Current Biology, Vol. 15, 1839–1846, October 25, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.08.066

Hypomorphic Mutation of PDK1 Suppresses Tumorigenesis in PTEN$^{+/-}$ Mice

Jose R. Bayascas,[1,*] Nick R. Leslie,[2] Ramon Parsons,[3] Stewart Fleming,[4,*] and Dario R. Alessi[1]

[1]MRC Protein Phosphorylation Unit
School of Life Sciences
University of Dundee
Dundee DD1 5EH
United Kingdom

[2]Division of Molecular Physiology
School of Life Sciences
University of Dundee
Dundee DD1 5EH
United Kingdom

[3]Integrated Program in Cellular, Molecular, and Biophysical Studies
Institute for Cancer Genetics
College of Physicians and Surgeons
Columbia University
New York, New York 10032

[4]Department of Molecular Pathology
Ninewells Hospital
University of Dundee
Dundee DD1 9SY
United Kingdom

Summary

Many cancers possess elevated levels of PtdIns(3,4,5)P_3, the second messenger that induces activation of the protein kinases PKB/Akt and S6K and thereby stimulates cell proliferation, growth, and survival [1, 2]. The importance of this pathway in tumorigenesis has been highlighted by the finding that PTEN, the lipid phosphatase that breaks down PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, is frequently mutated in human cancer [3, 4].Cells lacking PTEN possess elevated levels of PtdIns(3,4,5)P_3, PKB, and S6K activity [5–8] and heterozygous PTEN$^{+/-}$ mice develop a variety of tumors [9–11]. Knockout of PKBα in PTEN-deficient cells reduces aggressive growth and promotes apoptosis [12], whereas treatment of PTEN$^{+/-}$ mice with rapamycin, an inhibitor of the activation of S6K, reduces neoplasia [13]. We explored the importance of PDK1, the protein kinase that activates PKB and S6K [14], in mediating tumorigenesis caused by the deletion of PTEN. We demonstrate that reducing the expression of PDK1 in PTEN$^{+/-}$ mice, markedly protects these animals from developing a wide range of tumors. Our findings provide genetic evidence that PDK1 is a key effector in mediating neoplasia resulting from loss of PTEN and also validate PDK1 as a promising anticancer target for the prevention of tumors that possess elevated PKB and S6K activity.

*Correspondence: j.bayascas@dundee.ac.uk (J.R.B.); s.fleming@dundee.ac.uk (S.F.)

Results and Discussion

Generation of PDK1 Hypomorphic PTEN$^{+/-}$ Mice

In this study we explore whether suppression of PDK1 expression inhibits tumorigenesis in PTEN$^{+/-}$ mice. As PDK1$^{-/-}$ mice fail to develop beyond embryonic day (E)9.5 of embryogenesis, we generated hypomorphic PDK1$^{fl/fl}$ mice in which the neomycin resistance gene is inserted into an intron sequence between exons 2 and 3 of the *PDK1* gene in order to interfere with its expression (Figure 1A) [15]. The presence of the neomycin gene reduces the expression of PDK1 by 80%–90% in all tissues, and its removal using the CRE recombinase restores normal levels of PDK1 [15]. PDK1$^{fl/fl}$ mice, as well as PDK1$^{-/fl}$ mice possessing even lower levels of PDK1, are viable and fertile, and the only marked phenotype is that they are 30%–40% smaller than control littermates [15].

We crossed the hypomorphic PDK1$^{-/fl}$ mice with PTEN$^{+/-}$ mice as described in Figure 1B in order to generate a group of experimental PDK1$^{-/fl}$PTEN$^{+/-}$ and littermate control PDK1$^{+/fl}$PTEN$^{+/-}$mice. PDK1 hypomorphic mice, which are wild-type for PTEN (PDK1$^{-/fl}$ PTEN$^{+/+}$), are born at a reduced Mendelian frequency (Figure 1B, cross 3 and [15]), indicating that reduced PDK1 expression affects the development of some mice. Interestingly however, the PDK1$^{-/fl}$PTEN$^{+/-}$ mice are born at the expected frequency. There is evidence that PTEN$^{+/-}$ cells possess enhanced activity of the PI 3-kinase pathway [16, 17], which might be capable of rescuing the loss of viability resulting from a reduction in PDK1 expression. It is not possible to generate PTEN$^{-/-}$ mice as they die early in embryogenesis [9–11], and we have also been unable to generate viable PTEN$^{-/-}$PDK1$^{-/fl}$ mice (Figure 1B, cross 4), indicating that a reduction in PDK1 cannot rescue embryonic lethality resulting from complete loss of PTEN.

The group of experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice are ~30% smaller than their littermate control PDK1$^{+/fl}$ PTEN$^{+/-}$ mice at 6 weeks of age and remain smaller until 12 months of age (Figure 1C). Thus any increase in PtdIns(3,4,5)P_3 in the PTEN$^{+/-}$ mice does not compensate for the effect on cell size resulting from the hypomorphic mutation of PDK1. We measured PDK1 activity and its expression in the liver (Figure 1D), as well as muscle and adipose tissue (data not shown) and found that, as expected, PDK1 activity and protein levels in the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ animals are markedly reduced. We also found that PTEN activity and expression of the protein is the same in the liver (Figure 1E) and muscle and adipose tissues obtained from both the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ and the control PDK1$^{+/fl}$PTEN$^{+/-}$ animals (data now shown).

