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**PTEN protein phosphatase activity correlates with control of invasion, gene expression and tumour suppression, but not AKT**

**One sentence summary:** The lipid and protein phosphatase activities of PTEN must combine to control invasion, gene expression and tumour suppression

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**Abstract:** The tumour suppressor, Phosphatase and Tensin homolog deleted on chromosome ten (PTEN), has a well characterised and important lipid phosphatase activity and a poorly characterised protein phosphatase activity. We show that both activities are required together for the regulation of cellular invasion and most of its largest effects on gene expression. PTEN appears to dephosphorylate itself at Thr366 and mutation of this site makes lipid phosphatase activity sufficient for the regulation of invasion. We propose that the dominant role for PTEN's protein phosphatase activity is autodephosphorylation-mediated regulation of its lipid phosphatase activity. Since the regulation of invasion and these large gene expression changes do not correlate with total cellular levels of its PtdInsP<sub>3</sub> substrate and AKT activity, we speculatively propose a role for localised PtdInsP<sub>3</sub> signalling in the PTEN-mediated regulation of the former processes. Finally, in identifying a tumour-derived PTEN mutant selectively lacking protein phosphatase activity, we show that in some circumstances these processes, and not AKT, can correlate with PTEN-mediated tumour suppression.

## **Introduction**

Loss of function of the PTEN phosphatase is one of the most common events in the development of human cancers (1, 2). This loss of PTEN function occurs through mutation of one or both alleles of the tumour suppressor and through a variety of non-genomic mechanisms (3). In normal physiology, PTEN plays a role in the regulation of many diverse processes, but key to its tumour suppressor functions appear to be highly conserved effects on the growth, proliferation and survival of many cell types (2, 4).

Biochemically, PTEN's best recognised function is as a lipid phosphatase, metabolising phosphatidylinositol 3,4,5-trisphosphate (PtdInsP<sub>3</sub>), the primary product of the class I phosphoinositide 3-kinases (PI3Ks). This phosphatase activity of PTEN can directly oppose the effects of PI3K on the many diverse PtdInsP<sub>3</sub> binding effector proteins that mediate downstream PI3K dependent signalling. These lipid binding proteins include the well-studied AKT group of PtdInsP<sub>3</sub>-activated serine/threonine protein kinases, but also many others, including several groups of regulators of the membrane-anchored ADP ribosylation factor (ARF) and RAC families of small GTPases (5). There are numerous examples of biological responses that appear to be regulated by the amplitude of PI3K-dependent signals, many related to cell growth and proliferation. However, there are also settings, such as cell polarisation during chemotaxis and in some epithelia, in which the subcellular localisation of PtdInsP<sub>3</sub> appears to play a key role (6-10). In these cases, localised PTEN activity appears to play a positive role in the establishment of PtdInsP<sub>3</sub> signal gradients or pools (11-14).

This evolutionarily conserved role as a core component of the PI3K signalling network provides a mechanistic explanation for many of the effects of PTEN on cell growth, proliferation, survival, metabolism and polarity. However, PTEN has other potential mechanisms of action, including protein phosphatase activity and proposed non-catalytic actions (15-20), but the importance of these alternative mechanisms is currently unclear. In recent work, we developed a PTEN mutant that retains wild-type lipid phosphatase activity, yet lacks activity against peptide substrates. We used this mutant to show that both lipid and protein phosphatase activities are required together for PTEN to inhibit glioma cell invasion (21). Here we provide a mechanistic basis for the concerted actions of PTEN's two activities and show that they are also required together to mediate many of the effects of PTEN on gene expression. Finally, we show that a mutation of PTEN, identified in a small cell lung carcinoma, selectively ablates PTEN's protein phosphatase activity, indicating for the first time a requirement for this activity in tumour suppression.

