. 3*F*), whereas OSE-PB cells failed to form any tumor-like lesions, with only the matrigel plug containing macrophages and cellular debris visible at the injection sites (Fig. 3*F*). It has been reported that local microenvironment may modulate tumor growth in vivo. Thus, we also transplanted these OSE cells into the mammary glands of recipient mice, trying to provide a stromal condition more supportive of tumor growth. Consistent with the findings from s.c. transplantation, both P and PA OSE cells grew into substantial tumor masses, but PB OSE cells did not form palpable masses (Fig. S7). Whereas allograft of these OSE cells in mammary fat pad did not significantly increase the tumor volumes of the Pten-null OSEs in vivo, these data provide further evidence that p110β is important for the growth of Pten-null OSE cells in vivo.

To determine whether the dependence on p110β is preserved in larger Pten-null tumors, we attempted to generate more aggressive Pten-null tumors. To this end, we isolated OSE cells from mice with homozygous floxed alleles of both *Pten* and *p53* [$Pten^{loxP/loxP}$; $trp53^{loxP/loxP}$ (PP)], and from mice with additional homozygous alleles of floxed p110α [$Pten^{loxP/loxP}$; $trp53^{loxP/loxP}$; $p110\alpha^{loxP/loxP}$ (PPA)], or floxed p110β [$Pten^{loxP/loxP}$; $trp53^{loxP/loxP}$; $p110\beta^{loxP/loxP}$ (PPB)] (Fig. 3*G*). Our hope was that genetic deletion of p53 would be superior to the dominant negative construct used in the original model. We immortalized these cell lines in vitro with CDK4-R24C and treated them with Ad-Cre to excise *Pten* and *p53*, alone or in combination with *p110α* or *p110β* (Fig. 3*G*). We then injected these cells s.c. into the flanks of nude mice. The resulting PP and PPA tumors did grow faster and to a larger size, although the resulting tumors were still smaller than OSE-PK or OSE-K tumors (Fig. 3*H*). Consistently, loss of p110β significantly reduced tumor sizes in this genetic background, similar to our results with OSE-PB cells (Fig. 3*F*). Taken together, these results suggest that tumorigenesis induced by *Pten* loss in OSE cells depends on the p110β isoform of PI3K. However, in the presence of oncogenic Kras, these cells shift their isoform dependence from p110β to p110α, even when *Pten* is deleted.

Pharmacological Inhibition of p110α Effectively Blocks Tumorigenic Growth of OSE-PK Cells. Because p110 isoform-selective inhibitors are available, and some of them are entering clinical trials (33), we sought to verify the isoform dependence shown in the genetic model using pharmacological inhibitors. For this study we used BYL719, a p110α-selective inhibitor currently in clinical trials for cancer patients (34), and KIN193, a p110β-selective inhibitor that effectively reduces PI3K signaling in some PTEN-deficient cancer cells (23).

We injected OSE-PK cells s.c. into the flank of nude mice before randomization. One cohort of mice was treated with BYL719, another with KIN193, and the third group with vehicle control. Tumor volumes in mice of each cohort were measured

