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Both p110α and p110β isoforms of PI3K can modulate the impact of loss-of-function of the PTEN tumour suppressor

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The PI3K (phosphoinositide 3-kinase) pathway is commonly activated in cancer as a consequence of inactivation of the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), a major negative regulator of PI3K signalling. In line with this important role of PTEN, mice that are heterozygous for a PTEN-null allele (PTEN$^{+/-}$ mice) spontaneously develop a variety of tumours in multiple organs. PTEN is a phosphatase with selectivity for PtdIns(3,4,5)P_3, which is produced by the class I isoforms of PI3K (p110α, p110β, p110γ and p110δ). Previous studies indicated that PTEN-deficient cancer cell lines mainly depend on p110β, and that p110β, but not p110α, controls mouse prostate cancer development driven by PTEN loss. In the present study, we investigated whether the ubiquitously expressed p110α can also functionally interact with PTEN in cancer. Using genetic mouse models that mimic systemic administration of p110α- or p110β-selective inhibitors, we confirm that inactivation of p110β, but not p110α, inhibits prostate cancer development in PTEN$^{+/-}$ mice, but also find that p110α inactivation protects from glomerulonephritis, pheochromocytoma and thyroid cancer induced by PTEN loss. This indicates that p110α can modulate the impact of PTEN loss in disease and tumourigenesis. In primary and immortalized mouse fibroblast cell lines, both p110α and p110β controlled steady-state PtdIns(3,4,5)P_3 levels and Akt signalling induced by heterozygous PTEN loss. In contrast, no correlation was found in primary mouse tissues between PtdIns(3,4,5)P_3 levels, PI3K/PTEN genotype and cancer development. Taken together, our results from the present study show that inactivation of either p110α or p110β can counteract the impact of PTEN inactivation. The potential implications of these findings for PI3K-targeted therapy of cancer are discussed.

Key words: cancer, kinase isoform, lipid signalling, phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), tumour suppressor.

INTRODUCTION

PI3Ks (phosphoinositide 3-kinases) are a family of lipid kinases that catalyse the production of the lipid second messenger PtdIns(3,4,5)P_3 in cellular membranes [1]. PtdIns(3,4,5)P_3 and its degradation product, PtdIns(3,4)P_2, interact with a variety of intracellular downstream protein effectors such as the Akt protein kinase, adaptor proteins and regulators of small GTPases, which ultimately control cell proliferation, survival and migration [2,3]. Overactivation of the PI3K signalling cascade leads to deregulation of these cellular processes, contributing to cancer development and progression. A common mechanism of constitutive PI3K activation in cancer results from loss or loss-of-function of the PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor, a lipid phosphatase that converts PtdIns(3,4,5)P_3 into PtdIns(4,5)P_2, hereby terminating the PI3K signal [4–7]. Heterozygous PTEN-null mice (PTEN$^{+/-}$ mice) spontaneously develop many different tumours [8–10]. In addition to its lipid phosphatase activity [11], there is increasing evidence that a PI3K-independent protein phosphatase activity of PTEN [12–14] and/or phosphatase-independent activities in the nucleus [15,16] also contribute to the tumour suppressor function of PTEN.

Among the eight isoforms of PI3K in mammals, only class I PI3Ks generate PtdIns(3,4,5)P_3, the lipid substrate for PTEN [17]. Class I PI3Ks consist of a p110 catalytic subunit (p110α, p110β, p110γ or p110δ) complexed to a regulatory subunit that targets the p110 subunit to the plasma membrane upon stimulation of tyrosine kinase or G-protein-coupled receptors [3]. *PIK3CA*, the gene encoding p110α, is the only PI3K catalytic subunit gene found to be mutated in cancer [18], leading to its overactivation. Initial studies showed that inactivation of PTEN and the presence of oncogenic *PIK3CA* mutations did not co-occur in cancer [19–21]. However, additional work has shown that these two genetic alterations can co-exist, for example in endometrial carcinoma, where PTEN loss and mutant *PIK3CA* lead to a higher activation of the PI3K pathway and a possible co-operation towards a more invasive phenotype [22].

Previous studies have indicated that aberrant PI3K signalling and tumorigenesis due to PTEN deficiency mainly rely on p110β activity. This is the case in several PTEN-deficient cancer cell lines that seem to depend on p110β for proliferation [23,24]. In addition, conditional knockout of p110β, but not of p110α, was found to impair cancer development induced by prostate-specific PTEN loss in a mouse model [24,25].

In the present study, we used PTEN$^{+/-}$ mice and cells as a broader genetic screen of PTEN inactivation to assess the role of p110α and p110β in PTEN-induced tumourigenesis. Although global inactivation of p110α in PTEN$^{+/-}$ mice did not protect from a wide spectrum of tumours, including prostate cancer, the

Abbreviations used: FBS, fetal bovine serum; FOX, forkhead box; FRET, Förster resonance energy transfer; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; p, phosphorylated; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PIN, prostatic intra-epithelial neoplasia; PTEN, phosphatase and tensin homologue deleted on chromosome 10; shRNA, short hairpin RNA; TCA, trichloroacetic acid; WT, wild-type.

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incidence of other pathologies such as glomerulonephritis, thyroid tumours and phaechromocytoma was reduced, documenting that p110α can also control biological effects induced by PTEN loss. p110α could also dampen enhanced PtdIns(3,4,5)P_3 production and PI3K signalling in cultured PTEN$^{+/-}$ cell lines. The implications of these findings for PI3K-targeted cancer therapy are discussed.

EXPERIMENTAL

Small molecule inhibitors

TGX-221 and LY294002 were from Calbiochem.

Isolation and immortalization of MEFs (mouse embryonic fibroblasts)

E13.5 (embryonic day 13.5) embryos were homogenized, dissociated with trypsin and cells allowed to adhere on tissue culture dishes for 2–3 days in DMEM (Dulbecco's modified Eagle's medium) containing 10 % FBS (fetal bovine serum) until confluent. Early passage MEFs (passage 2) were immortalized by transduction with a puromycin resistance-containing retrovirus expressing a microRNA-based shRNA (short hairpin RNA) against p53 [26]. After retroviral infection, MEFs were selected with 1 μg/ml puromycin (Sigma) for 10 days.