## Results

### **Requirements for both the protein and lipid phosphatase activities of PTEN in the control of invasion and gene expression**

Almost all of the data that imply important effects of PTEN's protein phosphatase activity rely upon a functionally selective mutant, PTEN G129E, originally identified in two Cowden syndrome families, that lacks lipid phosphatase activity, yet retains protein phosphatase activity (22, 23). We recently systematically developed a PTEN mutant that selectively retains wild-type activity against vesicular lipid substrates, but lacks substantial protein phosphatase activity, PTEN Y138L. Critically, when expressed at near physiological levels in PTEN null cells, PTEN Y138L strongly reduces total cellular PtdInsP<sub>3</sub> levels and AKT phosphorylation and activity as the wild-type enzyme does (21). Here we extend this comparison to show indistinguishable effects of wild-type (WT) and Y138L PTEN expression level on AKT phosphorylation in 3 PTEN null glioma cell lines and that the strength of this effect is dependent on the level of PTEN but does not vary between PTEN WT and Y138L (Figs. 1, 2 and S1). Both PTEN proteins also suppress platelet derived growth factor (PDGF) stimulated P-AKT in U87MG cells ((21) and Fig S1). These mutants together therefore provide unique tools to investigate the contributions of these two catalytic activities to PTEN's biological functions. We used lentiviruses to express these two untagged PTEN mutants in PTEN null U87MG or DBTRG-05MG glioblastoma cells at levels similar to those found in cells expressing endogenous PTEN, alongside wild-type PTEN and a catalytically dead mutant, PTEN C124S. Expression of wild-type PTEN suppresses the invasion of U87MG cells in 3D

matrigel assays, but neither PTEN G129E nor PTEN Y138L alone, nor the two proteins expressed together was able to suppress invasion ((21) and Fig. 1). To ensure that both proteins were being expressed in these experiments, we performed co-expression of green fluorescent protein (GFP)-PTEN G129E with untagged PTEN Y138L, still with no effect on cellular invasion (Fig. 1). This argues that both activities must exist in the same protein molecule to mediate the suppression of invasion.

To extend our analysis of PTEN's separable activities to a more global and unbiased analysis, we performed whole genome microarray analysis of gene expression changes occurring in U87MG cells transduced with the different PTEN proteins and grown for 16 hours in 3D matrigel cultures. We analysed this data initially by making pairwise comparisons with sham transduced controls. The expression of GFP or a catalytically inactive PTEN mutant had no significant effect on gene expression in these cells (statistical analyses are described in a supplementary methods section), which gives us confidence that our lentiviral expression system has few artefactual effects on cell behaviour. However, wild-type PTEN altered the expression of a very large number of transcripts (Fig. 2). The expression of PTEN G129E, with only protein phosphatase activity, caused modest changes in the abundance of a small number of transcripts, but PTEN Y138L, which is able to suppress cellular PtdInsP<sub>3</sub> levels and AKT activity, shared a large set of transcriptional responses with wild-type PTEN, yet failed to affect a large group of transcripts that were the most highly responsive to the wild-type enzyme (Figs. 2A, 2B, 2C). Detailed comparisons of the transcriptional responses to wild-type and Y138L PTEN showed that almost all probes either responded similarly to both enzymes,

or responded only to wild-type PTEN (Fig. 2D). There were almost no probes that were affected strongly by wild-type and weakly by Y138L, indicating a qualitatively different signalling mechanism mediated only by PTEN WT, not a quantitative difference in the ability of these proteins to control one pathway or set of responses. KEGG and GeneOntology group analysis correlates both wild-type and Y138L PTEN transcriptional responses with several recognised pathways (Fig. S2), in agreement with ability of both proteins to control AKT and indeed cell proliferation in these cultures (21).

The set of transcripts (Table S1) most strongly induced selectively by wild-type PTEN activity, but not by PTEN Y138L, was compared with available gene expression data from human glioblastoma samples. 510 of the 517 tumour samples analysed have more than 25% of the top 169 PTEN-responsive genes down-regulated and 81 of these 169 genes are down-regulated in more than 25% of the 517 tumour samples. Both of these results show significant overlap and suggest that this transcriptional control pathway may exist in a clinically relevant setting ( $p=0+0.01$  and  $p=0.019\pm 0.01$  Figure 2E and Supplementary Methods). We also verified that PTEN Y138L was able to suppress AKT phosphorylation in U87MG cells cultured in 3D matrigel (Fig. 2F).

