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Important role of the LKB1-AMPK pathway in suppressing tumourigenesis in PTEN deficient mice

By

Xu Huang¹, Stephan Wullschleger¹, Natalia Shpiro¹, Victoria A McGuire², Kei Sakamoto¹, Yvonne L Woods³, Wendy McBurnie³, Stewart Fleming³ and Dario R Alessi¹.

1. MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.

2. College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.

3. Department of Molecular Pathology, University of Dundee, Ninewells Hospital, Dundee DD1 9SY, UK.

Running Title: AMPK activators suppress tumourigenesis.

Correspondence XH (x.huang@dundee.ac.uk) or DRA (d.r.alessi@dundee.ac.uk).

Abstract. The LKB1 tumour suppressor phosphorylates and activates AMPK when cellular energy levels are low, thereby suppressing growth through multiple pathways, including inhibiting the mTORC1 kinase that is activated in the majority of human cancers. Blood glucose lowering type-2 diabetes drugs also induce LKB1 to activate AMPK, indicating that these compounds could be used to suppress growth of tumour cells. In this study we investigated the importance of the LKB1-AMPK pathway in regulating tumourigenesis in mice resulting from deficiency of the PTEN tumour suppressor, which drives cell growth through over-activation of the Akt and mTOR kinases. We demonstrate that inhibition of AMPK resulting from a hypomorphic mutation that decreases LKB1 expression does not lead to tumourigenesis on its own, but markedly accelerated tumour development in PTEN^{+/-} mice. In contrast, activating the AMPK pathway by administration of PTEN^{+/-} mice metformin, phenformin or A-769662, significantly delayed tumour onset. We demonstrate that LKB1 is required for activators of AMPK to inhibit mTORC1 signalling as well as cell growth in PTEN deficient cells. Our findings highlight in an animal model relevant to understanding human cancer, the vital role that the LKB1-AMPK pathway plays in suppressing tumourigenesis resulting from loss of PTEN tumour suppressor. They also suggest that pharmacological inhibition of LKB1 and/or AMPK would be undesirable, at least for the treatment of cancers in which the mTORC1-pathway is activated. Most importantly our data demonstrate the potential of AMPK activators such as clinically approved metformin as anti-cancer agents, which will suppress tumour development by triggering a physiological signalling pathway that potently inhibits cell growth.

Introduction.

The majority of cancers possess mutations that stimulate proliferation, growth and survival by activating the PI 3-kinase-Akt-mTOR signalling pathways [1, 2]. A frequent mutation leading to the activation of this pathway, results in loss of the PTEN lipid phosphatase, which breaks down the phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) second messenger formed following activation of PI 3-kinase [3, 4]. Heterozygous PTEN^{+/-} mice spontaneously develop a variety of tumours [5-11] and tumorigenesis in these mice is suppressed by reducing expression of PDK1 an upstream activator of Akt [12] or by deletion of the Akt1 isoform [13]. Akt drives cell proliferation, at least in part, through its ability to activate mTOR complex-1 (mTORC1), via phosphorylation of tuberous sclerosis complex-2 (TSC2) and PRAS40 (reviewed in [14, 15]). Proliferation and growth of PTEN-deficient tumours is inhibited by treatment with the mTORC1 inhibitor rapamycin or by knock-down of Raptor, a regulatory subunit of mTORC1 [16, 17]. Furthermore, overexpression of the Rheb GTPase, an activator of mTORC1 stimulates growth and proliferation of Akt1/Akt2 deficient cells [17].

There has been much interest in dissecting the role of other pathways that modulate mTOR signalling. One of these comprises the AMP-activated protein kinase (AMPK), that is phosphorylated and activated by the LKB1 tumour suppressor protein kinase when cellular energy levels are low [18, 19]. Under these conditions 5'-AMP levels are elevated and bind to AMPK, inducing a conformational change. This protects AMPK from dephosphorylation by an as yet unidentified phosphatase, thereby promoting its phosphorylation and activation by LKB1 [20, 21]. Once activated, AMPK functions to restore energy levels by phosphorylating key cellular substrates leading to the stimulation of ATP generating pathways. Activation of AMPK also inhibits energy consuming processes such as protein synthesis and growth. These effects are mediated in part through inhibition of mTORC1 resulting from phosphorylation of TSC2 by AMPK [22].

Pathways controlled by mTORC1 are activated in cells and tumours possessing reduced AMPK activity as a result of loss of LKB1 [2, 23]. Moreover, low energy stress or oral hypoglycaemic anti-type 2 diabetes drugs, such as metformin or phenformin that activate AMPK, fail to inactivate mTORC1 or inhibit cell growth and proliferation in LKB1-deficient cells [2, 23, 24]. In addition, humans lacking one allele of the LKB1 gene develop Peutz-Jeghers cancer syndrome, that bears phenotypic resemblance to other conditions resulting from inappropriate activation of mTORC1, including Cowden's syndrome (loss of PTEN) or tuberous sclerosis (loss of TSC1 or TSC2) [25, 26]. LKB1 is also mutated in certain sporadic human cancers and with particularly high frequency in lung cancer [27, 28].

These findings suggest that inhibition of the LKB1-AMPK signalling axis in cancer is undesirable, as it would be expected to promote tumourigenesis. However, it has also been reported that LKB1-deficient fibroblasts undergo a greater degree of cell death under energy deprived conditions [2]. Thus cancer cells lacking the ability to control their energy levels through the AMPK pathway may be more susceptible to stresses that lower energy levels. Furthermore, inhibition of AMPK has recently been shown to markedly sensitize a variety of cancer cells to cisplatin, a chemotherapeutic agent [29]. Thus inhibition of the LKB1-AMPK kinase pathway might actually be beneficial for the treatment of cancer, promoting apoptosis of cancer cells by interfering with their ability to manage energy stress and by increasing the effectiveness of cytotoxic compounds.

In this study we explore the effects of lowering the expression of LKB1 and stimulating AMPK on tumour development in PTEN^{+/-} mice. Our findings demonstrate the crucial role that the LKB1-AMPK signalling network plays in controlling the tumourigenesis that results from the loss of PTEN. The data suggest that the use of clinically approved AMPK activators, such as metformin may be useful for the treatment or prevention of cancer in humans. Our observations also demonstrate that inhibiting LKB1-dependent pathways for the treatment of tumours in which the mTOR pathway is activated is highly undesirable.

Experimental Procedures

Materials. Protease-inhibitor cocktail tablets, Taq Polymerase and precast SDS polyacrylamide Bis-Tris gels were from Invitrogen; Protein G-Sepharose, $\gamma^{32}\text{P}$ -ATP were purchased from Amersham Bioscience; phosphocellulose P81 paper was from Whatman; Triton X-100, dimethyl pimelimidate were from Sigma. 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) from Toronto Research Chemicals Inc; Phenformin hydrochloride and 1,1-Dimethyl-biguanid Hydrochlorid (Metformin) were from Sigma- Aldrich; A-769662 was synthesized as described previously [30].

Antibodies. Anti-AMPK α 1 was raised in sheep against the peptide (CTSPDSDLDDHHLTR, residues 344-358 of rat AMPK α 1), anti-LKB1 for immunoprecipitation and immunoblotting was raised in sheep against the peptide (TFIHRIDSTEVYQPR, residues 24-39 of mouse LKB1). The anti-LKB1 (#3050) for immunohistochemistry, phospho-AMPK α Thr172 (#2535), phospho-acetyl-CoA carboxylase (ACC) Ser79 (#3662), pan-ACC (#3661), S6 ribosomal protein (#2212), phospho-S6 ribosomal protein Ser235 (#2211), S6K1 (#9202), phospho-S6K1 Thr389 (#9205), phospho-Akt Thr308 (#9275), phospho-Akt Ser473 (#9271), FOXO-1 (#9462), and PTEN (#9559) were purchased from Cell Signalling Technology. The anti-Akt1 (#05-591) was from Upstate. The anti-Ki67 (#VP-K452) was from Vector Labs. Secondary antibodies coupled to horseradish peroxidase were from Pierce.

Mice Breeding, Genotype Analysis and Tumour Analysis. All animal studies were approved by the University of Dundee Ethics Committee and performed under a UK Home Office project licence. The generation and genotyping of the PTEN $^{+/-}$ mice [7] and the LKB1 hypomorphic mice [31] have been described previously. The parental LKB1 $^{fl/+}$ and PTEN $^{+/-}$ used for these experiments had been backcrossed with C57BL/6J for over 7 generations prior to initiating the crosses for this study. Littermates with different genotypes of LKB1 and PTEN were derived as described in Figure 1A and maintained under standard husbandry conditions for a period of up to 14 months of age. During this period mice were monitored weekly for tumour development. According to our UK Home Office licence, any animal that displayed an obvious external tumour of over 1.44cm 2 or showed signs of sickness was culled and subjected to necropsy and pathological analysis after tissue fixation in 10% formalin as described previously [12].