Quantification of PtdIns(3,4,5)P_3

PtdIns(3,4,5)P_3 was measured in extracts from MEFs or tissue samples using a time-resolved FRET (Förster resonance energy transfer) assay [27]. For MEFs, medium was aspirated from 10 cm^2 dishes of cells and 0.5 ml of ice-cold 0.5 M TCA (trichloroacetic acid) was added immediately and allowed to stand on ice for 5 min. The cells were then scraped off, the wells rinsed with additional TCA if required, and the precipitate was pelleted. Neutral lipids were extracted from the pellet with 1 ml of methanol/chloroform (2:1, v/v) by vortex-mixing 3–4 times over a 10 min period at room temperature (22 °C) and the solvent was discarded. The acidic lipids were then extracted as follows: 500 μl of chloroform/methanol/12 M HCl (40:80:1, by vol.) was added to the pellet and vortexed occasionally over a 15 min period at room temperature. Chloroform (180 μl) and 0.1 M HCl (300 μl) were then added, vortex-mixed and centrifuged (2000 ***g*** for 1 min) to separate the organic phase. The organic phase was then collected into a clean tube and dried in a speed vacuum centrifuge. The lipids were resuspended by sonication [using a Sonics Vibra-Cell™ set to 42 W output; 15 s pulse per sample in a water-cooled cup sonicator probe at 5 °C (Sigma)] in 60 μl of assay buffer (50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EGTA and 1.2 % sodium cholate). The mass of inositol lipid was estimated by adding 25 μl of the re-suspended lipid to 25 μl of detection mix as described previously [27].

The extracts from tissue samples were prepared in a similar manner, with the biopsy samples being snap frozen in liquid nitrogen and stored at − 80 °C until required. The tissue samples were thawed into 0.5 M TCA, centrifuged and the pellet extracted as described for the MEFs above. The protein content of the samples (both MEFs and tissue) was estimated by adding 1 ml of acetone to the acidic solvent extraction after removal of the lipid-containing lower phase and the protein was pelleted by centrifugation (10 000 ***g*** for 5 min). After removal of the acetone and air drying, the protein pellet was re-dissolved by incubation overnight at 50 °C in 5 % (w/v) SDS and 0.2 M NaOH. The dissolved protein was diluted as required and the protein concentration was estimated using a Pierce micro BCA (bichinchonic acid) kit as per the manufacturer's instructions. The mass of PtdIns(3,4,5)P_3 estimated by the time-resolved-FRET assay was then expressed as pmol PtdIns(3,4,5)P_3/mg of total protein.

Cell lysis and immunoblot analysis

Cells lysis buffer contained 50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 50 mM NaF and 1 % Triton X-100, supplemented with 2 mg/ml aprotinin, 1 mM pepstatin, 1 ng/ml L-leupeptin, 1 mM PMSF and 1 mM Na_3VO_4. Lysates were analysed by SDS/PAGE and immunoblotting using antibodies against pThr^{24}/Thr^{32}-Foxo3a (where p is phosphorylated and Fox is forkhead box) (9464), pThr^{308}-Akt (4056), pSer^{473}-Akt (4058), total Akt (9272), pSer^{240}/Ser^{244}-S6 (2215) and total ribosomal S6 (2217), all from Cell Signaling Technology. Quantitation of immunoblot signal intensity was performed with a Bio-Rad GS-800 densitometer and QuantityOne software.

Mice

Mice were kept in individually-ventilated cages and cared for according to U.K. Home Office regulations. The generation and genotyping of PTEN$^{+/-}$ [10] and the p110$\alpha^{D933A/WT}$ (where WT, wild-type) mice have been described previously [28]. These mouse lines were backcrossed to the C57BL/6 background for >10 generations before initiating the intercrosses for the present study. p110$\beta^{D931A/WT}$ mice were on a 129/Sv background backcrossed for six generations onto the C57BL/6 background. The difference in genetic background between the p110α and p110β experimental groups was controlled for by using two independent PTEN$^{+/-}$ control cohorts, made up of respective littermates of the PI3K × PTEN intercrosses. Mice were maintained under standard husbandry conditions for a period of up to 16 months of age. During this time, animals were monitored weekly and killed if they became unwell, exhibited reduced body weight or an obvious external tumour of over 1.44 cm^3. Killed animals were subjected to necropsy and pathological analysis after fixation of tissues in 4 % paraformaldehyde.

Histological analysis of tumours and immunohistochemistry

For histological analysis, tissues were immersion-fixed in either 4 % paraformaldehyde or 10 % neutral-buffered formalin. Small blocks from all tissues were sampled following dissection, further fixed and processed. They were then processed to paraffin wax blocks, where sections of 6 microns were cut and stained with haematoxylin and eosin. Further sections were stained with periodic acid Schiff without prior diastase digestion as required. Primary antibodies were used to detect B220/CD45R (RA3-6B2, BD Pharmingen), CD79αcy (HM57, Dako), CD3 (F7.2.38, Dako) and Ki67 (VP-K452, Vector Laboratories) to characterise lymphomas. Antibodies against PTEN (9188), FOXO1 (9462), pSer^{473}-Akt (3787) and pSer^{235}/Ser^{236}-S6 (4857) were purchased from Cell Signaling Technology and used to probe the tumours. Antibody binding was visualized using Vectastain reagents and protocols performed on a Dako Immunostainer. Sections were viewed on a Nikon Eclipse E600 microscope and images captured on a Nikon DXM1200 digital camera.