### **Evidence for PTEN autodephosphorylation of Thr366**

This analysis of PTEN mediated gene regulation shows that the protein phosphatase activity of PTEN correlates with very modest effects acting independently, yet that in the wild-type enzyme, protein phosphatase activity acts together with lipid phosphatase activity to mediate many of PTEN's greatest effects on gene expression. One simple

hypothesis to explain the requirement for both activities within the same molecule is the previous proposal that PTEN may autoregulate through the action of its protein phosphatase activity on its own C-terminal phosphorylation sites (19, 24). We suggest that this leads to control of its lipid phosphatase activity.

Therefore, we chose to test the hypothesis that PTEN might dephosphorylate itself, using a range of phospho-specific antibodies raised against sites in the PTEN C-terminus. Thr366 (T366) has been shown to be phosphorylated by Glycogen Synthase Kinase 3 (GSK3), which requires prior priming phosphorylation of Ser370 (S370) by Protein Kinase CK2 (25, 26). Also phosphorylated by CK2, usually to high stoichiometry, is a cluster of four sites between Ser380 and Ser385 (27, 28). We found that wild-type and catalytically dead PTEN had the same level of phosphorylation on this cluster of residues (Figs. 3A, 3B). This analysis included a de-phospho-specific antibody raised against these 380-385 sites. This antibody gives a strong signal with unphosphorylated bacterially expressed PTEN, but with PTEN expressed in U87MG cells, gives a weak signal that is unaffected by the catalytic activity of the PTEN protein. However, both T366 and Ser370 displayed much lower levels of phosphorylation in active PTEN than in catalytically impaired proteins (Figs. 3A, 3B). This implied that they could be sites of autodephosphorylation. We also observed that when the cellular phosphorylation of T366 is blocked by the application of the well validated selective GSK3 kinase inhibitor, CT99021 (29), the phosphorylation of T366 on wild-type PTEN falls over a period of 1-2 hours, yet catalytically impaired PTEN proteins remained phosphorylated for many hours (Figs. 3C, S3). We tested the ability of purified PTEN to dephosphorylate T366 in vitro,

using as a substrate the purified C-terminal tail of PTEN phosphorylated with GSK3. In these experiments, PTEN WT had robust activity, PTEN G129E was somewhat weaker and both PTEN C124S and Y138L had little or no activity (Figs 3D and S3C).

Consistent with the autodephosphorylation of T366, we found that when PTEN activity was inhibited by cellular treatment with hydrogen peroxide or hyperosmotic stress (30, 31), this stimulated an increase in PTEN P-T366 (Figs. 4A and S4). The phosphorylation of T366 that was stimulated by treatment with 0.5M sorbitol could be inhibited by CT99021, indicating that phosphorylation is mediated by GSK3 and also consistent with the hypothesis that phosphorylation increases due to reduced dephosphorylation rather than simply through increased activity of an alternate kinase activated by osmotic stress (Fig. S4C).

To further test the hypothesis that PTEN would dephosphorylate its own C-terminus intramolecularly, predicted by the failure of co-expressed PTEN Y138L and PTEN G129E to inhibit cellular invasion, we analysed P-T366 PTEN in cells co-expressing the active and inactive proteins. Wild-type PTEN had no effect on the high P-T366 level of co-expressed inactive GFP-PTEN C124S, yet itself displayed low T366 phosphorylation (Fig. 4B). Similarly, further data showing that low T366 phosphorylation of wild-type PTEN is not simply the result of low downstream PI3K-dependent phosphorylation in cells expressing this active PtdInsP<sub>3</sub> phosphatase, is provided by the lack of effect of a PI3K inhibitor on the high T366 phosphorylation of catalytically dead PTEN C124S (Fig. S4D). A further prediction of PTEN C-terminal intramolecular autodephosphorylation is