Immunohistochemistry Analysis. Tumour slices were generated as described previously [12]. Immunohistochemical staining was performed with automated procedures at the University of Dundee Tissue Bank at Ninewells Hospital. The antibodies used in the staining as described above. Antibody staining was detected using the rabbit Vectastain ABC kit (VectorLabs). Sections were viewed on a Nikon Eclipse 600 microscope and images captured on a Nikon DXM1200 digital camera with EclipseNet software.

Assay of LKB1 and AMPK. The LKB1 and AMPK activities were assayed as described previously [31]. In brief, 500 μg of different mouse tissues and ES cell lysates were used to immunoprecipitate LKB1 and 50 μg of lysates were used for AMPK α 1 immunoprecipitation. Kinase activities were measured employing the LKBtide peptide [32] for LKB1 or AMARA peptide for AMPK α 1 [33]. One milli-Unit (mU) of activity was defined as that which catalysed the incorporation of 1pmol of ^{32}P into the substrate per min.

Generation of LKB1-PTEN knockout ES cells. Female LKB1 $^{fl/+}$ PTEN $^{+/-}$ mice were induced to superovulate by the injection of PMSG. This was followed 48 hours later by the injection of HCG. These mice were then mated with male LKB1 $^{fl/+}$ PTEN $^{+/-}$ mice. Blastocysts were removed at 2.5 days post coitus (dpc) and cultured on 24-well plates on a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts for 1-2 weeks to allow the ES cells to grow. Wells were trypsinised and 80% of the aliquot frozen in two batches while the remaining 20% was used to grow cells for DNA preparation. Cells were genotyped by PCR for PTEN [7] or LKB1 [31]. In order to generate LKB1 knock-out ES cell lines, LKB1 $^{fl/fl}$ PTEN $^{-/-}$, LKB1 $^{fl/fl}$ PTEN $^{+/+}$ ES cells obtained as above were subjected to Cre-mediated deletion to remove the floxed LKB1 allele as described previously [34]. After transfection by

electroporation with 30 μ g pMC-CrePuro, the cells were seeded onto feeder layers under puromycin (1 μ g/ml) selection and colonies were picked in duplicate into 96-well tissue culture plates. One of each selected colonies was subjected to G418 selection (0.2mg/ml). The sensitive colonies were expanded and analyzed by PCR and immunoblotting to confirm the deletion of LKB1 alleles. LKB1^{+/+}PTEN^{-/-} ES cell line, was generously provided to us by Dr. Pier P. Pandolfi (Harvard medical school) [6].

Cell culture, stimulation and cell lysis. LKB1^{+/+}PTEN^{+/+}, LKB1^{+/+}PTEN^{-/-}, LKB1^{-/-}PTEN^{-/-}, LKB1^{-/-}PTEN^{-/-} ES cells were grown on gelatinized tissue culture plastic in DMEM (Sigma, #D6046) containing 10% Fetal Bovine Serum (Hyclone, #SH300710.02) supplemented with 1000mg/L glucose, 0.1 mM non-essential amino acids, antibiotics (100 units penicillin G, 100 μ g/ml streptomycin), antimycotic (1 μ g/ml Ciproxin Infusion), 2 mM L-glutamine, 0.1 mM β -mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor. The ES cells were cultured to 80% confluence on 10 cm diameter dishes and then stimulated with the indicated agonists as described in the figure legends. The cells were lysed in 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and 'Complete' proteinase inhibitor cocktail (one tablet per 50 ml)) and centrifuged at 4°C for 15 minutes at 13,000 rpm. The supernatants were aliquoted, frozen in liquid nitrogen, and stored at -80°C until use. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

Cell growth assay. ES cell lines were seeded in 12 wells plates (30,000 cells per well) in DMEM containing 10% FBS in the presence or absence metformin or phenformin. The cell medium was changed every 24 hours with freshly dissolved metformin or phenformin added. After 72 hours, the cells were washed once with 1 \times Phosphate Buffered Saline (PBS), and fixed in 4% (v/v) paraformaldehyde in PBS for 15 min. After washing once with water, the cells were stained with 0.1% crystal violet in 10% ethanol for 20 min and washed three times with water. Crystal violet was extracted from cells with 0.5 ml of 10% (v/v) acetic acid for 20 min. The eluate was then diluted 1:10 in water and absorbance at 590 nm quantitated.

Administration of mice with AMPK activators. The indicated strains of mice were administered with different AMPK activators in their drinking water (metformin 300mg/kg/day, phenformin 300mg/kg/day, A-769662 30mg/kg/day). Drugs were dissolved from powder freshly every day into the drinking water taking into account the mouse body weight and the volume of water the animal consumes each day. No other water was available for the mice to drink and the volume of water consumed by each mouse per day was noted and used to calculate the dose of drugs required. A-769662 was dissolved in DMSO to 1 M, prior to diluting into drinking water. The LKB1^{fl/+} PTEN^{+/-} mice were maintained under the standard husbandry conditions described above and monitored weekly for tumour development as well as body weight and food intake. Blood glucose levels were measured using the Ascensia Breeze blood glucose meter (Bayer), blood lactic acid using the Accutrend Lactate Meter (Roche) and serum insulin using an ELISA Kit (Crystal Chem).

Results.

Generation of LKB1 hypomorphic PTEN^{+/-} mice. To investigate how suppression of LKB1 pathway affected tumourigenesis, we crossed PTEN^{+/-} mice which spontaneously develop tumours in multiple tissues after ~8 months of age [5-11], with LKB1^{fl/fl} hypomorphic mice in which the expression of LKB1 is reduced 5-10-fold in most tissues analysed [31]. Employing crosses described in Figure 1A, we generated littermate strains of mice expressing normal levels of LKB1 and PTEN (LKB1^{+/+}PTEN^{+/+}), reduced LKB1-normal PTEN (LKB1^{fl/fl}PTEN^{+/+}), normal LKB1-deficient PTEN (LKB1^{+/+}PTEN^{+/-}), moderately reduced LKB1-deficient PTEN (LKB1^{fl/+}PTEN^{+/-}) and reduced LKB1-deficient PTEN (LKB1^{fl/fl}PTEN^{+/-}). All mouse strains were of normal size and apart from the LKB1^{fl/fl} PTEN^{+/-} mice that were born at ~50% of the expected number, the other mouse strains were recovered in the expected Mendelian frequency (Fig 1B Cross 2). In the PTEN^{+/-} mice, the level of the PTEN protein in liver (Fig 1C), heart, spleen and intestine (Supplementary Fig 1A) was about half that observed in tissues derived from PTEN^{+/+} mice. The LKB1^{fl/fl} mice had markedly reduced levels of LKB1 protein and activity in all tissues examined (Fig 1C and Supplementary Fig 1A). In tissues derived from LKB1^{fl/+} mice, intermediate levels of LKB1 protein and activity were observed. In the LKB1^{fl/fl} hypomorphic mice, AMPK activity and its phosphorylation at the residue phosphorylated by LKB1 (Thr172), as well as phosphorylation of the AMPK substrate acetyl-CoA-carboxylase (ACC), were markedly reduced in liver (Fig 1D), spleen and intestine (Supplementary Fig 1B), establishing that reduction of LKB1 expression resulted in inhibition of AMPK activity in vivo. Consistent with the decreased AMPK activity, LKB1^{fl/fl} hypomorphic mice displayed increased mTORC1 activity, assessed by enhanced phosphorylation of S6K1 at Thr389 and the ribosomal S6 protein at Ser235 (Fig 1D and Supplementary Fig 1B). The LKB1^{fl/+} mice with only moderately reduced LKB1 protein, possessed levels of AMPK activity and phosphorylation of ACC similar to wild type mice (Fig 1D and Supplementary Fig 1B).

Analysis of tumour formation. Groups of ~30 mice of each genotype were monitored for tumours and culled when they exhibited large externally palpable tumours, reduced body weight, became unwell or reached 14 months of age. A necropsy was performed, the tissues fixed in 10% formalin and subjected to detailed histopathological examination (Table 1). By 14 months of age, the LKB1 hypomorphic mice not deficient in PTEN (LKB1^{fl/fl}PTEN^{+/+}), displayed no significant tumours (Fig 2A and Table 1). Therefore, reduction in LKB1 expression alone is not sufficient to induce tumourigenesis. Consistent with previous work [5-11], tumour development in LKB1^{+/+}PTEN^{+/-} mice was observed at ~7 months of age and by 14 months ~70% had one or more tumours. Strikingly, PTEN^{+/-} mice with moderately reduced LKB1 expression (LKB1^{fl/+}PTEN^{+/-}), developed tumours earlier, commencing at 4 months and by 9 months ~70% of mice developed tumours (Fig 2A). Consistent with reduction of LKB1 levels accelerating tumourigenesis in PTEN^{+/-} mice, animals with the most pronounced reduction of LKB1 expression (LKB1^{fl/fl}PTEN^{+/-}), developed tumours at 3 months and by 6 months ~70% of animals displayed tumours (Fig 2A).