RESULTS

Inactivation of either p110α or p110β reduces PtdIns(3,4,5)P_3 and Akt activation upon PTEN loss in MEFs

We first investigated whether the broadly expressed p110α or p110β isoforms of PI3K regulate signalling induced by PTEN

loss in MEFs, which mainly express p110α and p110β, with little or no expression of p110γ and p110δ [29]. p110α was inactivated genetically, using MEFs derived from intercrosses of p110$\alpha^{D933A/WT}$ mice, which are heterozygous for a kinase-inactivating germline knockin mutation in the ATP-binding site of p110α (D933A; [28]) and PTEN$^{+/-}$ mice. p110β in PTEN$^{+/-}$ MEFs was inactivated using the p110β-selective small molecule inhibitor TGX-221 [30]. We tested both primary cells as well as immortalized MEFs (stably expressing a shRNA-mir against p53), the latter being more representative of a cancer cell status.

As expected, heterozygous PTEN inactivation in MEFs gave rise to higher steady-state levels of PtdIns(3,4,5)P_3, both in primary and immortalized cells, under exponential growing conditions (Figure 1A). This enhanced PtdIns(3,4,5)P_3 level was decreased to the same extent by inactivation of either p110α or p110β (Figure 1A). In agreement with the higher levels of PtdIns(3,4,5)P_3, inactivation of PTEN led to enhanced phosphorylation of Akt on both Ser^{473} and Thr^{308} (Figure 1B). Phosphorylation on both sites was reduced by inactivation of p110α or p110β (Figure 1B).

Taken together, these data suggest that both p110α and p110β can contribute to enhanced PtdIns(3,4,5)P_3 synthesis and downstream signalling upon inactivation of PTEN at the cellular level.

Partial inactivation of p110α or p110β does not prolong survival of PTEN$^{+/-}$ mice

PTEN$^{+/-}$ mice develop tumours in many different tissues [8–10]. We investigated whether inactivation of p110α or p110β in PTEN$^{+/-}$ mice would affect overall survival, by crossing PTEN$^{+/-}$ mice either with p110$\alpha^{D933A/WT}$ mice [28] or p110$\beta^{D931A/WT}$ mice (D931A is an inactivating mutation in the ATP-binding site of p110β; J. Guillermet-Guibert and B. Vanhaesebroeck, unpublished work). Homozygous p110$\alpha^{D933A/D933A}$ mice or p110$\beta^{D931A/D931A}$ mice are embryonic lethal ([28] and J. Guillermet-Guibert and B. Vanhaesebroeck, unpublished work). Heterozygosity for these p110 alleles results in a partial inactivation of these PI3Ks [28,29], similar to what could be realistically achieved by systemic administration of a PI3K p110 isoform-selective pharmacological inhibitor.

Inactivation of p110α or p110β did not prolong overall survival of heterozygous PTEN$^{+/-}$ mice, compared with control PTEN$^{+/-}$ mice (Figure 2). The main reason for death was killing the mice, necessitated by the development of lymphoma, a predominant cancer in the PTEN$^{+/-}$ model [8,31], which occurred with a similar incidence and kinetics within the p110α and p110β cohorts.

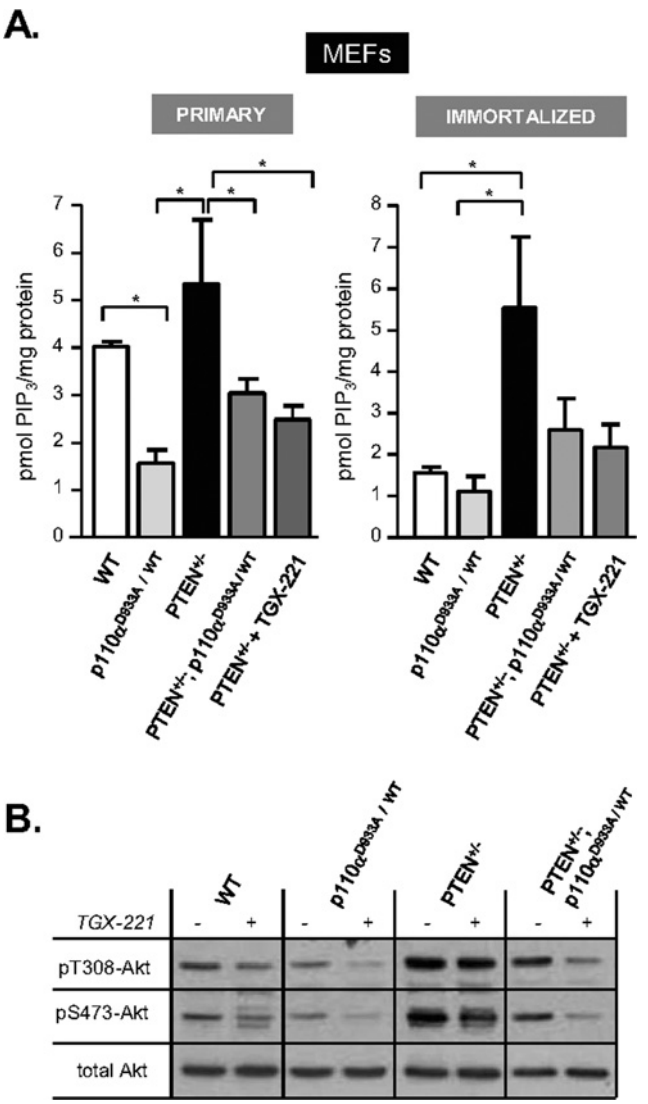

Figure 1 PI3K signalling upon inactivation of p110α or p110β in MEFs

(**A**) Cellular PtdIns(3,4,5)P_3 (PIP$_3$) levels in primary (left-hand panel) and immortalized (right-hand panel) MEFs of the indicated genotype. Where indicated, TGX-221 was added at 500 nM for 16 h, with control PTEN$^{+/-}$ cells incubated with vehicle only. Results from one experiment performed in triplicate are shown. The results shown are the means $\pm$ S.E.M. *$P < 0.05$ as obtained by Student's *t* test (Mann–Whitney). (**B**) MEFs of the indicated genotype were cultured in 10 % FBS in the presence of 500 nM TGX-221 or vehicle for 16 h, followed by cell lysis and immunoblotting with the indicated antibodies against Akt.