that blocking the recognised interaction of the C-terminal tail with the phosphatase domain (32, 33) should increase T366 phosphorylation, due to reduced association of T366 with the active site. Accordingly, alanine mutation of 3 clustered C-terminal phosphorylation sites (S380A, T382A, T383A, termed PTEN A3) which promotes an open conformation, was found to dramatically increase the T366 phosphorylation of PTEN (Fig. S5A). The correlation between PTEN activity and low T366 phosphorylation was also extended by analysis of four further PTEN mutations identified in human tumours. The catalytically active PTEN mutants, PTEN L42R and X404L (34, 35) were found to display low levels of T366 phosphorylation similar to wild-type PTEN, whereas mutants lacking protein phosphatase activity, G129R (34) and Y138C (see below), showed much higher levels (Fig. S5B). Finally, we chose to investigate whether there was a difference between the dephosphorylation in vitro of immunoprecipitated wild-type and catalytically dead PTEN by another protein phosphatase, lambda phosphatase (Fig 4C). In these experiments, similar dephosphorylation was observed with both proteins, suggesting that T366 was similarly accessible for dephosphorylation by exogenous phosphatases.

Together these data strongly indicate that PTEN is able to autodephosphorylate its own C-terminus at T366, and possibly also S370. The observed similarity in the dynamic phosphorylation of both sites in several experiments implies that both sites may be substrates for PTEN autodephosphorylation (Figs. 3A, 3B, 4B and S4 and S5). However, we have found that mutation of T366 to alanine in catalytically dead PTEN greatly reduces the phosphorylation of the adjacent S370 (Fig. S5), implying that T366

phosphorylation may itself affect the adjacent phosphorylation status of S370 and therefore that autodephosphorylation may be limited to T366.

### **Action of PTEN protein phosphatase activity through T366 to regulate invasion independently of AKT**

The de-phosphorylation of its C-terminus by PTEN could explain why both activities appear to be required together for PTEN to regulate invasion and gene expression. This predicts that blocking the phosphorylation of the auto-dephosphorylated residues by mutation might rescue the ability of lipid phosphatase activity to act without protein phosphatase activity. We therefore tested the ability of a PTEN Y138L T366A double mutant to inhibit invasion. Although PTEN Y138L was unable to strongly suppress U87MG cell invasion in a transwell assay, the wild-type enzyme and both PTEN Y138L T366A and PTEN Y138L S370A were active in this assay (Fig. 5). Also consistent with this hypothesis was the finding that a PTEN mutant mimicking phosphorylation at this site, PTEN T366D, was unable to suppress invasion, despite having both lipid and protein phosphatase activity. These effects on cellular behaviour mediated through T366 dependent lipid phosphatase activity are not limited to specific effects on cellular invasion, as observation of these U87MG cells expressing different PTEN proteins also revealed differences in cellular morphology when seeded into matrigel (as previously described (21)). Cells with correctly targeted lipid phosphatase activity, uninhibited by Thr366 phosphorylation, were viable and proliferative, yet displayed a lengthy delay in their extension of processes and spread into the surrounding matrix (Figs. S6 and 1D).

These data suggests that de-phosphorylation of T366 is required for the correct targeting of PTEN's lipid phosphatase activity. The separation of PTEN's activity to suppress invasion from its ability to suppress AKT phosphorylation was further tested using the well validated selective AKT inhibitor, Akti-1/2, which had no effect on U87MG cell invasion in this assay (Fig. 5C). Additionally, active mutants of AKT1, 2 and 3 were co-expressed alongside wild-type PTEN in U87MG cells and despite driving the phosphorylation of substrates such as GSK3, had no significant effect on the suppression of invasion caused by PTEN (Fig 5C p=0.61, 0.84 and 0.91 for AKT1, 2 and 3 respectively, t test). We tested the pan-PI3K inhibitor PI-103, which at 1 $\mu$ M suppressed AKT phosphorylation, but also had no effect on invasion. It was impossible to test the activity of PI-103 at higher concentrations due to cell death of these cultures at higher concentrations. The lack of effect of PI3K inhibition on invasion is consistent with the hypothesis that the role of PTEN in this assay is to act locally to generate a gradient of PtdInsP<sub>3</sub> and is not simply to reduce total cellular levels of this lipid signal.