Analysis of Survival

Up to 9 months of age there is no significant difference in the overall survival of the experimental PDK1$^{-/fl}$

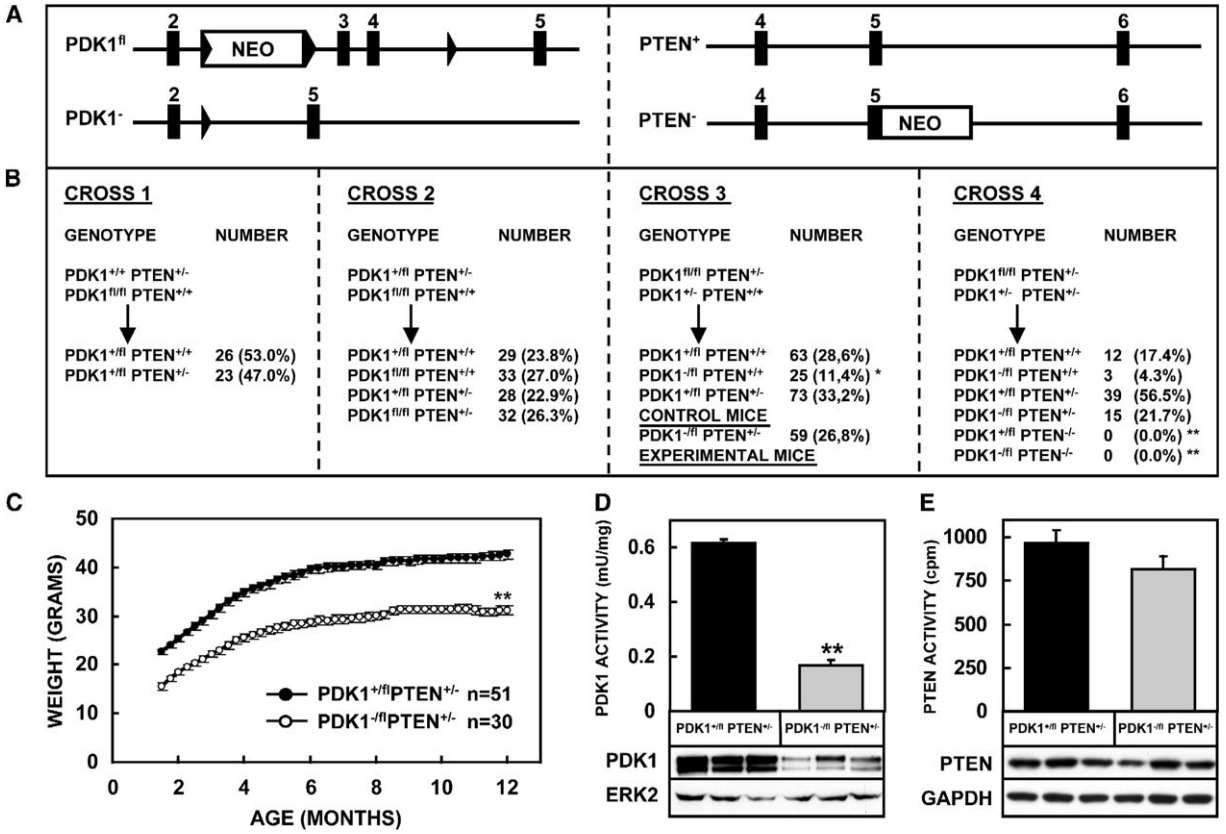

Figure 1. Generation and Analysis of PDK1 Hypomorphic PTEN$^{+/-}$ Mice

(A) Diagram illustrating the positions of the exons 2 to 5 of the *PDK1* gene [15] and 4 to 6 of the *PTEN* gene [11], depicting the different alleles that we have utilized. The black boxes represent exons, the continuous lines introns, and triangles represent CRE loxP excision site and NEO the neomycin resistance gene cassette.

(B) Breeding strategy employed to generate the control PDK1$^{+/fl}$PTEN$^{+/-}$ and experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice used in this study. The number and percentage of each genotype obtained after weaning are indicated. An asterisk indicates lower frequency than expected of PDK1$^{-/fl}$PTEN$^{+/+}$ obtained is statistically significant (χ^2 Test and Fisher's Exact Test $p < 0.05$).

(C) The mean body weight of the control PDK1$^{+/fl}$PTEN$^{+/-}$ and experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice at the indicated age, where n corresponds to the number of mice in each group. Values represent the mean ± SEM for each data point.

(D and E) Liver extracts for the indicated mice were prepared and PDK1 (D) or PTEN (E) were immunoprecipitated and assayed. The results shown are the average ± SEM corresponding to samples derived from three independent mice assayed in triplicate. Samples from three different mice of each genotype were also immunoblotted with the indicated antibodies. The double asterisks in (C) and (D) indicates $p < 0.005$ as obtained by the Student's t test.

PTEN$^{+/-}$ and control PDK1$^{+/fl}$PTEN$^{+/-}$ mice. We observed that ~20% of both groups of animals died spontaneously (Figure 2A). Pathological analysis of the mice that died during this period revealed that only a single 7-month-old PDK1$^{+/fl}$PTEN$^{+/-}$ control mouse had lymphoma, whereas the other animals possessed no detectable tumors or preneoplastic lesions (data not shown). It has been reported previously that PTEN$^{+/-}$ mice have a tendency to develop lethal polyclonal autoimmune disorder, resulting in renal failure [17]. We observed that several of the PDK1$^{-/fl}$PTEN$^{+/-}$ or PDK1$^{+/fl}$PTEN$^{+/-}$ mice that died within the first 9 months displayed kidney disease, predominantly glomerulonephritis, similar to that previously reported [17] (data not shown).

After 9 months of age, a significant number of control PDK1$^{+/fl}$PTEN$^{+/-}$ mice developed large, externally visible tumors, and by 12 months of age 40% of these mice displayed such tumors (Figure 2B). In striking contrast, none of the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice displayed externally visible tumors up to 14–15 months of age when the study was terminated. At this stage, only 2 out of 23 experimental animals were observed to possess obvious tumors, which were only visible following dissection (Figure 2B). In contrast, 68% of the control PDK1$^{+/fl}$PTEN$^{+/-}$ mice possessed at least 1 obvious tumor by 14–15 months of age.