Partial inactivation of p110α or p110β reduces PtdIns(3,4,5)P_3 levels without affecting lymphoma incidence

Despite the reduced levels of PtdIns(3,4,5)P_3 in lymphoma tissue isolated from PTEN$^{+/-}\times$p110$\alpha^{D933A/WT}$ mice (Figure 3B), inactivation of p110α did not affect lymphoma development (Figure 3A), correlating with a minor or no impact on signalling in tumour tissue derived from individual mice (Figure 3C). Immunohistochemical analysis also showed no differences in FOXO1 localization or intensity of reactivity for either phosphorylation of Akt (pSer^{473}) or S6 (pSer^{235}/Ser^{236}) in lymphomas arising in PTEN$^{+/-}$ and PTEN$^{+/-}\times$p110$\alpha^{D933A/WT}$ mice (results not shown).

Inactivation of p110β also did not affect lymphoma development (Figure 3D), but substantially reduced PtdIns(3,4,5)P_3 levels in PTEN$^{+/-}$ lymphoma tissue (Figure 3E), with a variable impact on Akt/mTOR (mammalian target of rapamycin) signalling, which was often reduced (Figure 3F).

It is possible that the observed level of reduction in PI3K pathway signalling by p110α or p11β inactivation is insufficient to impact on lymphoma development, or that these lymphomas are/have become PI3K-independent and are unaffected by the reduction in PtdIns(3,4,5)P_3 levels induced by p110α or p110β inactivation.

Inactivation of p110β, but not of p110α, delays the development of PIN (prostatic intra-epithelial neoplasia) and prostate cancer induced by PTEN loss

We next assessed the impact of inactivation of p110α or p110β on prostate cancer and PIN, a premalignant proliferation arising

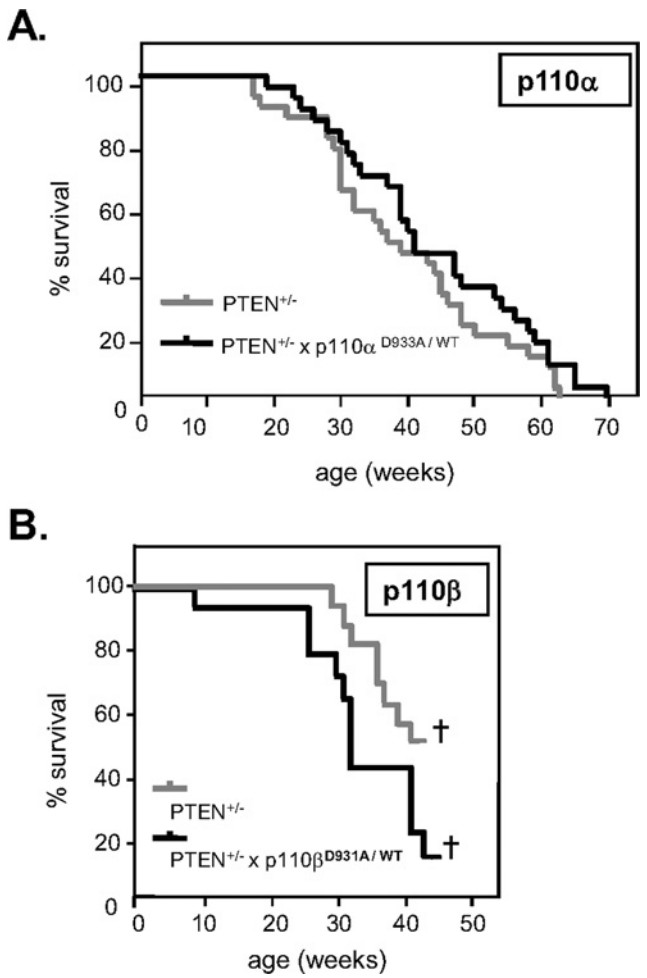

Figure 2 Impact of inactivation of one allele of p110α or p110β on survival of PTEN$^{+/-}$ mice

(**A**) Survival curves of PTEN$^{+/-}$ and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice. The number of mice used were: PTEN$^{+/-}$, $n = 31$ (16 females, 15 males); and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$, $n = 29$ (12 females, 15 males) (Mantel–Cox test, $P = 0.2036$; Gehan–Breslow–Wilcoxon test, $P = 0.2617$). (**B**) Survival curve of PTEN$^{+/-}$ and PTEN$^{+/-}$ ×p110$\beta^{D931A/WT}$ mice. The number of mice used were: PTEN$^{+/-}$, $n = 16$ (6 females, 10 males); and PTEN$^{+/-}$ ×p110$\beta^{D933A/WT}$, $n = 14$ (8 females, 6 males) (Mantel–Cox test, $P = 0.3380$; Gehan–Breslow–Wilcoxon test, $P = 0.1279$). †the p110β cohort was terminated at 42 weeks of age.

within the prostate. Inactivation of p110β, but not of p110α, has previously been shown to protect from this pathology induced by prostate-specific loss of PTEN [25]. In line with this previous study, in which p110α or p110β were homozygously inactivated, we observed a clear reduction in the frequency of PIN (from 10 % to 0 %) and prostate cancer (from 40 % to 17 %) upon heterozygous inactivation of p110β, but not of p110α (Figure 4A).

We next investigated whether the isoform-specific impact of PI3K inactivation on prostate cancer development correlated with an isoform-specific effect on PtdIns(3,4,5)P_3 levels in this tissue. This was analysed in young (8–10-week-old) mice, before they develop PIN or prostate cancer.