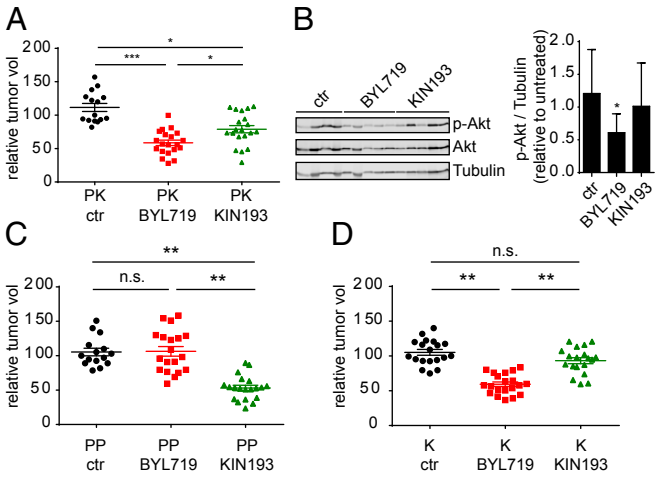

Fig. 4. Pharmacological inhibition of p110α effectively blocks tumorigenic growth of OSE-PK cells. (*A*) Ovarian surface epithelial (OSE) cells from mice with expression of oncogenic *Kras* and deletion of *Pten* (PK) were injected s.c. into the flank of NCrNu recipient mice. Treatment with pharmacological inhibitors (BYL719, 45 mg/kg once daily p.o. and KIN193, 20 mg/kg once daily i.p.) was started 1 d after injection. After 3 wk, mice were killed and tumor sizes were measured with a caliper. Shown are single tumor sizes with mean values and SEMs. $*P < 0.001$; $**P < 0.0001$. (*B*) Tumors from Fig. 4*A* were homogenized, lyzed, and analyzed by Western blot using the indicated antibodies. Shown is one representative Western blot (*Left*); quantifications represent mean values and SEMs from eight independent tumors per group (*Right*). $*P < 0.05$. (*C* and *D*) Ovarian surface epithelial (OSE) cells from mice with deletion of *Pten* and *trp53* (PP) (*C*) or expression of oncogenic *Kras* and deletion of *Pten* (PK) (*D*) were injected s.c. into the flank of NCrNu recipient mice and treated as described in *A*. $**P < 0.0001$, n.s., not statistically significant.

at the end of the treatment course of 3 wk. Consistent with our genetic findings, the tumor volumes in the BYL719-treated mice were significantly smaller than those of mice in the control group or KIN193-treated group (Fig. 4*A*). We further carried out a similar drug trial experiment using established tumors to more closely mimic a clinical setting. Again the tumors in mice treated with BYL719 had much reduced sizes than tumors in mice treated with either vehicle or KIN193 (Fig. S8). We also noticed that the tumor volumes in KIN193-treated mice were somewhat reduced or showed a trend toward decrease compared with tumors in the control group (Fig. 4*A* and Fig S8), perhaps due to an off-target effect of the inhibitor. Analyses of tumor specimens prepared from mice in the different groups showed a significant reduction in p-Akt levels in BYL719-treated tumors compared with either control tumors or tumors treated with KIN193 (Fig. 4*B*).

In parallel, we performed the same drug testing experiment on mice bearing allograft tumors of OSE-K and OSE-PP cells. Consistent with the results from our genetic models, BYL719 significantly inhibited OSE-K tumors, whereas KIN193 markedly blocked OSE-PP tumors (Fig. 4 *C* and *D*). Taken together, our results suggest that ovarian tumors with PTEN loss and wild-type Kras are dependent on the p110β isoform of PI3K, indicating such tumors might benefit from treatment with a p110β-selective inhibitor. However, tumors with combined lesions of PTEN loss and Kras activation are dependent on the p110α isoform of PI3K. Our results suggest that the p110α-selective inhibitor BYL719 may be effective in treating cancers with coexisting PTEN deficiency and Kras activation.

Discussion

The data presented here establish that altering the genetic background of a PTEN-null tumor can change its PI3K isoform dependence. The assumption that loss of PTEN in an incipient tumor acts to remove a "brake" from PI3K signaling raises the question of what signals provide the initial "foot on the accelerator." Because p110β is thought to be primarily required not for receptor tyrosine kinase (RTK) signaling but rather for G protein-coupled receptor (GPCR) or integrin signaling (22, 35–37), it is possible that the in vivo tumors studied here are responding to a local GPCR ligand, such as lysophosphatidic acid (LPA), arising either from the surrounding stroma or from an autocrine loop. However, we have no evidence for this in our system and, indeed, we found that LPA did not stimulate growth of the Pten-null OSE cells in vitro (Fig. S9). There is also evidence that the background signaling state in the absence of ligands for PI3K might be p110β dependent (38–40). In this theory the background p110β signaling would be deregulated in Pten-null tumors. Finally it is possible that there exists a direct molecular mechanism linking p110β to Pten. In any case, an activated allele of Kras would be expected to directly engage p110α (18–20, 41) providing a new "accelerating" signal in the PK tumors.