Analysis of Tumors

Mice were culled when they exhibited large external tumors, lost weight, became sick or reached 15 months of age. A necroscopy was performed, the tissues fixed in 10% formalin, and subjected to detailed histopathological examination. The types of tumors and the proportion of mice displaying these lesions are summarized in Table 1 and representative histopathological sections are shown in Figure 3. Similar to previous reports for the PTEN$^{+/-}$ mice [9–11], we observed that

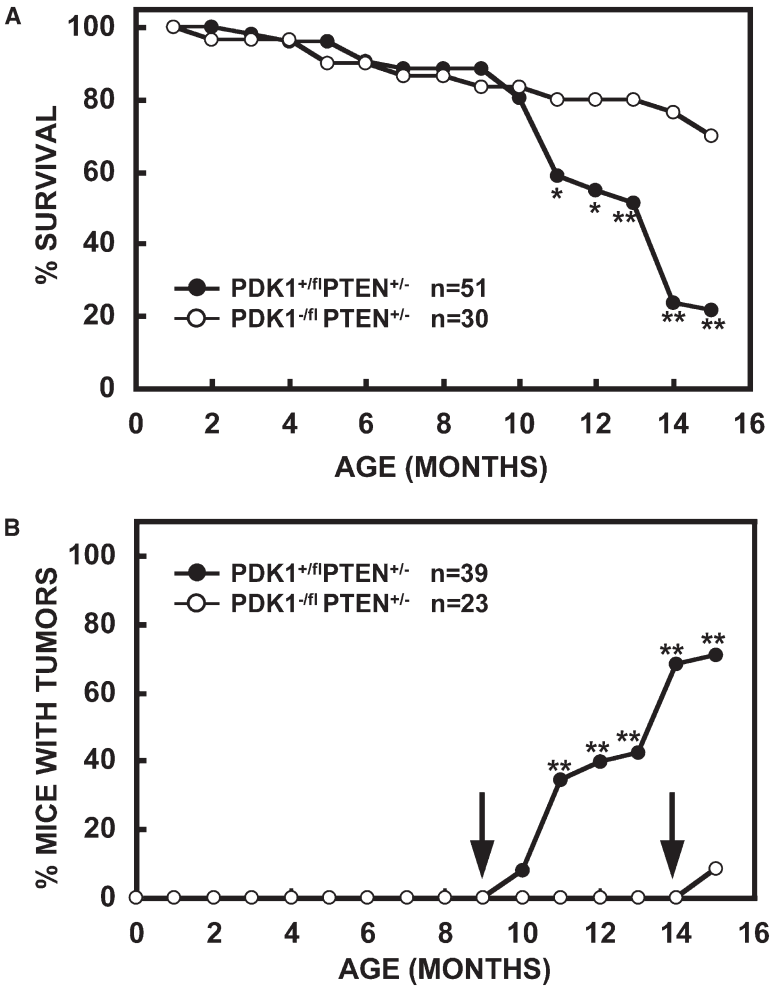

Figure 2. Analysis of Survival and Tumor Formation

(A) Mice were maintained under standard husbandry conditions and the percentage of surviving mice is indicated.

(B) The percentage of mice with visible tumors is indicated. For mice up to 12 months of age, these tumors only comprise those that were externally visible. At 14–15 months of age when the study was terminated, tumors that were visible internally after dissection are also included in the analysis. n corresponds to the number of mice of each genotype. Arrows in (B) indicate the onset of tumor development for each group of mice. An asterisk indicates $p < 0.05$, and a double asterisk, $p < 0.005$ obtained by both the χ^2 Test and the Fisher's Exact Test.

72% of the control PDK1$^{+/fl}$PTEN$^{+/-}$ mice developed tumors (Table 1). These comprised a range of tumors frequently observed in human disease and which exhibit hormone or cytokine dependent growth (lymphoma (Figure 3A), endometrial carcinoma (Figure 3C), prostate carcinoma (Figure 3D), phaeochromocytoma (Figure 3F), breast adenocarcinoma (Figure 3H), or testicular tumor (Figure 3J). Other types of tumors including, colon adenoma, thyroid carcinoma, liver hepatoma, and lung adenocarcinoma are also observed but with lower incidence (Table 1). In addition to these primary tumors, pulmonary metastases were also seen.

In contrast, a markedly lower proportion of the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice had developed tumors by 15 months of age. Although 42% of control female and 20% of control male PDK1$^{+/fl}$PTEN$^{+/-}$ mice had endometrial carcinoma or testicular carcinoma, no such tumors are observed in any of the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ animals (Table 1). The proportion of PDK1$^{-/fl}$PTEN$^{+/-}$ mice that developed lymphoma (Figure 3B), prostate carcinoma (Figure 3E), phaeochromocytoma (Figure 3G), and breast adenocarcinoma (Figure 3I) is 2.5- to 7.5-fold lower than observed in the control PDK1$^{+/fl}$PTEN$^{+/-}$ animals (Table 1). One experimental PDK1$^{-/fl}$PTEN$^{+/-}$ animal developed pancreatic carcinoma, whereas none of the control animals developed this lesion. In total, 64 tumors were detected in 39 control PDK1$^{+/fl}$PTEN$^{+/-}$ mice, compared with only 7 tumors in 23 experimental PDK1$^{-/fl}$PTEN$^{+/-}$ animals.