Pathological analysis indicated that the types and morphology of tumours arising in PTEN^{+/-} mice with normal and reduced levels of LKB1 were broadly similar (Table 1). Representative histopathological sections of these tumours are shown in Figure 2B. The tumours comprised lymphoma, intestinal polyps, prostate carcinoma, breast adenocarcinoma, pheochromocytoma and prostatic intraepithelial neoplasia (Fig 2B). The proportion of mice displaying lymphoma, intestinal polyps, pheochromocytoma, and prostate carcinoma were moderately (< 2-fold) higher in the LKB1 hypomorphic PTEN^{+/-} mice compared to the PTEN^{+/-} mice expressing normal levels of LKB1 (Table 1). The intestinal polyps were found in both the small and large intestine, but were more numerous in the small intestine. In some animals single polyps were observed, but in others multiple polyps, up to 10, were found. They ranged in size from small pedunculated polyps projecting into but not obstructing the lumen and measuring less than one millimetre in diameter to very large extensive

sessile growths occupying more than one centimetre of bowel wall and obstructing the lumen (Fig 2B). The larger and more numerous polyps were seen in animals with LKB1^{fl/fl}PTEN^{+/-} genotype. Architecturally the polyps were biphasic complex growths with distorted glands and a cellular stroma (Fig 2B & Supplementary Fig 2). The gland epithelium was composed of crypts, absorptive epithelium mostly on the surface, numerous mucin-secreting cells and Paneth cells. There was no significant atypia and the mitotic figures were mostly within the crypt like population. These surface epithelial cells but not the stromal component stained with a broad-spectrum cytokeratin antibody. The stromal component had the morphology of smooth muscle, with spindle shaped cells with abundant eosinophilic cytoplasm and cigar shaped nuclei lying within a loose extracellular matrix. This stromal component stained in immunocytochemistry experiments with an antibody to the alpha-smooth muscle isoform of actin and the intermediate filament desmin confirming the smooth muscle phenotype. As with the epithelium there was no atypia and mitoses were infrequent. 15-30% of LKB1^{+/+}PTEN^{+/-} and LKB1^{fl/fl}PTEN^{+/-} animals also developed a number of minor pre-neoplastic lesions the most common being prostatic intraepithelial neoplasia and intratubular germ cell neoplasia (Table 1).

We examined intestinal polyps from the LKB1^{+/+}PTEN^{+/-} and LKB1^{fl/fl}PTEN^{+/-} animals and found that in polyps of both genotypes, FOXO1 was predominantly in the cytosol (Fig 2C), indicating that Akt was activated. This was confirmed by the finding of high levels of phosphorylation of Akt at Thr308 and phosphorylation of the S6 protein at Ser235 predominantly in the epithelial cells of the polyps from both genotypes (Fig 2C). Comparable levels of Ki67 staining were also seen in the epithelium and smooth muscle cells of the polyps from LKB1^{+/+}PTEN^{+/-} and LKB1^{fl/fl}PTEN^{+/-} animals. The staining with Ki67 showed regional variation in the epithelium being most marked in the crypts and sparse on the surface epithelium. The staining of the smooth muscle cells was more randomly distributed. Analogous analysis was undertaken for the lymphoma and pheochromocytoma and no significant differences in the staining patterns were detected between the tumours arising in the control and experimental mice (data not shown).

Role of the LKB1-AMPK pathway in modulating mTOR signalling and growth of ES cells. We next explored the role that the LKB1-AMPK pathway plays in regulating Akt and mTOR utilising single LKB1^{+/+}PTEN^{-/-} or LKB1^{-/-}PTEN^{+/-} as well as double LKB1^{-/-}PTEN^{-/-} knockout embryonic stem cells, generated as described in the Experimental Procedures. The PTEN-deficient (LKB1^{+/+}PTEN^{-/-} and LKB1^{-/-}PTEN^{-/-}) ES cells displayed elevated levels of phosphorylation of Akt and mTORC1 activity as judged by increased phosphorylation of S6K1 and S6 protein (Fig 3A). LKB1^{-/-}PTEN^{+/-} cells possessed low levels of Akt phosphorylation, but elevated levels of phosphorylation of S6K at Thr389, similar to those observed in LKB1^{+/+}PTEN^{-/-} deficient cells (Fig 3A). We also investigated how treatment of these ES cells with activators of AMPK affected Akt and mTORC1. Treatment of wild type LKB1^{+/+}PTEN^{+/+} or LKB1^{+/+}PTEN^{-/-} cells with the AMP mimetic AICAR or either of the anti-type-2 diabetes drugs metformin or phenformin, stimulated phosphorylation of AMPK as well as ACC. There was also decreased phosphorylation of S6K1 as well as its substrate S6 protein suggestive of inhibition of mTORC1 (Fig 3B). In contrast, treatment of LKB1-deficient (LKB1^{-/-}PTEN^{+/+} or LKB1^{-/-}PTEN^{-/-}) cells with AMPK activators, failed to induce phosphorylation of AMPK or ACC or inhibit phosphorylation of S6K1 or S6 protein, indicating that in LKB1-deficient cells AMPK was not activated, nor was mTORC1 inhibited (Fig 3B). Treatment of ES cells with AMPK activators did not inhibit phosphorylation of Akt at Thr308 (Fig 3B).

We also analysed the effect of metformin and phenformin on the growth of ES cell lines. Consistent with studies performed in other cell lines [24], the growth of wild type LKB1^{+/+}PTEN^{+/+} as well as LKB1^{+/+}PTEN^{-/-} ES cells was significantly inhibited in a dose-dependent manner by metformin (Fig 3C) or phenformin (Fig 3D). In contrast, the growth of LKB1-deficient LKB1^{-/-}PTEN^{+/+} or LKB1^{-/-}PTEN^{-/-} ES cells, was inhibited to a markedly lower extent by metformin or phenformin (Fig 3C & 3D).

These observations support the notion that LKB1 is required for the growth suppressing effects of metformin or phenformin.

Effect of AMPK activating drugs on PTEN-tumourigenesis. The finding that reduction in LKB1 expression accelerated tumour development in PTEN^{+/-} animals and that AMPK is required to inhibit mTORC1 and cell growth in PTEN-deficient cells, led us to hypothesize that activation of AMPK would suppress tumourigenesis in mice. To test this, we studied how administration of PTEN^{+/-} mice with AMPK activating agents affected tumour development. We utilised metformin or phenformin that activate AMPK indirectly, most likely by decreasing ATP regeneration through the inhibition of mitochondrial respiration (reviewed [18, 19]). We also used a recently formulated compound A-769662, that activates AMPK both allosterically and by inhibiting dephosphorylation of AMPK at Thr172, the residue phosphorylated by LKB1 [20, 35, 36]. Control experiments indicated that mice administered any of these drugs over a two-week period, possessed significantly elevated levels of AMPK activity/phosphorylation and ACC phosphorylation in liver (Fig 4A), spleen (Fig 4B) and intestine (Fig 4C). A-769662 stimulated AMPK activity to a similar extent in all tissues as observed following an intraperitoneal injection of the AMP mimetic drug AICAR. Phenformin and metformin also enhanced AMPK activity in the liver, but were less potent than A-769662 or AICAR at stimulating AMPK in the spleen or intestine (Fig 4).

To investigate the effects of AMPK activators on tumour development, we utilised LKB1^{fl/+}PTEN^{+/-} animals, as these develop tumours within 4 months. Control studies revealed that administering LKB1^{fl/+} mice with phenformin, metformin or A-769662, induced similar level of AMPK activation as observed in LKB1^{+/+} mouse tissues (Fig 4). We also found that AMPK activators induced similar activation of AMPK in LKB1^{fl/+} and LKB1^{+/+} ES cells (supplementary Fig 3). Thus the slight reduction in LKB1 levels in LKB1^{fl/+} animals does not significantly interfere with the ability of phenformin, metformin or A-769662 to stimulate AMPK. Groups of ten LKB1^{fl/+}PTEN^{+/-} mice from 4 weeks of age were given either no drug or prescribed one of the AMPK activating drugs. The mice tolerated long-term administration of AMPK activating compounds, and their body weight, levels of blood glucose, insulin, lactate, and food intake were not affected significantly (Supplementary Fig 4). In the control group given no drug, tumour formation commenced as expected by 4 months of age and 100% of mice developed tumours by 8 months (Fig 5A). Strikingly, tumour formation was markedly delayed in all mice administered one of the AMPK activators. By 6 months of age none of the animals on phenformin or A-769662 had developed tumours at a stage when 60% of the control animals had tumours. Development of tumours in mice treated with phenformin and A-769662 started at 7 months of age. By 10 months of age 60% of mice on phenformin and 80% on A-769662 possessed tumours. After 12 months, 20% of animals treated with phenformin or 10% with A-769662 displayed no tumours (Fig 5A). Consistent with metformin being a weaker activator of AMPK than phenformin or A-769662, we observed that metformin was less effective at inhibiting tumourigenesis. For mice prescribed metformin, tumour development commenced at 5 months, with all animals developing tumours by 12 months. We have also repeated this study with another set of ten LKB1^{fl/+}PTEN^{+/-} mice for each experimental condition. These mice are currently 10 months old, and the data obtained so far is similar, with all the control mice having developed tumours and tumour development being markedly delayed in mice administered AMPK activators. Pathological investigation of the tumours indicated that the types and morphology of tumours arising in animals administered AMPK activators were similar to those observed in control animals not given a drug (Table 2). Representative histopathological sections of intestinal polyps and lymphoma and prostatic intraepithelial neoplasia are shown in Figure 5B.