### **A tumour-derived PTEN mutant that controls AKT, but has selective lost protein phosphatase activity**

These experiments indicate that the key role for PTEN's protein phosphatase activity in the regulation of glioma cell invasion is autodephosphorylation of T366. However, the importance of PTEN's protein phosphatase activity in tumour suppression is only indirectly reflected in these experiments through the pathological significance of tumour cell invasion and the gene expression signature shared in human tumour samples. The PTEN Y138L mutant used in this study was developed in a systematic screen informed

by studies of the PTEN related proteins, transmembrane phosphatase with tensin homology (TPTE) and TPTE and PTEN homologous inositol lipid phosphatase (TPIP) (21). To our knowledge, the only mutation of Tyr138 reported in a human tumour sample is a Tyr138Cys (Y138C) mutation identified in a metastatic small cell lung cancer cell line, NCI-H196 (36). To test for a more direct correlation between PTEN's protein phosphatase activity and tumour suppression, we performed an analysis of this mutant enzyme. When expressed, purified and assayed in vitro, PTEN Y138C had the same catalytic properties as PTEN Y138L, displaying lipid, but not protein phosphatase activity (Fig. 6). Similarly, expression of PTEN Y138C at physiological levels in PTEN null U87MG cells reduces cellular PtdInsP<sub>3</sub> levels and AKT phosphorylation as efficiently as either wild-type or Y138L PTEN, yet (like Y138L) failed to suppress cellular invasion (Fig. 6). We therefore investigated the function of PTEN Y138C in NCI-H196 cells, where it is expressed endogenously. RT-PCR validation confirmed the expression of this mutant allele but failed to detect wild-type PTEN or other secondary mutations (Figs. 7A, 7B). Accordingly, phosphatase assays of immunoprecipitated PTEN showed that these cells express PTEN with robust lipid phosphatase activity, but little or no protein phosphatase activity (Fig. 7C). As would be predicted, we also found that the mutant PTEN Y138C and C124S proteins expressed endogenously in NCI-H196 and U343MG cells respectively, both displayed elevated phosphorylation of Thr366 relative to wild-type protein in HEK293T cells (Fig. 7D). Notably, knockdown of PTEN expression in NCI-H196 cells using lentiviral shRNA led to an increase in cellular AKT phosphorylation (Fig. 7E). This shows that the PTEN Y138C mutant is stably expressed and is active in the suppression of the AKT branch of PI3K dependent signalling in these

cells and implicates another signalling mechanism as being the driver behind selection for the mutation of PTEN in this tumour.

## Discussion

We show that suppression by PTEN of total cellular PtdInsP<sub>3</sub> levels and AKT activity is not sufficient to mediate most of its greatest effects on gene expression and cellular invasion in 3D cultured U87MG cells. We also show that a mutation identified in a small cell lung cancer, PTEN Y138C, selectively lacks protein phosphatase activity, but retains the ability to control cellular PtdInsP<sub>3</sub> and AKT. This implicates other PTEN regulated signalling mechanisms in the regulation of cellular invasion, gene expression and tumour suppression. Our data also indicate that PTEN autodephosphorylates T366 in its C-terminal tail and that this autodephosphorylation is required together with lipid phosphatase activity, for PTEN to control invasion, cell morphology and probably many gene expression responses.

The AKT kinases have received a great deal of attention as likely mediators of pro-oncogenic signalling downstream of PTEN and PI3K. In *Pten* heterozygous mice that develop tumours in multiple tissues, deletion of *Akt1* blocks tumorigenesis (37) and selectively reducing Akt activity (caused by mutation of the Pdk1 PH domain) leads to delayed and slower growing tumours (38). These data indicate that AKT activity is necessary for tumour formation in many tissues, and are consistent with the hypothesis that the elevated AKT activity driven by PTEN loss promotes tumour formation. Accordingly, cancer drug discovery programmes to develop AKT inhibitors are well progressed and show some promise (39, 40). However, the importance in tumours, and

their dependence on, the other additional signalling pathways activated downstream of PTEN and PI3K is poorly understood.