Employing immunohistochemistry analysis, we investigated the subcellular localization of the FOXO1 transcription factor, which is localized in the cytosol rather than the nucleus after phosphorylation by PKB [18], the phosphorylation of S6 protein by S6K [19], and monitored cell proliferation by using the Ki67 cell division marker in the mouse tumors. We first examined prostate carcinoma (Figure 4A) and breast adenocarcinoma (Figure 4B) from the PDK1$^{-/fl}$PTEN$^{+/-}$ and PDK1$^{+/fl}$PTEN$^{+/-}$ animals. In the tumors of both genotypes, FOXO1 was predominantly in the cytosol (Figures 4A and 4B, panels 1 and 2), indicating that PKB was activated. High levels of phospho-S6 protein were also observed (Figure 4A and 4B, panels 3 and 4), suggesting that S6K was active in the tumors of either genotype. Comparable levels of Ki67 staining were also seen in the prostate carcinoma and breast adenocarcinoma from PDK1$^{+/fl}$PTEN$^{+/-}$ and PDK1$^{-/fl}$PTEN$^{+/-}$ animals in which ~25% of cells were stained (Figures 4A and 4B, panels 5 and 6). Analogous analysis was undertaken for the lymphoma and phaeochromocytoma and no signifi-

Table 1. Incidence of Tumors in PDK1$^{+/fl}$ PTEN$^{+/-}$ and PDK1$^{-/fl}$ PTEN$^{+/-}$ Mice

	PDK1$^{+/fl}$ PTEN$^{+/-}$ (n = 20 males, 19 females)	Percentage (%)	PDK1$^{-/fl}$ PTEN$^{+/-}$ (n = 14 males, 9 females)	Percentage (%)	P Value: χ^2 Test (Fisher's Exact Test)
Tumors					
Lymphoma	21	53.8	2	8.7	0.0004 (0.0003)**
Endometrial carcinoma (female)	8	42.1	0	0	0.0213 (0.0243)*
Phaeochromocytoma	15	38.4	1	4.3	0.0030 (0.0022)*
Prostate carcinoma (male)	7	35.0	1	7.1	0.0368 (0.0326)*
Breast adenocarcinoma (female)	5	26.3	1	11.1	0.3598 (0.3498)
Testicular carcinoma (male)	4	20.0	0	0	0.0748 (0.1045)
Colon adenoma	2	5.1	0	0	0.2696 (0.3918)
Lung metastasis	2	5.1	0	0	0.2696 (0.3918)
Lung adenocarcinoma	1	2.5	0	0	0.4388 (0.6290)
Thyroid carcinoma	1	2.5	0	0	0.4388 (0.6290)
Liver hepatoma	1	2.5	0	0	0.4388 (0.6290)
Pancreatic carcinoma	0	0	1	4.3	0.1892 (0.3710)
Number of mice displaying tumors	28	71.8	5	21.7	0.0001 (0.0001)**
Preneoplastic Lesions					
Prostatic intraepithelial neoplasia (male)	10	50.0	3	21.4	0.0915 (0.0909)
Endometrial hyperplasia (female)	5	26.3	2	22.2	0.8153 (0.6023)
Intratubular germ cell neoplasia (male)	1	5.0	0	0	0.4129 (0.7212)
Hyperplasia seminal vesicle (male)	0	0	1	7.1	0.2250 (0.4117)
Mice without tumors displaying preneoplastic lesions	4	10.2	6	26.1	0.1016 (0.1958)
Mice displaying no tumor-associated pathology	7	17.9	12	52.2	0.0047 (0.0058)**
Other Nonneoplastic Lesions					
Persistent trophoblast	7	36.8	1	11.1	0.1228 (0.1228)
Glomerulonephritis	2	5.1	0	0	0.2696 (0.3918)
Hydronephrosis	1	2.6	0	0	0.4388 (0.6290)
Salivary gland abscess	1	2.6	0	0	0.4388 (0.6290)
Breast abcesses (female)	0	0	1	11.1	0.1892 (0.3710)
Pancreatitis	0	0	2	8.7	0.0612 (0.1338)

n corresponds to the total number of mice and the percentages indicate the number of mice displaying the indicated pathologies. *p < 0.05; **p < 0.005.

cant differences in the staining patterns were detected between the tumors arising in the control and experimental mice (data not shown). Taken together the data indicate that the few tumors that develop in the PDK1$^{-/fl}$ PTEN$^{+/-}$ mice are similar to those in the control animals and have elevated PKB and S6K activity, leading to increased proliferation of cells within the tumor.

Some differences in staining between tumor types were however observed. S6 protein was highly phosphorylated in all of the carcinomas but was low in the phaeochromocytomas. In the lymphomas, which were all of follicular origin, the S6 protein phosphorylation was seen in centroblasts, but not in centrocytes. The pattern of FOXO1 cytosolic staining closely matched that seen for phospho-S6 protein. There was strong FOXO1 cytoplasmic staining of the tumor cells of both breast and prostate carcinoma, whereas in lymphoma cytoplasmic staining of centroblasts was detected but with weaker nuclear staining of some centrocytes. We were unable to detect FOXO1 in the phaeochromocytomas. Ki67 staining was observed in ~10% of lymphomas (again mostly centroblasts) and ~5% of phaeochromocytomas.

Analysis of Preneoplastic Lesions

A more equal proportion of minor preneoplastic lesions are observed in control PDK1$^{+/fl}$PTEN$^{+/-}$ and experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice. Thus ~25% of each of these genotypes developed endometrial hyperplasia (Figures 3M and 3N), whereas the proportion of PDK1$^{-/fl}$ PTEN$^{+/-}$ mice displaying prostatic intraepithelial neoplasia (Figures 3K and 3L) is only ~2-fold lower than those observed in the control PDK1$^{+/fl}$PTEN$^{+/-}$ animals (Table 1). By 15 months of age, only 18% of the control PDK1$^{+/fl}$PTEN$^{+/-}$ mice were free of tumors or preneoplastic lesions compared to 52% of the experimental PDK1$^{-/fl}$PTEN$^{+/-}$ animals.