There are several possible explanations for how Kras expression can change the p110 requirements for the growth of Pten-null tumors. The relative expression levels of the p110 isoforms might change in response to different genetic alterations—but the data in Fig. 3*B* clearly rule out this possibility. Another possibility arises from potential differences in the strengths of the signals activating the PI3K pathway in the different genetic backgrounds. It is conceivable that the signal intensity of the p110β-dependent input is much lower than the Kras-generated signal carried by p110α. This possibility, together with the fact that p110α has a stronger kinase activity than p110β (16, 39), would explain the shift toward a p110α requirement. Alternatively, it is possible that activation of Kras both activates p110α and shuts off the signal responsible for p110β activation. Finally, in addition to the ability of Kras to activate p110α directly, it might also generate an autocrine or paracrine loop leading to the activation of RTKs, as has been reported in other cell types (42, 43), which would strengthen the dependence of the signal on p110α.

It is notable the loss of *Pten* alone produces only weakly tumorigenic cells. The PTEN-null OSE cells invariably grew more slowly in vivo after transplantation than the K and PK cells, resulting in both smaller tumor size and longer apparent latency, suggesting that it may require additional "hit(s)" to cooperate with *Pten* loss for tumor progression to the more rapid growth seen in PK tumors. This is consistent with previously published studies showing that expression of an endogenous oncogenic allele of *p110α* or loss of *Pten* in the OSE via adeno-Cre injection produced only serous papillary hyperplasia of the OSE even upon long observation (44). However, combining the two PI3K pathway lesions produced frank tumors (44). Similarly, combining mutant Kras with Pten loss in OSEs produced aggressive tumors. In this case, the mutant Kras is able to contribute to tumorigensis not only via PI3K activation but via a number of other signaling pathways. In addition, Wu et al. showed that a combination of *Pten* loss and *Apc* loss, but not the single lesions, resulted in aggressive tumors (45).

The fact that a PTEN-null tumor can depend on either p110α or p110β has important implications for therapy. Clinical trials of PI3K inhibitors have begun and are showing modestly promising results. The earliest trials have largely featured so-called pan-PI3K inhibitors that block the action of all receptor-coupled class I PI3K isoforms. However, preclinical work and several clinical trials suggest that inhibitors of individual isoforms may be able to achieve greater efficacy with fewer side effects (46). For example, the compound GS1101, an inhibitor of the p110δ isoform, has proven extremely effective in certain B-cell neoplasias (47). Early unpublished results also suggest that p110α-selective inhibitors are outperforming pan-PI3K inhibitors in ER positive breast cancer (33). Thus, there is considerable need to determine the isoform dependence of a given tumor class. Recent work in model systems and human tumor cell lines has shown that tumors driven by oncogenes and activated receptors such as HER2 depend on p110α, suggesting that inhibitors targeting this isoform might be efficacious in this tumor class (16). Our original work and that of others has shown that many PTEN-null tumors depend on p110β (22–26). However, more recent work has shown that, in at least some tissues, PTEN-null tumors do not depend on p110β alone (26–28, 48).

Importantly, this study highlights that, even in tissues where PTEN loss leads to p110β dependence, tumors may still escape the dependence on that isoform by acquiring additional mutations. One solution to this problem might be to use a combination of selective p110α and p110β inhibitors, or a pan-PI3K inhibitor. However, given the important physiological roles of PI3K, pan inhibitors have limitations due to their side effects, such as defects in insulin signaling (3, 46). Because p110α is more important in insulin signaling, it may be advantageous to use p110β-selective inhibitors to treat PTEN-null tumors whenever possible. This suggests that a real emphasis must be placed on finding specific biomarkers or genetic characteristics that will predict the specificity for a given p110 isoform.

Materials and Methods

Mouse Strains. *LSL-Kras*$^{G12D/+}$ mice and *Pten*$^{loxP/loxP}$ were acquired from the National Cancer Institute Mouse Models of Human Cancers Consortium mouse repository. *p110α*$^{loxP/loxP}$ (17) and *p110β*$^{loxP/loxP}$ (22) mice were developed previously in this laboratory. All animals were on a mixed genetic background. NCrNu female mice (Taconic) were used for allograft transplantation. All animals were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committees of Dana–Farber Cancer Institute and Harvard Medical School.