Discussion.

Previous work has shown that heterozygous LKB1^{+/-} mice develop intestinal polyps resembling tumours seen in humans with Peutz Jeghers syndrome. However, it has been controversial whether the tumours resulted from LKB1 haploinsufficiency or loss of heterozygosity (reviewed in [26]). Interestingly, hypomorphic LKB1^{fl/fl}PTEN^{+/+} mice do not develop tumours, despite expressing lower levels of LKB1 in all tissues, than the tumour-developing LKB1^{+/-} animals. This suggests that loss of heterozygosity rather than haploinsufficiency accounts for the tumour formation observed in LKB1^{+/-} mice. It is possible that complete loss of cellular LKB1 and hence AMPK activity is required to induce tumourigenesis through the mTORC1 pathway. In contrast, the partial AMPK activity observed in LKB1^{fl/fl} mice may be sufficient to inhibit mTORC1 driven tumourigenesis. This would explain why the activity of mTORC1 pathway is not markedly elevated in the liver, spleen and intestine tissues of LKB1^{fl/fl} mice (Fig 1D & Supplementary Fig 1B), compared to previous work undertaken in LKB1^{-/-} deficient cell lines and tissues [2, 37]. It is also possible that loss of LKB1 alone without mutations in other oncogenes or tumour suppressors is insufficient to drive tumour formation. This might explain why in human cancer, loss of LKB1 is rarely found alone and frequently occurs in conjunction with mutations in other oncogenes such as Ras [28, 38]. Thus the LKB1-AMPK pathway might operate to modulate and suppress inappropriately high mTORC1 activity, rather than acting as a direct stimulator of mTORC1 in the absence of any other activating stimulus.

The acceleration of tumourigenesis in LKB1 hypomorphic PTEN^{+/-} mice provides a genetic demonstration of the cooperation between the LKB1 and PTEN pathways in a mouse model of cancer. Our data indicate that the rate and extent of tumour development in PTEN^{+/-} mice correlates with the level of LKB1 expression. Even LKB1^{fl/+} PTEN^{+/-} mice that only have a moderate ~40% decrease in LKB1 expression, develop tumours significantly earlier than the LKB1^{+/+}PTEN^{+/-} animals (Fig 2A). As mentioned previously, others have postulated that inhibition of LKB1 or AMPK might actually be therapeutically advantageous for the treatment of cancer in humans, as suppression of this pathway might reduce the ability of tumour cells to tolerate and survive low energy and/or chemotherapy [2, 29]. However our findings indicate that long-term inhibition of the LKB1-AMPK pathway would not be desirable at least for the treatment of tumours that possess elevated PI 3-kinase-mTOR pathway signalling. Moreover, we would recommend that protein kinase inhibitors being developed for cancer treatment are counter-screened to ensure that they do not inhibit the LKB1-AMPK signalling pathway. As LKB1 phosphorylates and activates other protein kinases related to AMPK [32, 39], we cannot rule out the possibility that reduction in the activity of one or more of these enzymes contributes to activation of mTORC1 and/or acceleration of tumourigenesis in LKB1 hypomorphic PTEN^{+/-} mice. The role of AMPK-related kinases in controlling mTOR has yet to be investigated, although metformin and phenformin do not stimulate the activity of AMPK-related kinases [32, 40]. A recent report demonstrates that heterozygous LKB1^{+/-} mice are markedly sensitised to the chemical carcinogen 7,12-dimethylbenz(α)anthracene although this effect was attributed to an AMPK-mTOR independent pathway that has yet to be characterised [41]. It would be interesting to investigate whether this was mediated through one or more of the AMPK-related kinases.

It is also likely that AMPK suppresses cell growth by phosphorylating substrates other than mTORC1 regulators. One study suggests that AMPK inhibits cell-cycle progression by controlling the phosphorylation and stability of p27(kip), a cell cycle inhibitor [42]. However, whether AMPK directly phosphorylates p27(kip) has yet to be established. The stabilisation of p27(kip) was postulated to enable cancer cells to better survive conditions of nutrient and energy stress [42]. In a similar vein, AMPK was claimed to enhance survival of hypoxic cancer cells by stabilising the hypoxia-inducible factor-1α [43]. Taken together, these findings indicate that in addition to suppressing proliferation and growth, activation of the AMPK pathway may also facilitate the survival of some tumour-cells. If this were the case, AMPK

activators when used in cancer therapy might have more profound tumour regression effects if utilised in combination with apoptosis-inducing therapies. Other potential AMPK anti-cancer targets include p53, which supposedly following phosphorylation by AMPK, induces apoptotic cell death rather than enhancing survival [44, 45]. Recent work also demonstrates that AMPK regulates epithelial cell polarity in part by controlling the assembly and disassembly of tight junctions [46, 47]. The ability of AMPK to maintain cell polarity under situations of energy stress is likely to also contribute to its anti-tumour effects. Loss of LKB1 in the pancreas has also been demonstrated to lead to marked disorganisation of acinar polarity as well as cytoskeletal and tight junction defects [48]. Whether this is mediated through AMPK and/or AMPK-related kinases is unknown. Much more work is required to delineate the diverse effects of activating AMPK activity in cancer cells and to define the substrates that AMPK phosphorylates to regulate growth, proliferation, survival and polarity.

Loss of function mutations in LKB1 are particularly common in human lung cancers [27, 28]. Consistent with observations of previous studies [5-11], we found that PTEN^{+/-} mice whether hypomorphic for LKB1 or not, failed to develop lung cancer. In human lung cancer, loss of LKB1 is frequently associated with gain of function mutations in Ras [27, 28]. This indicates that lung tumour formation may be driven by cooperation between the LKB1/mTOR and Ras pathways. It has also been proposed that exposure of lung cells to inhaled carcinogens plays a role in promoting tumour development in LKB1-deficient lung tissue [41]. Further work is required to understand why mutations of LKB1 are so prevalent in lung cancer and the role that Ras and/or carcinogens play in promoting transformation of LKB1-deficient lung cells. It would also be interesting to investigate how modulating the levels of LKB1 expression or activating AMPK affects lung cancer as well as other tumours harbouring activating mutations of Ras. Mutation of LKB1 in lung cancers was also reported to enhance metastasis [28], however, we found no increased metastasis of tumours between the PTEN^{+/-} and normal or hypomorphic LKB1 mice.

Before the discovery that the LKB1 tumour suppressor activated AMPK, there was little interest in the role of AMPK in cancer. However, the ability of AMPK to gauge and control cellular energy places it in an ideal position to ensure that cell growth and proliferation is coupled to the availability of a sufficient supply of nutrients and energy. The finding that three distinct drugs that activate AMPK delayed tumourigenesis in PTEN^{+/-} mice, suggests that activators of AMPK could have therapeutic benefit for the treatment of cancer in humans. Metformin has been used as a frontline therapy for type 2 diabetes for over 50 years and is inexpensive and well tolerated in humans. Phenformin is a more potent activator of AMPK than metformin and is also more effective at reducing blood glucose levels in diabetic patients, but was withdrawn from clinical use in the USA in 1977, as it caused lactic acidosis in some patients [49]. For the treatment of some cancers, side effects such as lactic acidosis may be more acceptable than for a chronic disease like diabetes, and the reintroduction of phenformin for cancer therapy should be explored. It may also be possible to initiate research to define the patients who are at risk of developing phenformin-induced lactic acidosis. Metformin and phenformin both activate AMPK indirectly, probably by decreasing cellular energy levels by inhibiting mitochondrial respiration (reviewed [18, 19]). Since the discovery that metformin exerts its blood glucose lowering effects by activating AMPK, pharmaceutical companies have devoted significant resources to develop drugs that directly activate AMPK for the treatment of type 2 diabetes. The discovery of A-769662 was therefore a landmark finding [35], as it demonstrated that the development of such activators was feasible. The recent elucidation of the 3 dimensional structure of AMPK subunits [21, 50], coupled with further structural analysis to dissect the mechanism by which A-769662 interacts with AMPK, should accelerate the discovery of novel allosteric activators AMPK. The present study suggests that these compounds should not only be assessed for their anti-type 2 diabetes properties, but also for their ability to suppress and treat human cancer. A small-scale epidemiology, population-based case-control study was carried out to address whether diabetics prescribed metformin were protected from

developing cancer suggested that those taking metformin were 23% less likely to get cancer with the reduction in risk rising to 40% for people who had been administered the drug for a longer period of time [51]. In future work it would also be of considerable interest to investigate whether AMPK-activating drugs act synergistically with other anticancer agents including those that inhibit the mTOR-signalling pathway. It is possible that some of the inhibitors developed against the components of the mTOR pathway would be rendered more effective at suppressing cancer and prolonging lifespan, if prescribed in combination with metformin or phenformin. AMPK is also activated in humans during exercise and several studies have reported that exercise is beneficial in the prevention of certain cancers [26]. The effect of exercise in reducing cancer frequency is likely to be complex, but the depletion of whole-body ATP levels during exercise would raise AMPK activity and have the potential to inhibit cell growth and prevent cancer.