Persistent Trophoblast Formation in PTEN$^{+/-}$ Mice

Unexpectedly, during the pathological analysis of mouse tissues, we observed that 37% of the control female PDK1$^{+/fl}$PTEN$^{+/-}$ mice that had never been used for breeding displayed persistent trophoblast (Table 1). In contrast, trophoblast is not normally observed in mice that have not previously bred. Two separate events must have occurred to account for this observation, namely partenogenetic fertilization of an ovum and formation and persistence of trophoblast. Trophoblast development from oocytes is dependent upon expression from the paternal copy of the *IGF-2* gene, as the maternal copy of *IGF-2* gene is imprinted and not expressed [20]. The signaling pathway by which IGF-2 stimulates trophoblast development is unknown. How-

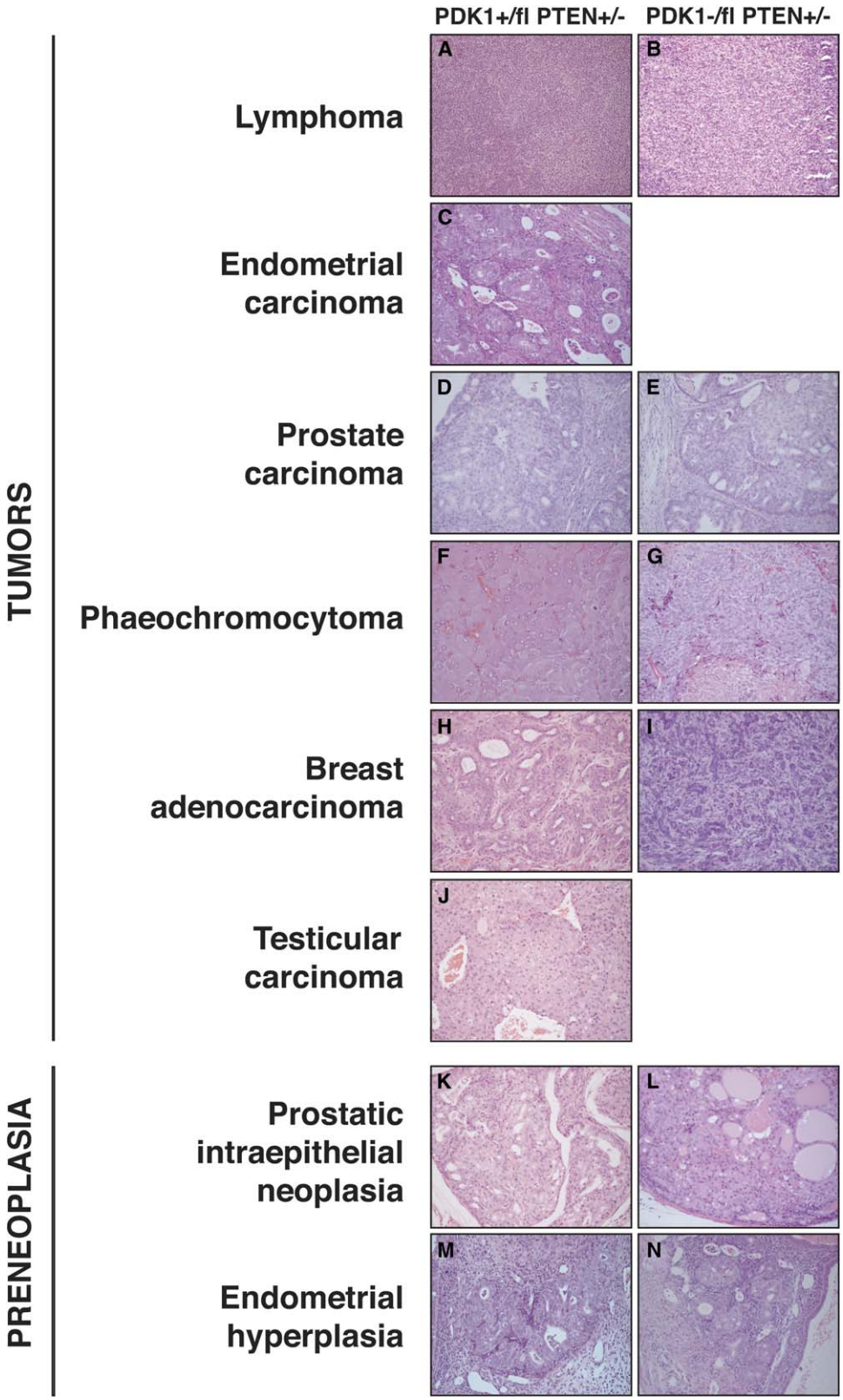

Figure 3. Histopathological Analysis of PDK1$^{+/fl}$PTEN$^{+/-}$ and PDK1$^{-/fl}$PTEN$^{+/-}$ Mice

The pathology of the several tumor types shows no differences between those occurring in PDK1$^{+/fl}$ PTEN$^{+/-}$ and those occurring in PDK1$^{-/fl}$ PTEN$^{+/-}$. (A and B) Non Hodgkin's lymphomas were of a diffuse predominantly small cell type with some plasmacytoid differentiation. (D and E) Prostatic carcinomas showed a cribriform architecture on occasion with comedo-type necrosis and invasion of the surrounding stroma. (F and G) Phaeochromocytomas were composed of large polygonal amphophilic cells with granular cytoplasm and a rich vascular supply. (H and I) The breast carcinomas were of an invasive ductal type eliciting a desmoplastic reaction. (J) Testicular tumors, in this example a Leydig cell tumor, were only seen in the control animals as were endometrial carcinomas (C); preneoplastic lesions of prostate amounting to high grade PIN (K and L) were seen in both groups of animals; as was atypical endometrial hyperplasia (M and N). Haematoxylin and eosin, original magnification ×120. (A), (C), (D), (F), (H), (J), (K), and (M), PDK1$^{+/fl}$ PTEN$^{+/-}$; (B), (E), (G), (I), (L), and (N), PDK1$^{-/fl}$ PTEN$^{+/-}$.