Compared with PTEN$^{+/-}$ prostates, inactivation of p110α in a PTEN$^{+/-}$ context did not noticeably affect PtdIns(3,4,5)P_3 levels (Figure 4B). The impact of p110β inactivation on PtdIns(3,4,5)P_3 levels was more variable. In fact, we frequently observed increased PtdIns(3,4,5)P_3 levels upon p110β inactivation compared with WT mice, not only in the prostate (Figure 4B, right-hand panel), but also in the other tissues where this parameter was analysed, namely in uterus (Figure 4C) and thyroid (Figure 4D). Increases in PtdIns(3,4,5)P_3 were not seen upon p110α inactivation, e.g. in prostate (Figure 4B) and thyroid (Figure 5C). In the context of PTEN inactivation, p110β co-inactivation led to a broad variability in PtdIns(3,4,5)P_3 levels, which were substantially increased in some of the mice (Figure 4B). It cannot be excluded that such enhanced PtdIns(3,4,5)P_3 production could contribute to protection from cancer, for example by induction of senescence due to high Akt activity [32,33].

p110α inactivation protects from the development of glomerulonephritis, pheochromocytoma and thyroid cancer upon PTEN inactivation

We next explored the impact of p110α inactivation on tumours in PTEN$^{+/-}$ mice other than prostate and lymphoma. Histopathological analysis showed that PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice developed a broad spectrum of tumours in different tissues, similar to PTEN$^{+/-}$ mice (Figure 5A).

Interestingly, the impact of p110α inactivation appeared to differ among tumour types. Indeed, whereas the incidence of several tumour types was unaffected (PIN, prostate cancer, lymphoma, thymic hyperplasia and breast cancer), that of others was either slightly increased (endometrial hyperplasia) or reduced (pheochromocytoma and thyroid tumours) (Figure 5A). The non-neoplastic immune-mediated glomerulonephritis was also reduced by p110α inactivation (Figure 5A). No clear correlation with time of tumour incidence was observed, although thymic hyperplasia and phaeochromocytoma may occur at a later age upon p110α inactivation (Figure 5B). Taken together, the changes in tumour incidence indicate that p110α can functionally interact with PTEN tumourigenesis *in vivo* in a tissue-specific manner.

In the thyroid, a tissue in which inactivation of p110α protects from PTEN loss-induced cancer (Figure 5A), PtdIns(3,4,5)P_3 levels in young (8–10 weeks of age) mice were similar in PTEN$^{+/-}$ and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice, again demonstrating the poor prognostic value of tissue PtdIns(3,4,5)P_3 levels for subsequent cancer development.

DISCUSSION

Ever since the discovery of PTEN as one of the most commonly inactivated genes in cancer, studies have been undertaken to define the effectors involved in tumourigenesis driven by PTEN inactivation. Mice with global or tissue-specific inactivation of PTEN have been a key tool in these investigations. Signalling proteins identified to play a role in the tumour-suppressive activity of PTEN include PDK1 (phosphoinositide-dependent kinase 1) [31], Akt1 [34], mTORC1 (mTOR complex 1) and mTORC2 [35–40] and LKB1 (liver kinase B1)–AMPK (AMP-activated protein kinase) [41].

Relatively less effort has been undertaken to assess the roles of the different class I PI3K isoforms in the signalling and biology induced by PTEN inactivation. This is surprising, given that these PI3Ks generate the PtdIns(3,4,5)P_3 lipid substrate for PTEN, and that they are ultimately the most proximal mediators of the lipid kinase-dependent impact of PTEN inactivation. Class I PI3Ks include the p110α, p110β, p110γ and p110δ isoforms, of which p110α and p110β are ubiquitously expressed, with p110δ and p110γ being mainly found in leucocytes. p110δ and p110γ have been functionally linked to PTEN in untransformed B-cells (p110δ; [42]) and endothelial cells (p110γ; [43]), but their roles in cancer induced by PTEN deficiency have not been investigated.

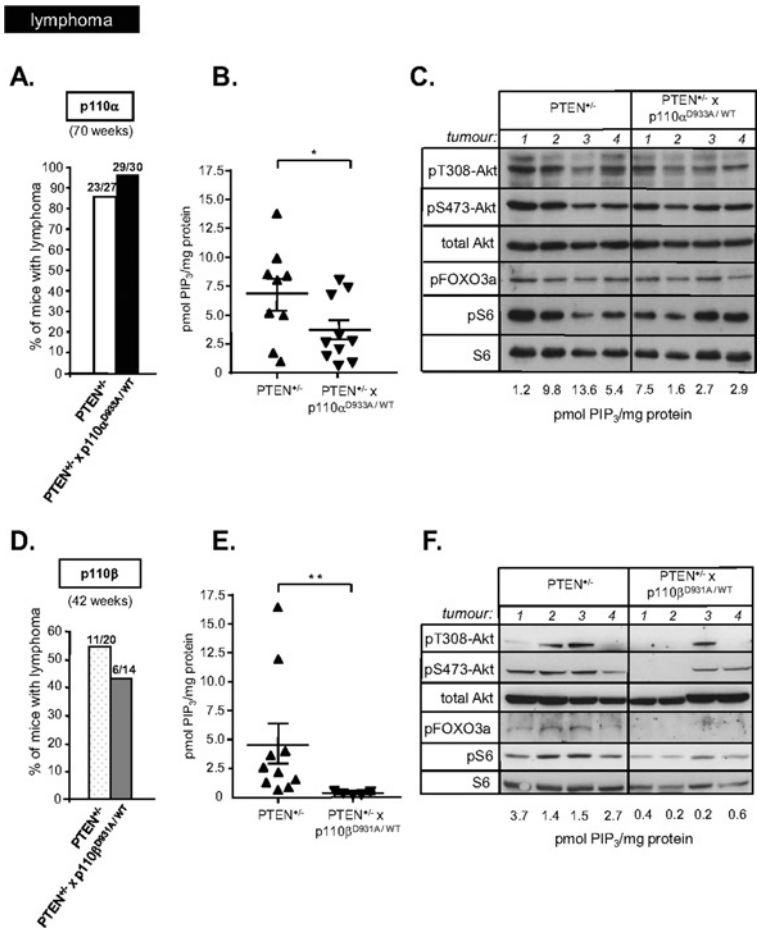

Figure 3 PtdIns(3,4,5)P_3 levels and PI3K/Akt signalling in PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ and PTEN$^{+/-}$ ×p110$\beta^{D931A/WT}$ lymphomas