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Figure Legends

Figure 1. Generation and analysis of LKB1 hypomorphic PTEN^{+/-} mice. (A) Diagram illustrating the positions of the exons 3 to 8 of LKB1 gene and 4 to 6 of the PTEN gene. 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 implemented to generate the different genotypes of LKB1 and PTEN mice used in this study. The number and percentage of each genotype obtained after weaning are indicated. An asterisk indicates reduced Mendelian frequency of LKB1^{fl/fl}PTEN^{+/-} mice obtained. (C) Liver extracts derived from two different mice of each genotype were subjected to immunoblotting with the indicated antibodies. LKB1 was also immunoprecipitated and its activity assayed employing the LKBtide peptide. The results are presented as the average specific activity \pm SEM of samples derived from three independent mice assayed in triplicate. (D) As in C except that AMPK α 1 and AMPK α 2 were immunoprecipitated and assayed employing the AMARA peptide.

Figure 2. Analysis of tumour formation in LKB1 hypomorphic PTEN^{+/-} mice. (A) Incidence curve of tumours in LKB1-PTEN mice. Mice were maintained under standard husbandry conditions and the percentage of mice at each age with visible tumours indicated. For mice up to 13 months of age, only externally visible tumours were accounted; At 14 months of age when the study was terminated, tumours that were visible internally after dissection are also included in the analysis. n corresponds to the number of mice of each genotype. The p values of a student t-test comparing the data to LKB1^{+/+}PTEN^{+/-} results are displayed. The dotted line indicates the mean time in which 50% of animals developed tumours. (B) Representative histopathological analysis of tumours from LKB1^{+/+}PTEN^{+/-} and LKB1^{fl/fl}PTEN^{+/-} mice. The tumours show the same morphology in the different genotypes. Lymphomas (H&E x20 objective) arise predominately in the sub maxillary and parotid nodes and have the features of marginal zone low-grade B cell non-Hodgkin's lymphoma. The intestinal polyps (H&E x2 and x20 objectives) are biphasic tumours comprising complex epithelial growth with irregular branched glands and a smooth muscle cell stroma. The polyps were larger and more numerous in the LKB1^{fl/fl}PTEN^{+/-} animals than the LKB1^{+/+}PTEN^{+/-}. Phaeochromocytoma (H&E x20 objective) was composed of sheets of polygonal cells, supported by a rich vascular supply replacing the adrenal medulla. Prostate carcinoma (H&E x20 objective) in these animals is a moderately well differentiated microglandular carcinoma with comedo features. Breast carcinoma (H&E x20 objective) in both genotypes is a tubulo-papillary carcinoma with focal poorly differentiated areas eliciting a brisk stromal desmoplasia. Prostatic intraepithelial neoplasia PIN (H&E x40 objective) is a cribriform high-grade dysplasia I both genotypes. (C) Immunocytochemistry of intestinal polyps. Akt-p-T308 staining with this antibody was confined to the epithelium of the polyp and showed increased intensity towards the surface. FOXO-1 staining was seen in the cytoplasm and nucleus of both the epithelial and smooth muscle cells of the polyp with stronger staining of the epithelial cells. Ki67 staining was seen in the nuclei as expected a rather patchy distribution. Most epithelial cells staining was in crypts but the smooth muscle staining was more randomly distributed. S6-p-S235 reactivity was most marked in the cytoplasm of epithelial cells towards the surface of the polyp. Staining for ACC-p-S79 was represented by a weak diffuse cytoplasmic reactivity in the epithelial cells. AMPK-p-T172 immunoreactivity was present throughout the epithelial component but weaker staining of the smooth muscle was also seen.

Figure 3. Requirement of LKB1 for AMPK-mediated inhibition of mTORC1 in PTEN-deficient cells. (A) The indicated strains of ES cells were generated as described in the experimental procedures and cultured to 80% confluence in 10% Fetal Bovine Serum. The cells were lysed and LKB1 or PTEN immunoprecipitated and subjected to immunoblot analysis. LKB1 immunoprecipitate were also assayed employing the LKBtide peptide the results displayed as the average \pm SEM

corresponding to two independent experiments performed in triplicate. Cell extracts derived from two different dishes of cells were also immunoblotted with the other indicated antibodies. (B) As in (A) except that the ES cells were left untreated (-) or treated with AICAR (2 mM), metformin (10 mM, MET) or phenformin (2 mM, PHEN) for 2 hours. Cells were lysed and subjected to the indicated immunoblot analysis. AMPK α 1 was also immunoprecipitated and assayed and the assay results shown are the average \pm SEM corresponding to two independent experiments assayed in triplicate. (C & D) ES cells were seeded in 12 wells plates (30,000 cells per well) in DMEM containing 10% FBS in the presence of indicated doses of metformin (C) or phenformin (D) for 72 hours with the medium being changed each day with freshly dissolved metformin or phenformin. The cells were stained with crystal violet and following extraction with acetic acid, cell growth assessed by measuring absorption of each sample at 590 nm. The data is presented relative to the absorbance of wild type LKB1^{+/+}PTEN^{+/+} ES cells that is given a value of 1. The data are the average \pm SEM corresponding to two independent experiments with each condition undertaken in triplicate.

Figure 4. Activation of AMPK in mouse tissues following administration of AMPK activators. Groups of 5 mice of the indicated genotype at 8 weeks of age were left untreated or treated with the indicated AMPK activators dissolved in their drinking water daily for a period of 14 days (metformin (MET) 300mg/kg/day, phenformin (PHEN) 300mg/kg/day, A-769662 30mg/kg/day). Another group of age-matched mice were injected intraperitoneally with AICAR (500 mg/kg) for 30min. Mice were culled and liver (A), intestine (B) and spleen (C) extracts generated and subjected to immunoblot analysis with the indicated antibodies. For each condition duplicate immunoblots are shown with samples derived from different mice. AMPK α 1 was also immunoprecipitated and assayed. The assay results shown are the average \pm SEM from samples derived from the five mice each assayed in triplicate.

Figure 5. Activating AMPK delays onset of tumourigenesis in PTEN^{+/-} animals. (A) LKB1^{fl/+}PTEN^{+/-} mice from 4 weeks of age were left untreated (control) or administered the indicated AMPK activators (metformin 300mg/kg/day, phenformin 300mg/kg/day, A-769662 30mg/kg/day) in their drinking water daily. Each day freshly dissolved AMPK activator was added to drinking water and mice were maintained under standard husbandry conditions. The percentage of mice at each age with visible tumours is indicated. n corresponds to the number of mice of each genotype. The p values of a student t-test comparing the data to control mice not administered AMPK activator are displayed. The dotted line indicates the mean time in which 50% of animals developed tumours. (B) Representative histopathology of tumours from LKB1^{fl/+}PTEN^{+/-} mice untreated or treated with metformin, phenformin or A-769662. The tumour pathology was similar in the different treatment groups. Lymphomas were seen, mostly affecting the submandibular and parotid nodes and with a marginal zone type morphology. Intestinal polyps were in general less numerous and smaller but showed a biphasic morphology of epithelial and smooth muscle components with minimal atypia. We identified high grade PIN with a cribriform architecture and in places comedo necrosis in all three treatment groups and controls.