Much of our data suggest that PTEN affects invasion and gene expression in U87MG cells through the targeting of its lipid phosphatase activity. We speculate that this is through activity against a specific pool of PtdInsP<sub>3</sub> or generating a gradient of the lipid and in turn regulating PI3K-dependent signalling events that do not correlate with P-AKT. We propose a model in which phosphorylation of T366 interferes with a specific mechanism of spatially localised PTEN activity, probably through controlling the interaction of PTEN with a particular protein or proteins. In contrast to other phosphorylation sites in the PTEN C-terminus, T366 appears not affect the ability of PTEN to access membranes through electrostatic interactions, metabolise lipid substrates and regulate AKT (26). It is known that only the minimal catalytic core of PTEN (its phosphatase and C2 domains) is required to interact with the plasma membrane and reduce total cellular levels of PtdInsP<sub>3</sub> and AKT activity (41). However, more complex targeting of PTEN is required for the control of other more spatially regulated PI3K dependent processes, such as membrane ruffling and cell polarity (35, 42). We suggest that this previously observed distinction between untargeted lipid phosphatase activity that controls total cellular levels of PtdInsP<sub>3</sub>, and more complex regulated PTEN activity, is closely related to our observation that P-T366 inhibited targeting is required for the regulation of cellular morphology and invasion.

We found that neither lipid nor protein phosphatase activity independently blocked invasion or could mediate the largest effects of wild-type PTEN on gene expression. However, a PTEN mutant with only lipid phosphatase activity, PTEN Y138L, acquired the ability to suppress cellular invasion when combined with mutation of Thr366, an inhibitory C-terminal phosphorylation site that appears to be dephosphorylated by the protein phosphatase activity of PTEN. This provides a mechanistic understanding of how the two activities could act in concert within the same molecule. Phosphorylation of Thr366 has already been shown to regulate the interaction of PTEN with Myosin V (43) and directly mediate binding to 58kD microspherule protein (MSP58) (18). Potentially controlled by such interactions, the role of spatially targeted PTEN activity and resultant PtdInsP<sub>3</sub> gradients in cell regulation is well appreciated (44, 45). Our data argue against important independent roles for PTEN's protein phosphatase activity, at least in the regulation of invasion and large gene expression responses in U87MG cells.

Our data suggest that the principal role for PTEN's protein phosphatase activity may be to directly dephosphorylate its own C-terminus. An alternative explanation for some of our T366 phosphorylation data is that catalytically inactive mutants such as PTEN C124S favour a closed conformation that protects P-T366 from dephosphorylation by another unknown phosphatase. However, the elevated phosphorylation of the open conformation mutant PTEN A3 and the similar dephosphorylation of wild-type and C124S PTEN by  $\lambda$ -phosphatase in vitro would argue against this. On the other hand, our proposal that Thr366 is a substrate for autodephosphorylation implies that this phosphorylated residue would, at least transiently, occupy the PTEN active site. One speculative interpretation of

the relatively slow apparent rate of autodephosphorylation (estimated cellular half-life measured in tens of minutes, Figs. 3C and S3) would imply that this threonine residue may be a very poor substrate for PTEN and through prolonged residence, occlude access to the active site.