(**A**) Incidence of lymphoma in PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ ($n = 30$) and littermate control PTEN$^{+/-}$ mice ($n = 27$). (**B**) PtdIns(3,4,5)P_3 levels in lipid extracts isolated from PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ and control PTEN$^{+/-}$ lymphoma tissue from age-matched mice. Number of tissue samples used: PTEN$^{+/-}$, $n = 9$; and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$, $n = 10$. *$P < 0.05$ as obtained by the Student's *t* test (Mann–Whitney). (**C**) Lymphoma protein extracts from the indicated mice were immunoblotted with the indicated antibodies. Tumour samples from four different mice were analysed per genotype. The PtdIns(3,4,5)P_3 (PIP$_3$) levels in each sample are indicated. (**D**) Incidence of lymphoma in PTEN$^{+/-}$ ×p110$\beta^{D931A/WT}$ mice ($n = 14$) and littermate control PTEN$^{+/-}$ mice ($n = 20$). (**E**) PtdIns(3,4,5)P_3 levels in lipid extracts isolated from PTEN$^{+/-}$ ×p110$\beta^{D933A/WT}$ and control PTEN$^{+/-}$ lymphoma tissue from age-matched mice. Number of tissue samples used: PTEN$^{+/-}$ ×p110$\beta^{D931A/WT}$, $n = 5$; and PTEN$^{+/-}$, $n = 10$. *$P < 0.05$ as obtained by the Student's *t* test (Mann–Whitney). (**F**) Lymphoma protein extracts from the indicated mice were immunoblotted with the indicated antibodies. Tumour samples from four different mice were analysed per genotype. The PtdIns(3,4,5)P_3 levels in each sample are indicated.

This also applies to p110α, whose role in this context has only been investigated in a model of selective PTEN loss in the prostate, where it does not appear to be of functional importance in blocking cancer development, in contrast with p110β [25]. PTEN-deficient cancer cell lines from different tissue origins (prostate, breast and glioblastoma) have also been shown to mainly depend on p110β and not p110α for their proliferation [23,24].

In order to investigate whether inhibition of p110α would impact on the development of cancer driven by PTEN loss, we crossed PTEN$^{+/-}$ mice with mice heterozygous for the p110α kinase-dead D933A knockin allele [28]. These p110$\alpha^{D933A/WT}$ mice have a 50 % reduction in p110α activity in all of the tissues where it is expressed [28], mimicking the impact of a systemically administered p110α-selective irreversible ATP-competitive inhibitor. MEFs derived from PTEN$^{+/-}$ and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ embryos were used as model cell lines to document that p110α inactivation can inhibit the impact of PTEN loss, namely by reducing the increased levels of PtdIns(3,4,5)P_3 and associated Akt signalling under steady-state growth conditions (Figure 1). At the organismal level, PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice developed a similar spectrum of tumours as PTEN$^{+/-}$ mice (Figure 5A) and had a similar lifespan, mainly because both groups developed lymphoma with a similar incidence as in PTEN$^{+/-}$ control mice (lymphoma is the most frequent cancer in the PTEN$^{+/-}$ model [8,31], becoming apparent within 15–20 weeks, often with large tumours that necessitated the early killing of the mice). In other cancer types, inactivation of p110α was found to have a differential impact. For example, it did not affect the incidence of PIN and prostate cancer, which we found to be reduced by ubiquitous heterozygous genetic inactivation of p110β, in agreement with previous findings of homozygous inactivation of p110β [25]. The incidence of glomerulonephritis, phaeochromocytoma and thyroid cancer in PTEN$^{+/-}$ mice, however, was reduced upon co-inactivation of one p110α allele. Taken together, these results show that p110α can functionally interact with PTEN in a signalling and cancer context, and that even partial inactivation of p110α can confer protection from cancer development in specific tissues.

The reason for a tissue-specific link of p110α and p110β with cancer due to PTEN loss is unclear at the moment. This observation might imply that treatment with p110 isoform-specific PI3K inhibitors could be therapeutically beneficial in