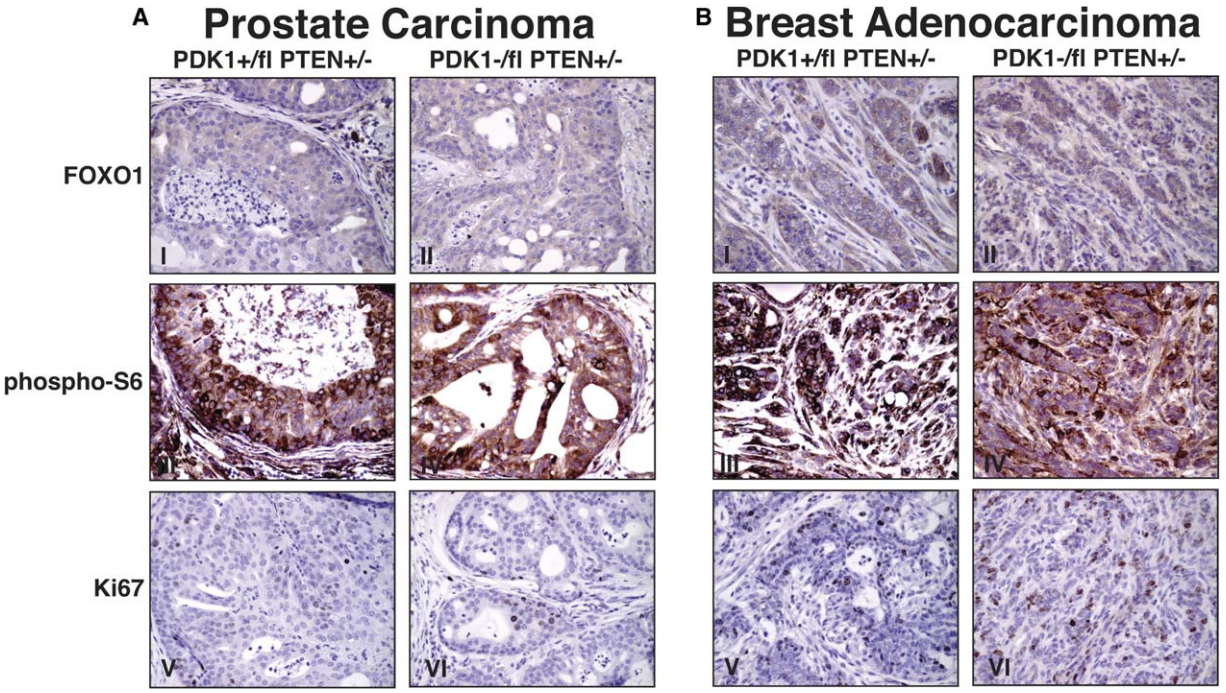

Figure 4. Immunohistochemistry Analysis of Tumors from PDK1$^{+/fl}$PTEN$^{+/-}$ and PDK1$^{-/fl}$PTEN$^{+/-}$ Mice

Sections derived from prostate carcinoma (A) and breast adenocarcinoma (B) of PDK1$^{+/fl}$PTEN$^{+/-}$ (panels I III, and V) and PDK1$^{-/fl}$PTEN$^{+/-}$ (panels II, IV, and VI) tumors were probed with the indicated antibody, counterstained with haematoxylin, and visualized with a magnification of ×160. In panels I and II, cytoplasmic reactivity of FOXO1 was mainly observed in tumor cells and to a lesser extent in fibroblasts of both breast adenocarcinomas. In panels III and IV, phospho-S6 protein phosphorylated at Ser235/Ser236 was seen by strong cytoplasmic reactivity in all tumor samples. In the breast adenocarcinoma, both tumor cells and the adjacent reactive stromal fibroblasts were observed to be stained. In panels V and VI, Ki67 staining showed that in both types of carcinoma about 25% of the tumor cells are in cell cycle with reactivity also noted in the stromal fibroblasts of the breast adenocarcinoma.

ever, if IGF-2 stimulated trophoblast development through the PI 3-kinase pathway, it is possible that the enhanced PtdIns(3,4,5)P_3 levels in PTEN$^{+/-}$ mice could account for the presence of trophoblast. It is also possible that deregulation of the imprinting of the maternal copy of the *IGF-2* gene in the PTEN$^{+/-}$ oocytes could account for the presence of trophoblast in these mice. We also observed that a lower proportion of experimental PDK1$^{-/fl}$PTEN$^{+/-}$ female mice demonstrated persistent trophoblast compared with the control PDK1$^{+/fl}$PTEN$^{+/-}$ mice, indicating that if trophoblast development and survival proceeds through the PI 3-kinase pathway, PDK1 may be involved in this process.

Potential Delay in the Onset of Tumorigenesis in PDK1$^{+/fl}$PTEN$^{+/-}$ Mice

The strain of PTEN$^{+/-}$ mice employed in our study [11], as well two other independently generated strains [9, 10], were reported to develop a significant number of tumors by 6–8 months of age. In contrast, when we analyzed a group of five control PDK1$^{+/fl}$PTEN$^{+/-}$ and five experimental PDK1$^{-/fl}$PTEN$^{+/-}$ mice 8 months of age, we were unable to detect any tumor or preneoplasia in these mice (data not shown), and apart from the single control PDK1$^{+/fl}$PTEN$^{+/-}$ mouse that developed lymphoma at 7 months of age, other tumors are only observed after 9 months of age (Figure 2B). One explanation for this observation is the difference in genetic background of our mice and those used in previous studies. The mice employed in our study had been backcrossed for at least three generations to C57BL/6J background (see Experimental Procedures), whereas the PTEN$^{+/-}$ mice used in other studies were of a more mixed genetic background. Another explanation is that the littermate control mice that we deployed are PDK1$^{+/fl}$ rather than PDK1$^{+/+}$, and our previous analysis demonstrated that PDK1 activity in several tissues derived from PDK1$^{+/fl}$ mice is 50%–85% of the level observed in wild-type PDK1$^{+/+}$ mice [15]. It is therefore possible that even a modest reduction in the expression of PDK1 in the control PDK1$^{+/fl}$PTEN$^{+/-}$ mice employed in this study could have delayed the onset of tumor formation. To address this question it would be necessary to compare tumor development in PDK1$^{+/fl}$ PTEN$^{+/-}$ and PDK1$^{+/+}$PTEN$^{+/-}$ mice.