We were unable to show *in vitro* that a phosphorylated full length PTEN protein would autodephosphorylate, which suggests the possibility that dephosphorylation may be mediated by another PTEN-associated phosphatase. However, since PTEN T366 dephosphorylation appears slow and because autodephosphorylation based control over PTEN function would seem likely to involve additional factors that influence the rate of this reaction, such as unidentified binding partners that promote or inhibit dephosphorylation we favour such a regulated direct mechanism. Some modest support for the inhibitory regulation of PTEN autodephosphorylation by factors such as oxidation or ubiquitination is provided by the observed increases in PTEN Thr366 phosphorylation observed in cells treated with hydrogen peroxide and hyperosmotic stress, that lead to PTEN oxidation and ubiquitination respectively (Figs. 4A, S4 and (31)). Our data also provide only some explanation for the elevated level of phosphorylation of T366 seen in the protein phosphatase active mutant PTEN G129E. Although we observed somewhat reduced dephosphorylation of a C-terminal tail substrate *in vitro* by PTEN G129E, it appears to us that the degree of cellular T366 elevation observed requires an unidentified mechanism, for example that this mutation favours a conformation in which the C-terminal tail is less closely associated with the phosphatase domain.

The strength of the comparison of the PTEN-responsive gene expression profiles we observed in vitro, with the clinical glioblastoma samples, supports the conclusion that the matrigel cultures used here are a reasonable model of the in vivo conditions. In particular, it confirms that the set of genes that we observe to be highly responsive to PTEN, but not correlating with PTEN's observable effects on AKT, accurately represent at least a subset of those genes that correlate with PTEN loss in glioblastoma tumors.

In the future it will be important to determine the signalling mechanisms responsible for the effects of PTEN on invasion and gene expression that do not correlate with AKT regulation. A deeper understanding of the downstream pathways activated in tumours with complete or selective loss of PTEN function should have important implications on the use of therapies targeting downstream events, some of which are already in clinical trials.

## **MATERIALS AND METHODS**

### **Cell culture and cell based assays**

U87MG glioblastoma and Human Embryonic Kidney 293T cells were cultured as previously described (Davidson et al.,2010). NCI-H196 cells were purchased from ATCC and cultured in RPMI-1640 medium, 10% fetal bovine serum (FBS). U343MG cells were purchased from CLS (Eppelheim, Germany) and cultured in Minimum Essential Medium, 10% FBS, and non-essential amino acid mix (Invitrogen GIBCO, Paisley, UK). DBTRG-05MG cells were cultured in RPMI medium supplemented with 10% FBS and non-essential aminoacids. Lentiviral particles were prepared as described elsewhere (Davidson et al.,2010). U87MG cells were transduced with lentiviruses in media supplemented with 16µg/ml of polybrene (hexadimethrine bromide, Sigma). The media was changed 24hrs post transduction and the cells were processed 48hrs post transduction.

3D matrigel cultures of U87MG cells were prepared as described elsewhere (Davidson et al., 2010). For these experiments, cells were transiently transduced in adherent culture 48 hours before seeding into matrigel. For the analysis of cell morphology, after 8hrs, the cultures were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS) for 30mins and then photographed by phase contrast microscopy. For each sample, 10 images were taken in random fields and the cells were counted using the NIH Image-J cell counter software.

For transwell invasion assays, U87MG cells were transduced with lentiviral particles encoding PTEN proteins. 48hrs post transduction, the cells were serum starved in migration buffer (DMEM, 1% bovine serum albumin (BSA), 0.5% FCS) for an hour. For inhibitor treatments, 1 $\mu$ M PI103 or 1 $\mu$ M Akti-1/2 was added to the migration buffer. The cells were then scraped off in migration buffer and 400,000 cells in 500 $\mu$ l were plated onto the upper chamber of matrigel transwell invasion chambers (BD Biosciences). 600 $\mu$ l of migration buffer was added to the lower chambers. The cells were allowed to invade for 16hrs and fixed and stained using a Diff-quick staining kit (Reagen). The chambers were dried, imaged at 10x magnification and the cells were counted using the NIH Image-J cell counter software.