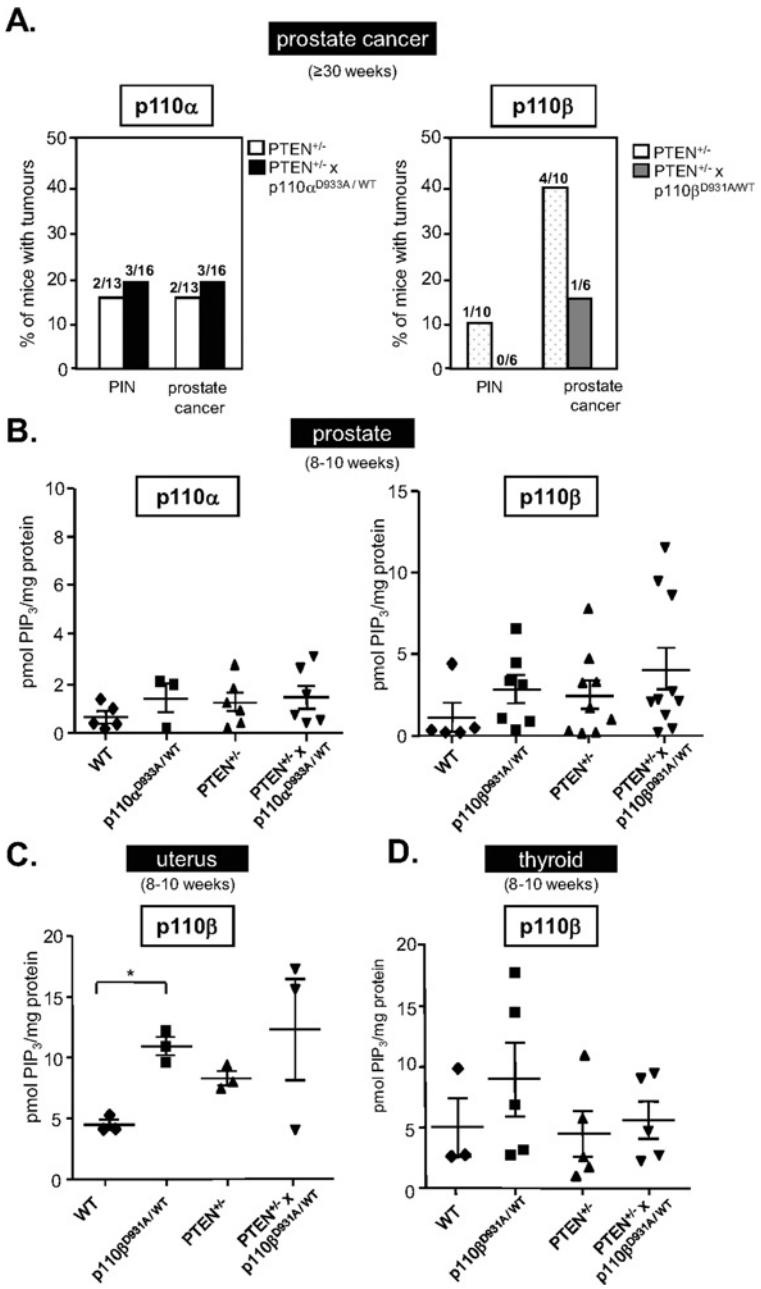

Figure 4 Impact of inactivation of one allele of p110α or p110β on the development of PIN and prostate cancer, and on PtdIns(3,4,5)P_3 levels in prostate, uterus and thyroid tissue

(**A**) Incidence of PIN and prostate cancer in PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice (left-hand panel, closed bars) and PTEN$^{+/-}$ ×p110$\beta^{D931A/WT}$ mice (right-hand panel, grey bars), compared with PTEN$^{+/-}$ mice. Two independent PTEN$^{+/-}$ control cohorts were used, for the p110α (open bars) and the p110β mice (dotted bars). (**B**, **C** and **D**) PtdIns(3,4,5)P_3 (PIP$_3$) levels in prostate, uterus and thyroid tissue from 8–10-week-old mice of the indicated genotypes. *$P < 0.05$ as obtained by Student's *t* test (Mann–Whitney).

the PTEN mutant cancer types in whose development these PI3K isoforms have been implicated. However, it remains to be documented whether the impact of PI3K inactivation on established cancer is similar to that on cancer development. Indeed, whereas germline organismal inactivation of PDK1 protects from cancer development in PTEN$^{+/-}$ mice [31], no such effect was observed when PDK1 was inactivated postnatally, either concomitantly with or subsequent to PTEN inactivation [44]. Moreover, although class I PI3K isoforms often have non-overlapping functions in untransformed cells [3], this non-redundancy is often lost in cancer cells [45,46]. For these reasons, therapeutic interference with PI3K signalling in cancer may have to target multiple PI3K isoforms, possibly simultaneously.

We hypothesized that a tissue-specific link of p110α and p110β with cancer development in a PTEN$^{+/-}$ context could be due to a differential impact of p110α and p110β inactivation on PtdIns(3,4,5)P_3 lipid levels in different tissues. Given the technical difficulties in measuring phosphoinositide lipids in general, and in tissues in particular, this key parameter of PI3K/PTEN signalling has, to the best of our knowledge, not