Table 1. Incidence of Tumours in LKB1 and PTEN deficient Mice

TUMOURS	LKB1 ^{+/+} PTEN ^{+/+} n=32 (12 males, 20 females)		LKB1 ^{fl/fl} PTEN ^{+/+} n=30 (13 males, 17 females)		LKB1 ^{+/+} PTEN ^{+/-} n=32 (15 males, 17 females)		LKB1 ^{fl/+} PTEN ^{+/-} n=30 (17 males, 13 females)		LKB1 ^{fl/fl} PTEN ^{+/-} n=30 (17 males, 13 females)	
	n	%	n	%	n	%	n	%	n	%
TUMOURS										
Intestinal polyps	1	3.13	0	0.00	12	37.50	18	60.00	18	60.00
Lymphoma	2	6.25	2	6.67	17	53.13	24	80.00	25	83.33
Phaeochromocytoma (male)	0	0.00	1	3.33	2	6.25	4	13.33	6	20.00
Prostate carcinoma (male)	0	0.00	0	0.00	4	12.50	3	10.00	4	13.33
Breast adenocarcinoma (female)	0	0.00	0	0.00	1	3.13	2	6.67	2	6.67
Pancreatic carcinoma	0	0.00	0	0.00	0	0.00	1	3.33	0	0.00
Number of mice displaying tumours	2	6.25	2	6.67	22	68.75	27	90.00	28	93.33
PRENEOPLASTIC LESIONS										
Prostatic intraepithelial neoplasia (male)	0	0.00	0	0.00	5	15.63	9	30.00	6	20.00
Intratubular germ cell neoplasia (male)	0	0.00	0	0.00	2	6.25	3	10.00	0	0.00
Prostatic hyperplasia	0	0.00	0	0.00	1	3.13	0	0.00	0	0.00
Reactive lymphoid hyperplasia	0	0.00	0	0.00	0	0.00	1	3.33	0	0.00
Uterine hyperplasia	1	3.13	0	0.00	0	0.00	0	0.00	2	6.67
Mice displaying no tumour-associated pathology	30	93.75	28	93.33	10	31.25	3	10.00	2	6.67
OTHER NON-NEOPLASTIC LESIONS										
Persistent trophoblast	0	0.00	1	3.33	0	0.00	5	16.67	0	0.00
Glomerulonephritis	0	0.00	0	0.00	1	3.13	1	3.33	0	0.00
Chronic pancreatitis	0	0.00	1	3.33	0	0.00	0	0.00	0	0.00
Massive thrombosis	0	0.00	1	3.33	0	0.00	0	0.00	0	0.00
Focal steato hepatitis	0	0.00	0	0.00	3	9.38	2	6.67	0	0.00
Plasma cell nest	0	0.00	0	0.00	0	0.00	1	3.33	0	0.00
Microcystis in kidney	0	0.00	0	0.00	0	0.00	1	3.33	0	0.00
Lung haemorrhage	0	0.00	0	0.00	0	0.00	2	6.67	0	0.00
Pulmonary oedema	0	0.00	0	0.00	0	0.00	0	0.00	1	3.33
Steatosis	2	6.25	2	6.67	0	0.00	1	3.33	0	0.00

n corresponds to the total number of mice and the percentages (%) indicate the number of mice displaying the indicated pathologies.

Table 2. Incidence of Tumours in LKB1^{fl/+} PTEN^{+/-} Mice

TUMOURS	Control		Metformin (300mg/kg/day)		Phenformin (300mg/kg/day)		A-769662 (30mg/kg/day)	
	n	%	n	%	n	%	n	%
Intestinal polyps	2	20.00	3	30.00	4	40.00	2	20.00
Lymphoma	10	100	10	100.00	7	70.00	8	80.00
Phaeochromocytoma	0	0.00	0	0.00	1	10.00	0	0.00
Number of mice displaying tumours	10	100	10	100.00	8	80.00	8	80.00
PRENEOPLASTIC LESIONS								
Prostatic intraepithelial neoplasia (male)	2	20.00	2	20.00	3	30.00	1	10.00
Hydronephrosis	0	0.00	0	0.00	1	10.00	1	10.00
Persistent trophoblastic disease	0	0.00	1	10.00	0	0.00	1	10.00
Cystic hyperplasia of endometrium	0	0.00	1	10.00	2	20.00	0	0.00
Mice displaying no tumour-associated pathology	0	0.00	0	0.00	2	20.00	2	20.00

n corresponds to the total number of mice and the percentages (%) indicate the number of mice displaying the indicated pathologies