OSE Isolation, Primary Culture, and Immortalization. For OSE isolation, ovaries were dissected and treated with collagenase (Sigma) and dispase (Life Technologies) for 1 h at 37 °C. The epithelial cells were pelleted by centrifugation at 800 × *g*. To immortalize the primary cells, OSEs were transduced

with p53-DD (6) and CDK4-R24C (Addgene plasmid 11254). OSE cells were treated in vitro with Ad-Cre (University of Iowa) for excision of floxed genes and kept in DMEM/F12 (Life Technologies) supplemented with 4% (vol/vol) FBS, 1% penicillin/streptomycin, 10 ng/mL EGF, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite.

In Vivo Drug Treatment. BYL719 (provided by Novartis Pharmaceuticals) was formulated in 0.5% methylcellulose and administered at 45 mg/kg orally (p.o.) once daily, KIN193 (purchased from MedChemexpress) was formulated in 7.5% (vol/vol) NMP, 40% (vol/vol) PEG-400 in H_2O and administered at 20 mg/kg i.p. once daily.

For detailed methods, please refer to *SI Materials and Methods*.

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1. Engelman JA, Luo J, Cantley LC (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 7(8):606–619.
2. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B (2010) The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 11(5):329–341.
3. Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 8(8):627–644.
4. Chang HW, et al. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science* 276(5320):1848–1850.
5. Zhao JJ, et al. (2003) Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. *Cancer Cell* 3(5):483–495.
6. Zhao JJ, et al. (2005) The oncogenic properties of mutant p110alpha and p110beta phosphatidylinositol 3-kinases in human mammary epithelial cells. *Proc Natl Acad Sci USA* 102(51):18443–18448.
7. Song MS, Salmena L, Pandolfi PP (2012) The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* 13(5):283–296.
8. Samuels Y, et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* 304(5670):554.
9. Bader AG, Kang S, Zhao L, Vogt PK (2005) Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 5(12):921–929.
10. Yuan TL, Cantley LC (2008) PI3K pathway alterations in cancer: Variations on a theme. *Oncogene* 27(41):5497–5510.
11. Thomas RK, et al. (2007) High-throughput oncogene mutation profiling in human cancer. *Nat Genet* 39(3):347–351.
12. Wood LD, et al. (2007) The genomic landscapes of human breast and colorectal cancers. *Science* 318(5853):1108–1113.
13. Boller D, et al. (2008) Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival in neuroblastoma cells. *Clin Cancer Res* 14(4):1172–1181.
14. Liu Z, et al. (2008) Highly prevalent genetic alterations in receptor tyrosine kinases and phosphatidylinositol 3-kinase/akt and mitogen-activated protein kinase pathways in anaplastic and follicular thyroid cancers. *J Clin Endocrinol Metab* 93(8):3106–3116.
15. Graupera M, et al. (2008) Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. *Nature* 453(7195):662–666.
16. Utermark T, et al. (2012) The p110α and p110β isoforms of PI3K play divergent roles in mammary gland development and tumorigenesis. *Genes Dev* 26(14):1573–1586.
17. Zhao JJ, et al. (2006) The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. *Proc Natl Acad Sci USA* 103(44):16296–16300.
18. Gupta S, et al. (2007) Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell* 129(5):957–968.
19. Ramjaun AR, Downward J (2007) Ras and phosphoinositide 3-kinase: Partners in development and tumorigenesis. *Cell Cycle* 6(23):2902–2905.
20. Fritsch R, et al. (2013) RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. *Cell* 153(5):1050–1063.
21. Hecht JL, Mutter GL (2006) Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol* 24(29):4783–4791.
22. Jia S, et al. (2008) Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. *Nature* 454(7205):776–779.