PDK1 as an Anticancer Target

Much effort is being devoted to developing anticancer drugs that inhibit components of the PI 3-kinase signaling pathway in order to treat tumors that have mutations in PTEN and other signaling networks that elevate the level of PtdIns(3,4,5)P_3 and/or enhance PKB and S6K activity. The PDK1 hypomorphic mice utilized in this study represent a genetic model that would be ex-

pected to mimic PTEN$^{+/-}$ mouse treated with a drug that reduces the endogenous activity of PDK1 by 80%–90%. The finding that a reduction in PDK1 activity by this amount markedly protects and/or delays tumor formation in PTEN$^{+/-}$ mice suggests that PDK1 is a promising anticancer target to prevent the formation of human tumors with elevated PKB and S6K activity.

Our results do not address the question of whether inhibiting PDK1 in an established tumor would prevent its growth or induce regression of the tumor. Recently however, relatively specific, small molecule inhibitors of PDK1 were described, which inhibited the growth and induced apoptosis of a wide range of cancer cell lines as well as suppressing the growth of melanoma tumors in a nude mice model [21]. Another drug with anticancer properties, 7-hydroxystaurosporine (also termed UCN-01), was originally reported to function as a CHK1 inhibitor but subsequently found to inhibit PDK1 with similar potency [22, 23], suggesting that some of the antitumorigenesis properties of this compound might be mediated through inhibition of PDK1. These findings, together with the genetic studies described in this paper, suggest that PDK1 inhibitors are likely to have utility in both the prevention and treatment of cancers that have elevated PtdIns(3,4,5)P_3 levels and/or PKB and S6K activity.

Conclusions

Several reports suggest that some of the tumor suppressor functions of PTEN are mediated independently of its ability to breakdown PtdIns(3,4,5)P_3, for example, by dephosphorylating other substrates (reviewed in [3, 4]) or through the ability of its C2 domain to control cell migration [24]. Moreover, elevated levels of PtdIns(3,4,5)P_3 resulting from a loss of PTEN, will activate several signaling processes in addition to PDK1-PKB-S6K pathway [1, 2]. The importance of these other networks in mediating the tumor suppressor activity of PTEN is unknown, but the results described in this study provides strong genetic evidence that PDK1 is a key effector in mediating tumorigenesis resulting from loss of PTEN. Our findings also support the notion that PDK1 is an attractive target for the development of drugs to prevent cancers that possess elevated PtdIns(3,4,5)P_3 levels and/or possess increased PKB and S6K activity. Further work will be required to establish the mechanism by which reduction in PDK1 levels markedly inhibits the onset of tumor formation and whether this involves decreased activation of PKB/S6K and/or other PDK1 targets.

Experimental Procedures

Materials

Taq DNA polymerase was purchased from Promega. Protein G-Sepharose and Streptavidin-Sepharose High Performance were purchased from Amersham Pharmacia Biotech. Roche supplied protease inhibitor cocktail tablets.

Antibodies

The PDK1 antibody used for immunoblotting and activity was raised in sheep against the sequence RKIQEVWRQQYQSNP DAAVQ (residues 540–559 of mouse PDK1). The ERK2 antibody was raised in sheep against the full-length human protein. Both antibodies were affinity purified with the appropriate antigen. The PTEN monoclonal antibody raised against amino acids 388–400 mapping at the C terminus of human PTEN was purchased from Santa Cruz Biotechnology (sc-7944). Monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (#SC5 clone). Secondary antibodies coupled to horseradish peroxidase were from Pierce.

Mice Breeding and Genotype Analysis

All animal studies and breeding was approved by the University of Dundee ethical committee and performed under a UK Home Office project license. The generation and genotyping of the PTEN$^{+/-}$ mice [11] and the PDK1 hypomorphic mice [15] have been described previously. The original PTEN$^{+/-}$ mice employed were from a mixed background [11] and were backcrossed for three generations with the C57BL/6J strain prior to crossing with the PDK1 hypomorphic mice. The PDK1$^{fl/fl}$ mice and PDK1$^{+/-}$ mice employed in the crosses described in Figure 1B were backcrossed for five to seven generations to the C57BL/6J strain prior to crossing with PTEN$^{+/-}$ mice. Littermates with genotypes PDK1$^{+/fl}$PTEN$^{+/-}$ (control) and PDK1$^{-/fl}$PTEN$^{+/-}$ (experimental) derived as described in Figure 1B were maintained under standard husbandry conditions for a period of up to 15 months of age. During this period mice were monitored weekly for tumor development and weight loss. In accordance with our home office license, any animal that displayed an obvious external tumor of over 1.44 cm^2, lost over 20% in body weight, or showed signs of sickness was culled and subjected to necropsy and pathological analysis after fixation of tissues in 10% formalin as described below.

Analysis of Tumors

After 15 months of age, surviving mice were culled, necropsy was performed, and all tissues were immediately fixed in 10% buffered formalin (Pre-filled Biopsy Pots, BIOS Europe, #BP120BF) and subsequently embedded in paraffin. 5 μm thick sections were cut, deparaffinized, and stained with haematoxylin and eosin for histological analysis employing standard protocols. Sections were viewed on a Nikon Eclipse 600 microscope and images captured on a Nikon DXM1200 digital camera supported by EclipseNet software.