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080557

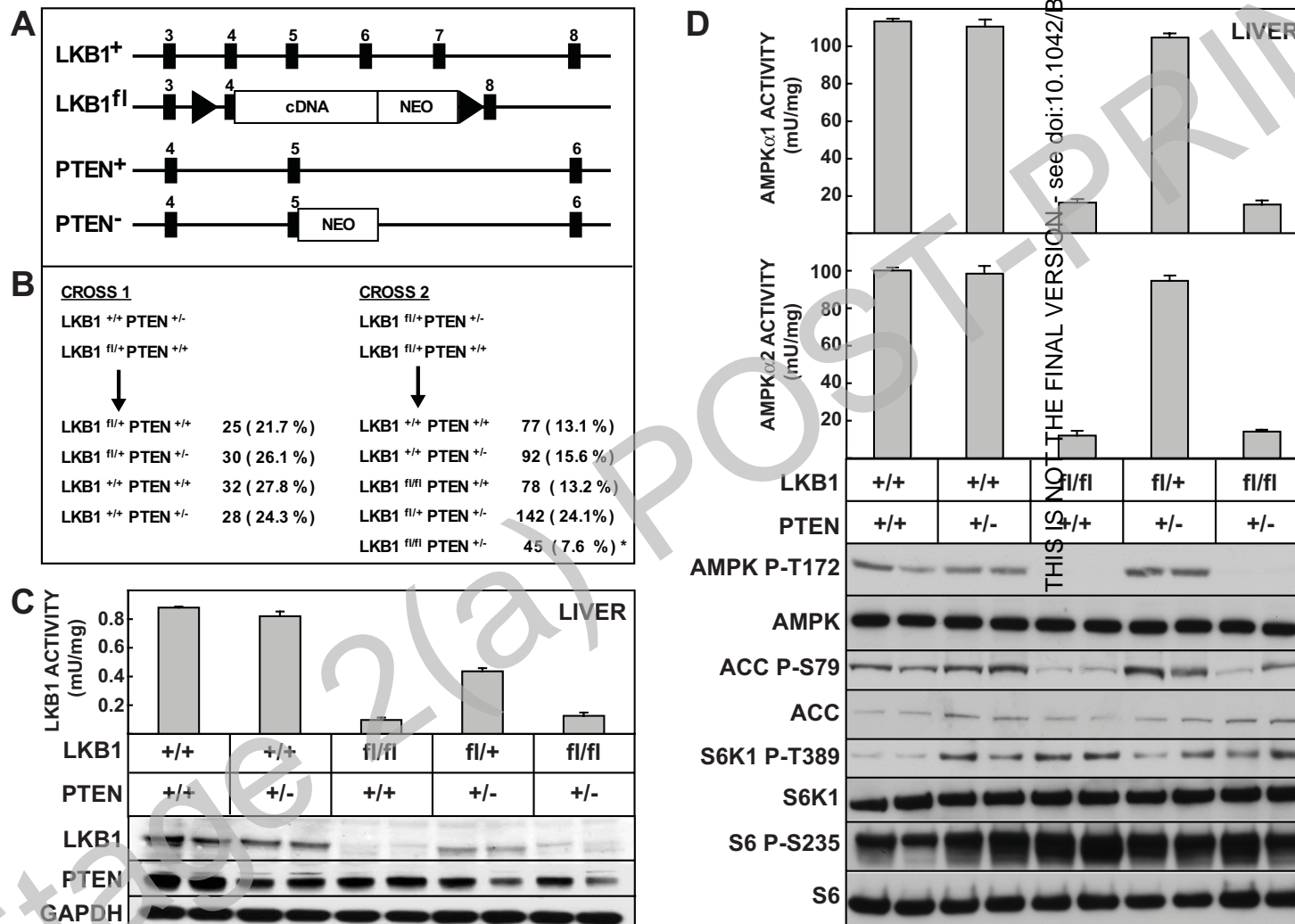


FIGURE 1

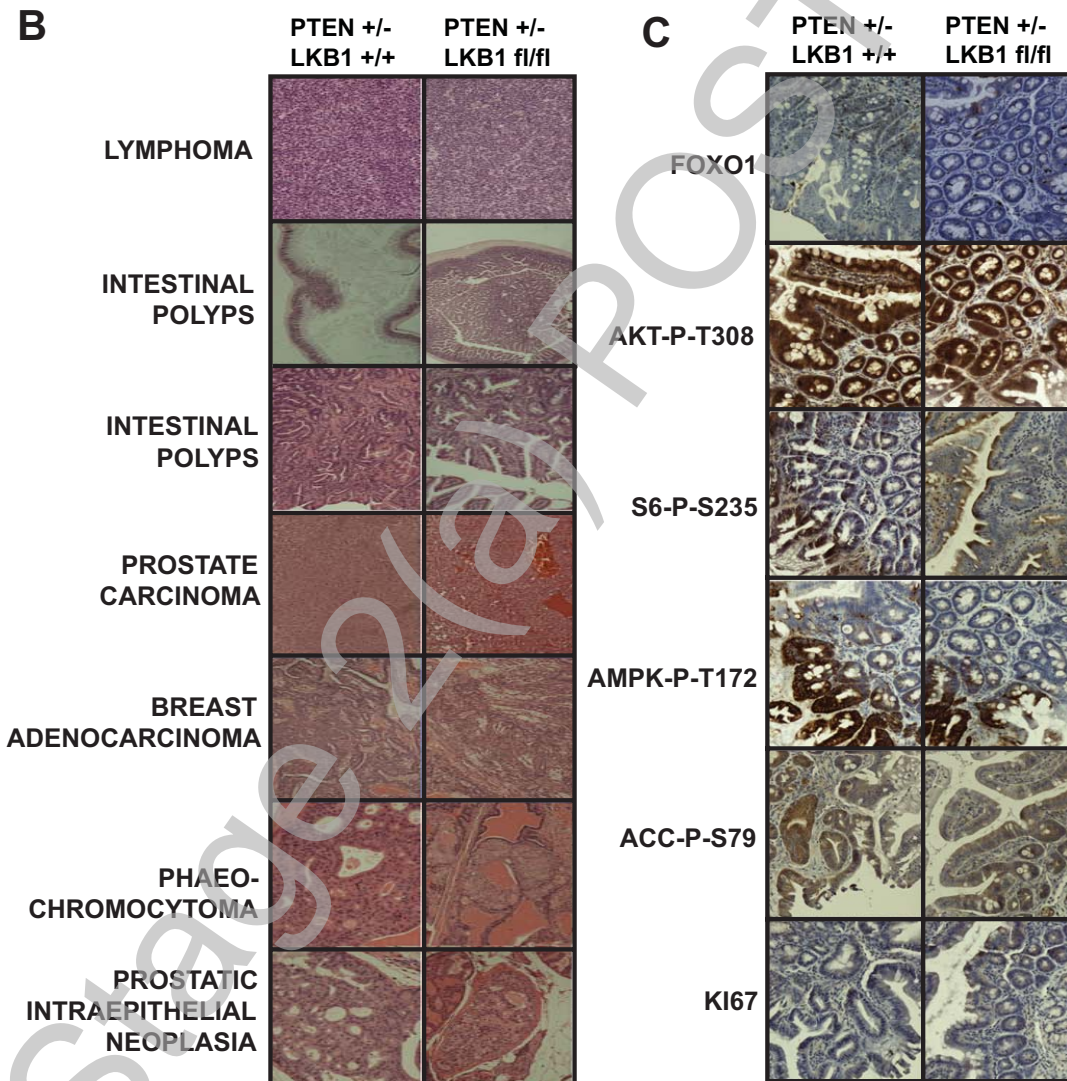
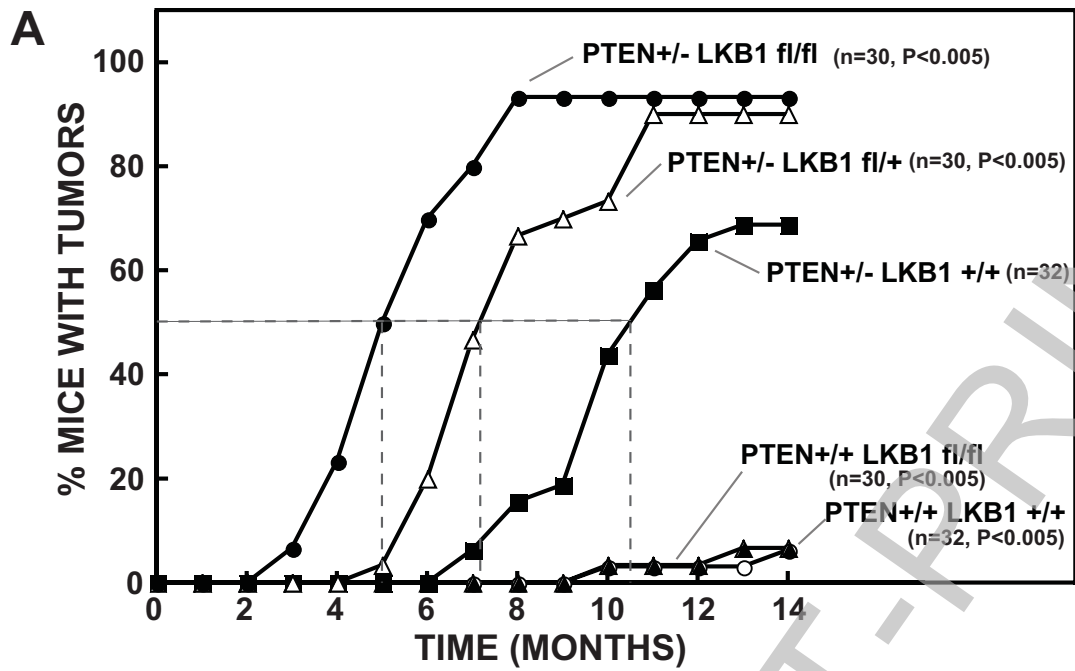


FIGURE 2

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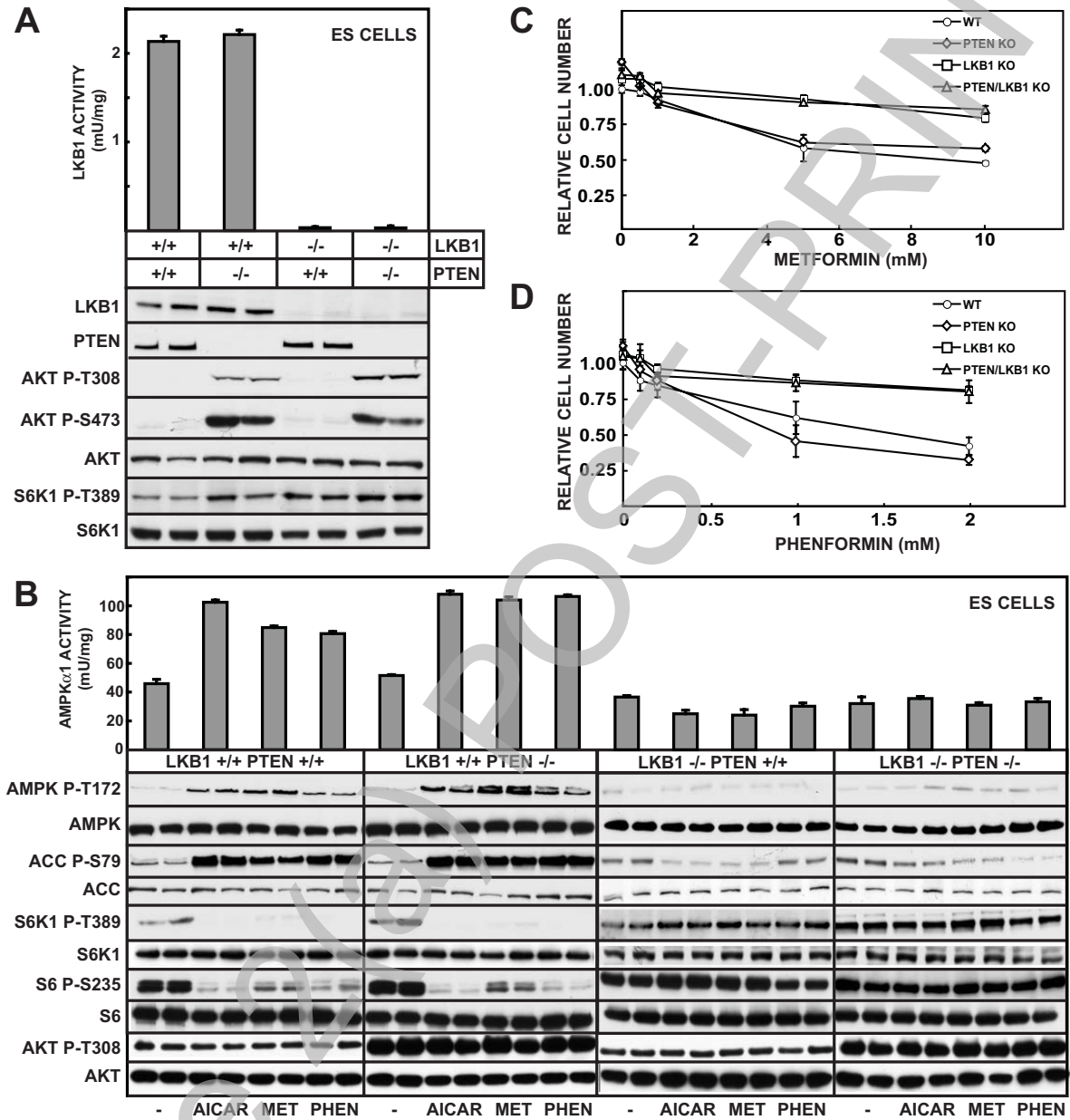