23. Ni J, et al. (2012) Functional characterization of an isoform-selective inhibitor of PI3K-p110β as a potential anticancer agent. *Cancer Discov* 2(5):425–433.
24. Torbett NE, et al. (2008) A chemical screen in diverse breast cancer cell lines reveals genetic enhancers and suppressors of sensitivity to PI3K isoform-selective inhibition. *Biochem J* 415(1):97–110.
25. Wee S, et al. (2008) PTEN-deficient cancers depend on PIK3CB. *Proc Natl Acad Sci USA* 105(35):13057–13062.
26. Berenjeno IM, et al. (2012) Both p110α and p110β isoforms of PI3K can modulate the impact of loss-of-function of the PTEN tumour suppressor. *Biochem J* 442(1):151–159.
27. Jia S, et al. (2013) Opposing effects of androgen deprivation and targeted therapy on prostate cancer prevention. *Cancer Discov* 3(1):44–51.
28. Wang Q, et al. (2013) Spatially distinct roles of class Ia PI3K isoforms in the development and maintenance of PTEN hamartoma tumor syndrome. *Genes Dev* 27(14):1568–1580.
29. Smolle E, et al. (2013) Targeting signaling pathways in epithelial ovarian cancer. *Int J Mol Sci* 14(5):9536–9555.
30. Dinulescu DM, et al. (2005) Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med* 11(1):63–70.
31. Jackson EL, et al. (2001) Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* 15(24):3243–3248.
32. Tuveson DA, et al. (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* 5(4):375–387.
33. Rodon J, Dienstmann R, Serra V, Tabernero J (2013) Development of PI3K inhibitors: Lessons learned from early clinical trials. *Nat Rev Clin Oncol* 10(3):143–153.
34. Furet P, et al. (2013) Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 kinase alpha inhibitor selected for clinical evaluation. *Bioorg Med Chem Lett* 23(13):3741–3748.
35. Ciraolo E, et al. (2010) Essential role of the p110beta subunit of phosphoinositide 3-OH kinase in male fertility. *Mol Biol Cell* 21(5):704–711.
36. Guillermet-Guibert J, et al. (2008) The p110beta isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110gamma. *Proc Natl Acad Sci USA* 105(24):8292–8297.
37. Martin V, et al. (2010) Deletion of the p110beta isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood* 115(10):2008–2013.
38. Dbouk HA, Pang H, Fiser A, Backer JM (2010) A biochemical mechanism for the oncogenic potential of the p110beta catalytic subunit of phosphoinositide 3-kinase. *Proc Natl Acad Sci USA* 107(46):19897–19902.
39. Knight ZA, et al. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* 125(4):733–747.
40. Vogt PK (2011) PI3K p110β: More tightly controlled or constitutively active? *Mol Cell* 41(5):499–501.
41. Castellano E, Downward J (2010) Role of RAS in the regulation of PI 3-kinase. *Curr Top Microbiol Immunol* 346:143–169.
42. Ebi H, et al. (2011) Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. *J Clin Invest* 121(11):4311–4321.
43. Molina-Arcas M, Hancock DC, Sheridan C, Kumar MS, Downward J (2013) Coordinate direct input of both KRAS and IGF1 receptor to activation of PI3 kinase in KRAS-mutant lung cancer. *Cancer Discov* 3(5):548–563.
44. Kinross KM, et al. (2012) An activating Pik3ca mutation coupled with Pten loss is sufficient to initiate ovarian tumorigenesis in mice. *J Clin Invest* 122(2):553–557.
45. Wu R, et al. (2007) Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/beta-catenin and PI3K/Pten signaling pathways. *Cancer Cell* 11(4):321–333.
46. Jia S, Roberts TM, Zhao JJ (2009) Should individual PI3 kinase isoforms be targeted in cancer? *Curr Opin Cell Biol* 21(2):199–208.
47. Castillo JJ, Furman M, Winer ES (2012) CAL-101: A phosphatidylinositol-3-kinase p110-delta inhibitor for the treatment of lymphoid malignancies. *Expert Opin Investig Drugs* 21(1):15–22.
48. Oda K, et al. (2008) PIK3CA cooperates with other phosphatidylinositol 3′-kinase pathway mutations to effect oncogenic transformation. *Cancer Res* 68(19):8127–8136.