Assay of PDK1 and PTEN

PDK1 was immunoblotted and assayed after its immunoprecipitation from tissue extracts employing the PDKtide peptide as described previously [15]. PTEN activity was measured following its immunoprecipitation from tissue extracts employing ^{33}P-labeled PtdIns(3,4,5)P_3 as described previously [25].

Immunohistochemistry Analysis

Tumor slices were generated as described above and subjected to heat-induced antigen retrieval. Immunohistochemical staining was performed with automated procedures at the Surgery and Molecular Oncology Department of the Ninewells hospital. The FOXO1 antibody (Cell Signaling #9462) was used at a dilution of 1/160, the Phospho-S6 Ser235/236 antibody (Cell Signaling #4857) used at a dilution of1/400 and the Ki67 antibody employed (VectorLabs clone SP6) at a dilution of 1/200. Antibody staining was detected using the rabbit Vectastain ABC Kit (VectorLabs).

Statistical Analysis

The chi-square test and the Fisher's Exact Test were performed to compare the incidence of the different types of tumors in the two genotypes analyzed, whereas the Student's t test was used to compare weights and activities measurements.

Acknowledgements

We thank the Tissue Bank of the University of Dundee for histological preparation and George Thompson for help with immunohistochemistry analysis, Leah Ronaldson and Gail Fraser for assistance with genotyping of mice and the antibody purification team (Division of Signal Transduction Therapy, University of Dundee) coordinated by Hilary McLauchlan and James Hastie for generation and purification of antibodies. A long-term fellowship from EMBO as well as a Marie Curie Fellowship supported J.R.B. We thank the

Philip Leverhulme Trust Prize as well as the Association for International Cancer Research (D.R.A.), Diabetes UK (D.R.A.) the Medical Research Council (D.R.A.), the Moffat Charitable Trust (D.R.A.), and the pharmaceutical companies supporting the Division of Signal Transduction Therapy (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck & Co., Inc, Merck KGaA, and Pfizer) for financial support.

Received: June 21, 2005
Revised: August 27, 2005
Accepted: August 30, 2005
Published: October 25, 2005

References

1. Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.D. (2001). Synthesis and Function of 3-Phosphorylated Inositol Lipids. Annu. Rev. Biochem. *70*, 535–602.
2. Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655–1657.
3. Leslie, N.R., and Downes, C.P. (2004). PTEN function: how normal cells control it and tumour cells lose it. Biochem. J. *382*, 1–11.
4. Sulis, M.L., and Parsons, R. (2003). PTEN: from pathology to biology. Trends Cell Biol. *13*, 478–483.
5. Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P., and Tonks, N.K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc. Natl. Acad. Sci. USA *95*, 13513–13518.
6. Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375–13378.
7. Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29–39.
8. Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G., and Stokoe, D. (1998). Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr. Biol. *8*, 1195–1198.
9. Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., et al. (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr. Biol. *8*, 1169–1178.
10. Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P.P. (1998). Pten is essential for embryonic development and tumour suppression. Nat. Genet. *19*, 348–355.
11. Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc. Natl. Acad. Sci. USA *96*, 1563–1568.
12. Stiles, B., Gilman, V., Khanzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and Wu, H. (2002). Essential role of AKT-1/protein kinase B alpha in PTEN-controlled tumorigenesis. Mol. Cell. Biol. *22*, 3842–3851.
13. Neshat, M.S., Mellinghoff, I.K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J.J., Wu, H., and Sawyers, C.L. (2001). Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc. Natl. Acad. Sci. USA *98*, 10314–10319.
14. Mora, A., Komander, D., Van Aalten, D.M., and Alessi, D.R. (2004). PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. *15*, 161–170.
15. Lawlor, M.A., Mora, A., Ashby, P.R., Williams, M.R., Murray-Tait, V., Malone, L., Prescott, A.R., Lucocq, J.M., and Alessi, D.R. (2002). Essential role of PDK1 in regulating cell size and development in mice. EMBO J. *21*, 3728–3738.
16. Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., et al. (2003). Pten dose dictates cancer progression in the prostate. PLoS Biol. *1*, e59. 10.1371/journal.pbio.0000059.
17. Di Cristofano, A., Kotsi, P., Peng, Y.F., Cordon-Cardo, C., Elkon, K.B., and Pandolfi, P.P. (1999). Impaired Fas response and autoimmunity in Pten+/– mice. Science *285*, 2122–2125.
18. Accili, D., and Arden, K.C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell *117*, 421–426.
19. Dufner, A., and Thomas, G. (1999). Ribosomal S6 kinase signaling and the control of translation. Exp. Cell Res. *253*, 100–109.
20. Constancia, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C., et al. (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature *417*, 945–948.
21. Feldman, R.I., Wu, J.M., Polokoff, M.A., Kochanny, M.J., Dinter, H., Zhu, D., Biroc, S.L., Alicke, B., Bryant, J., Yuan, S., et al. (2005). Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. J. Biol. Chem. *280*, 19867–19874.
22. Sato, S., Fujita, N., and Tsuruo, T. (2002). Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine). Oncogene *21*, 1727–1738.
23. Komander, D., Kular, G.S., Bain, J., Elliott, M., Alessi, D.R., and Van Aalten, D.M. (2003). Structural basis for UCN-01 specificity and PDK1 inhibition. Biochem. J. *375*, 255–262.
24. Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S., and Hall, A. (2004). Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. Science *303*, 1179–1181.
25. Leslie, N.R., Bennett, D., Lindsay, Y.E., Stewart, H., Gray, A., and Downes, C.P. (2003). Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J. *22*, 5501–5510.