FIGURE 3

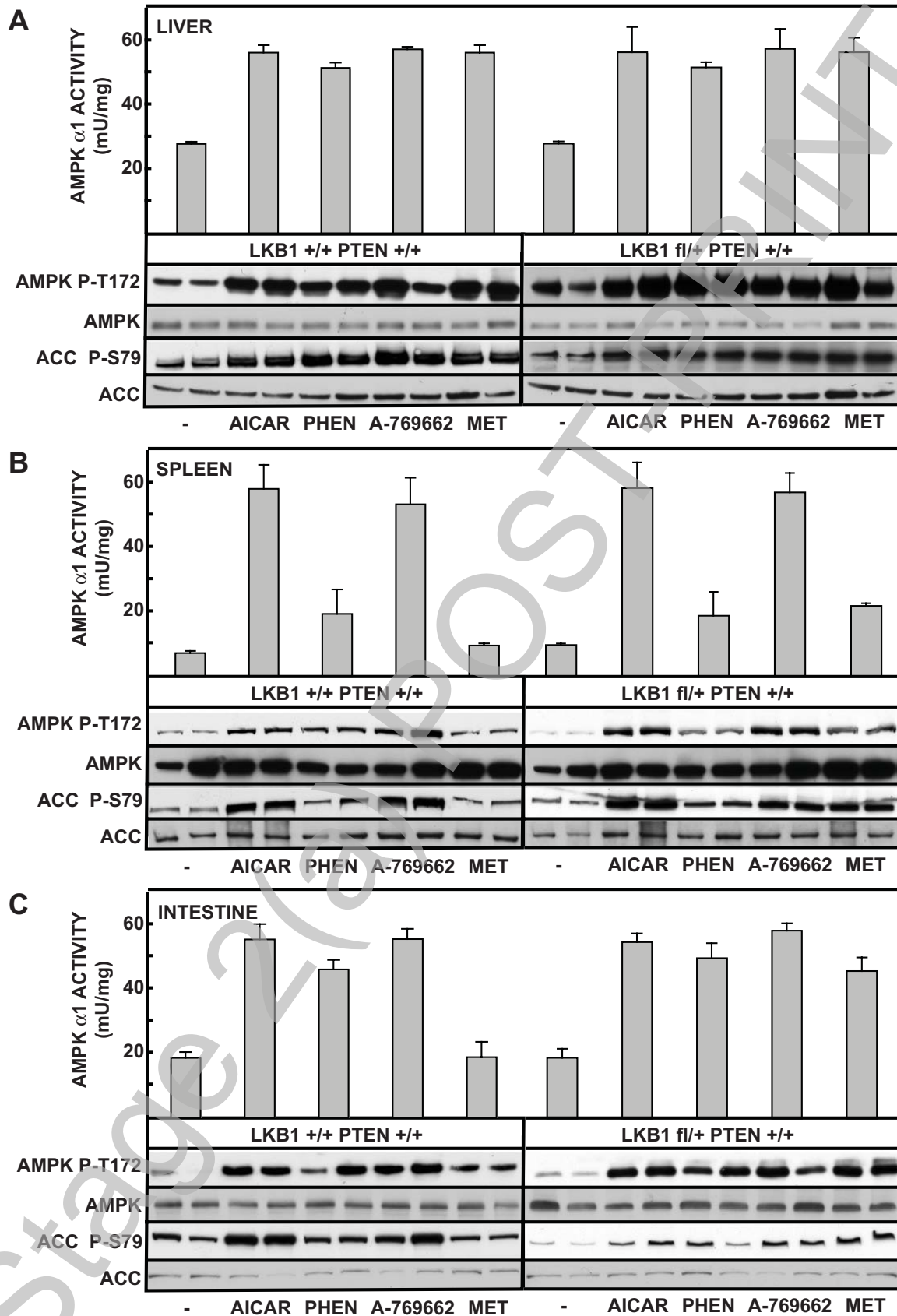


FIGURE 4

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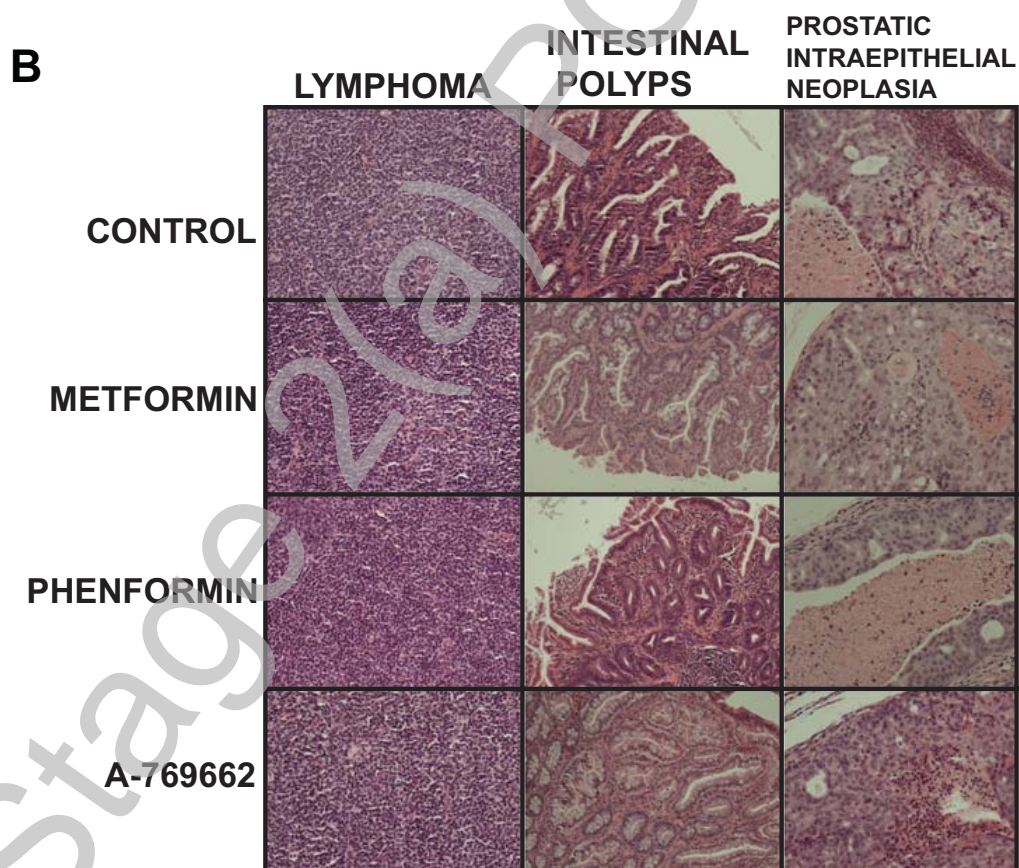
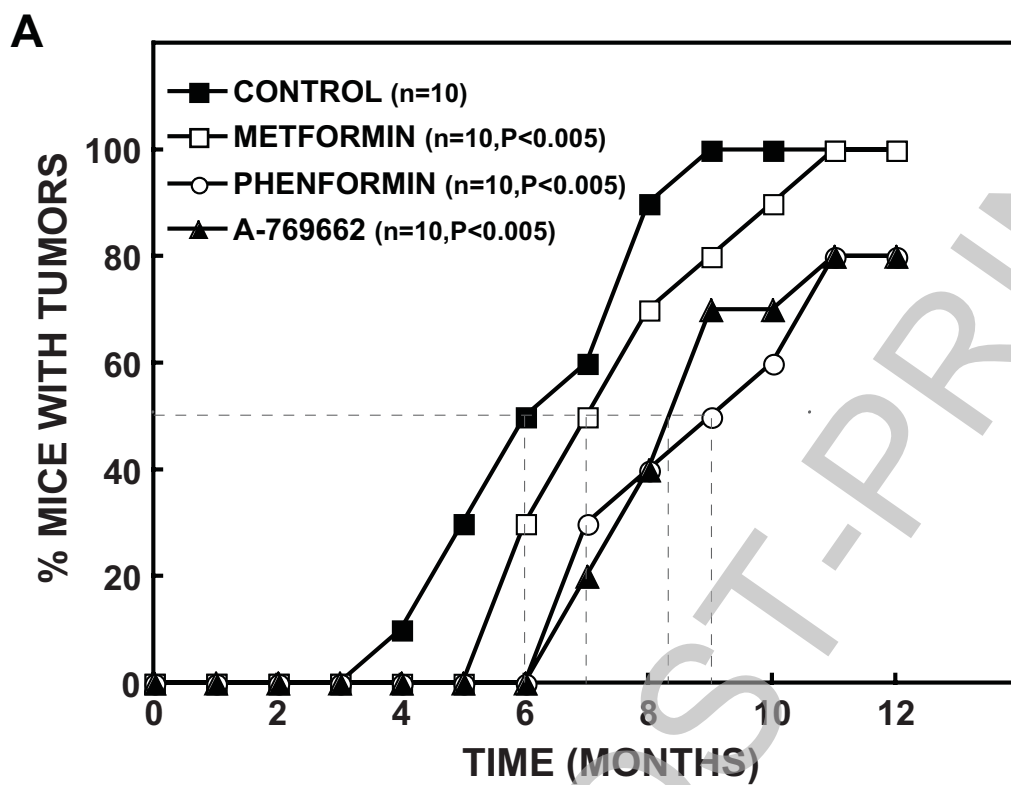


FIGURE 5