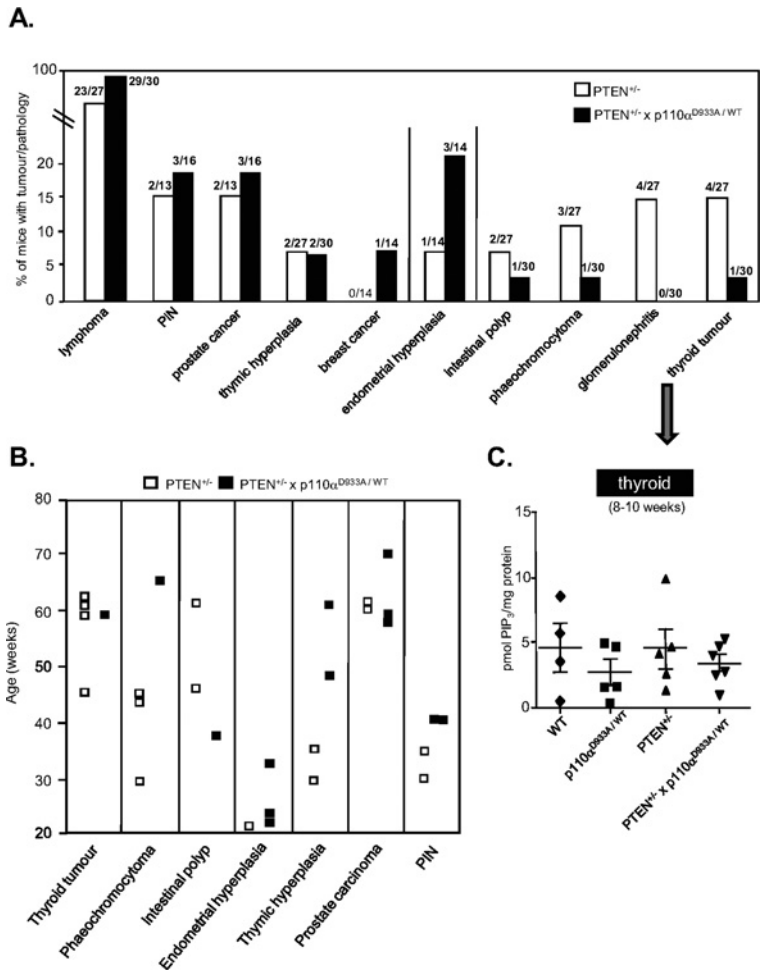

Figure 5 Impact of p110α inactivation on PTEN$^{+/-}$ tumours and pathologies

(**A**) Tumour spectrum and incidence in PTEN$^{+/-}$ and PTEN$^{+/-}$ ×p110$\alpha^{D933A/WT}$ mice. Number of mice used: PTEN$^{+/-}$, $n=27$ (14 females, 13 males); and PTEN$^{+/-}$ ×p110$\alpha^{+/-}$, $n=30$ (14 females, 16 males). The tumours/pathologies are grouped according to either no change, slightly increased or decreased upon p110α inactivation. (**B**) Timing of appearance of some of the tumours/pathologies shown in (**A**). (**C**) PtdIns(3,4,5)P_3 (PIP_3) levels in lipid extracts isolated from thyroid tissue from 8–10-week-old mice of the indicated genotypes.

been previously quantified in primary cancer tissues. Although we appreciate that a more detailed PtdIns(3,4,5)P_3 analysis is to be considered, including the measurement of lipid levels at different time points during tumour development, the results of the present study indicate that there might not be a strict correlation between tissue PtdIns(3,4,5)P_3 levels and cancer development or maintenance. Indeed, in many instances, PTEN$^{+/-}$ tissues did not have higher PtdIns(3,4,5)P_3 levels than WT tissues. Likewise, when PtdIns(3,4,5)P_3 was monitored in established PTEN$^{+/-}$ lymphoma, partial inactivation of p110α and p110β was found to decrease PtdIns(3,4,5)P_3 levels (Figure 3B), yet this had no discernable impact on tumour incidence (Figure 3A). There was also a poor correlation between the impact of PI3K isoform inactivation on tissue PtdIns(3,4,5)P_3 levels before cancer onset, and the cancer that eventually developed in these tissues. For example, in PTEN$^{+/-}$ thyroid tissues of 8–10-week-old mice, inactivation of p110α did not affect PtdIns(3,4,5)P_3 levels (Figure 5C); however, it reduced the incidence of this tumour at an advanced age (>50 weeks; Figures 5A and 5B). In the prostate, where inactivation of p110β provides some protection (Figure 4A), the impact of p110β inactivation was very variable, including increased PtdIns(3,4,5)P_3 levels in some cases (Figure 4B). It cannot be excluded that this enhanced PtdIns(3,4,5)P_3 production, although unexpected, may underpin protection from cancer, for example by induction of senescence due to high Akt activity [32,33].

Several possible explanations can be put forward for the apparent lack of correlation between PtdIns(3,4,5)P_3 levels and tumour development and maintenance. In the case of lymphoma, it is possible that the observed reduction in PtdIns(3,4,5)P_3 levels may not be sufficient to dampen signalling below the necessary threshold for growth inhibition. It is also possible that increases of PtdIns(3,4,5)P_3 levels in the stroma, i.e. not in the cancer cells themselves, may have facilitated lymphoma development. It is also possible that some PTEN$^{+/-}$ tumours do not depend on loss of the lipid phosphatase activity of PTEN, but on its protein phosphatase action [12–14] or its phosphatase-independent activities in the nucleus [15,16]. It is also possible that PtdIns(3,4,5)P_3 measured in crude tissue extracts is not the biologically relevant signalling pool at the subcellular level. Indeed, evidence for the existence of different cellular pools of PtdIns(3,4,5)P_3 has been reported [47], possibly residing in different subcellular compartments and with differential sensitivity to the PTEN and SHIP2 [SH2 (Src homology 2)-domain-containing inositol phosphatase 2] phosphatases, and with differential capacities to activate Akt. It is therefore possible

that inhibition of p110 isoforms in PTEN-null cells may alter the subcellular localization of PtdIns(3,4,5)P_3 pools, without inducing changes in the overall PtdIns(3,4,5)P_3 levels. Different p110 isoforms might also control basal compared with growth factor-stimulated Akt activity, as has been documented in PTEN-deficient prostate cancer cell lines in which p110α controls heregulin-induced Akt, whereas p110β, and to some extent p110δ, control basal Akt activity [48]. Our observations from the present study are reminiscent of the finding in colon cancer cell lines that activating mutations in p110α do not significantly alter growth factor-stimulated or steady-state PtdIns(3,4,5)P_3 levels under normal cell culture conditions [49]. Likewise, many *PIK3CA* (the gene encoding p110α) mutant cancer cell lines and human breast tumours exhibit only minimal Akt activation [50]. In other words, deregulation of p110α or PTEN in cancer does not necessarily result in changes in overall cellular PtdIns(3,4,5)P_3 levels or its downstream signalling pathways.

In summary, our data show that p110α can, similarly to p110β, functionally interact with PTEN in cultured cells, both in terms of PI3K lipid production and signalling. In addition, inactivation of p110α, in the context of a whole organism, can lead to reduced development of some tumour types. However, no direct correlation between steady-state levels of PtdIns(3,4,5)P_3 and cancer development was detected upon inactivation of p110α or p110β. It is hoped that new technological developments to monitor PtdIns(3,4,5)P_3 levels in primary tissues [51] will be instrumental in gaining further insight into the important question of when and where alterations in PtdIns(3,4,5)P_3 are of biological relevance.

AUTHOR CONTRIBUTION

Inma Berenjeno and Bart Vanhaesebroeck designed the experiments. Inma Berenjeno, Julie Guillermet-Guibert and Wayne Pearce performed the experiments. Stewart Fleming performed histology. Inma Berenjeno, Julie Guillermet-Guibert, Wayne Pearce, Stewart Fleming and Bart Vanhaesebroeck analysed the experiments. Inma Berenjeno and Bart Vanhaesebroeck wrote the paper. Bart Vanhaesebroeck obtained the funding.

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