### **Immunoblotting**

For protein analysis, adherent cells were washed twice in ice-cold PBS and lysed in ice-cold lysis buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 1mM EGTA, 1mM EDTA, 5mM sodium pyrophosphate, 10mM  $\beta$ -glycerophosphate and 50mM sodium fluoride,) containing freshly added 0.2mM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamidine, 10 $\mu$ g/ml aprotinin, 10 $\mu$ g/ml leupeptin, 1mM Sodium orthovanadate and 0.1% 2-mercaptoethanol. 1 $\mu$ M Microcystin was added when used for phospho-T366 analysis. Equal amounts of proteins were separated by SDS-PAGE using pre-cast 4–12% gradient gels (Invitrogen) and blotted onto PVDF membranes (Polyscreen; NEN/PerkinElmer). Most reagents for electrophoresis and blotting were purchased from Invitrogen, and standard manufacturers' protocols were followed. Western blotting analysis directly of lysates from cells cultured in matrigel was limited to antibodies with

the highest target selectivity by the high concentrations of sample protein derived from this matrix. Quantitation of blotting data was performed using AIDA densitometry software to analyse images obtained either directly from blots using a CDD camera or from processed film using a transmission scanner. Details regarding the specific antibodies used in this study are provided in a Supplementary Methods section.

### **Plasmids and lentiviruses**

Plasmid and lentiviral expression vectors for untagged and GST-tagged human PTEN (21, 31) and GFP-PTEN (46) have been previously described. The expression vectors pHR-SIN PTEN Y138C and pGEX6P1 PTEN Y138C were prepared by site directed mutagenesis of the corresponding PTEN wild type constructs using the following primers. F- GTGTAATGATATGTGCATGTTTATTACATCGGGGC and R- GCCCGATGTAATAAACATGCACATATCATTACAC. PTEN G129R was prepared by site directed mutagenesis of the pHR-SIN PTEN wild type constructs using the following primers F- GATTTCTGCAGAAAGACGTGAAGGCGTATACAGG and R- CCTGTATACGCCTTCACGTCTTTCTGCAGGAAATC. PTEN L42R was prepared by site directed mutagenesis of the pHR-SIN PTEN wild type constructs using the following primers F- CAATTCACCTGTAAAGCTGGAAAGAGACGAACTGGTGTAATG and R- CATTACACCAGTTCGTCTCTTTCCAGCTTTACAGTGAATTG. Plasmids encoding AKT1, AKT2 and AKT3 were kind gifts from Dario Alessi (University of Dundee). The E17K mutagenesis of AKT1 used the primers F- CTGCACAAACGAGGGAAGTACATCAAGACCTGG and R- CCAGGTCTTGATGTACTTCCCTCGTTTGTGCAG. The E17K mutagenesis of AKT2

used the following primers F- CACAAGCGTGGTAAA TACATCAAGACC and R- GGTCTTGATGTATTTACCACGCTTGTG. A FLAG tag and BamHI site was added upstream of the Akt2-E17K using the primers F- GTGGGATCCACCGCCATGGATTACAAGGATGACGACGATAAAAATGAGGTGTCTGTCATC and R- CACGGATCCTCACTCGCGGATGCTGGC and it was cloned into the BamHI site of pHR-SIN vector. The E17K mutagenesis of AKT3 was done using the following primers F- CAGAAGAGGGGAAAATATATAAAAAAC and R- GTTTTTTATATATTTTCCCCTCTTCTG. A FLAG tag and BglII site was added upstream of the Akt3-E17K using the primers F- GTGAGATCTACCGCCATGGATTACAAGGATGACGACGATAAAAGCGATGTTACCATTGTG and R- CACAGATCTCTTATTCTCGTCCACTTG and it was cloned into the BamHI site of pHR-SIN vector. An expression vector for GST-PTEN tail (351-403) was produced from a GFP-PTEN tail (351-403) vector (47) using the restriction enzymes EcoRI and NotI. For PTEN knockdown, pLKO.1-Puro lentiviral shRNA vectors were purchased from Sigma/TRC (Poole, Dorset, UK). The puromycin resistance gene was replaced with a GFP cDNA through a BamHI-KpnI cloning. All PCR clonings and mutagenesis were performed using the KOD Hot start polymerase (Novagen). DpnI enzyme was purchased from Promega.

### **Microarray analysis of gene expression in 3D cultures**

12 well plates were coated with 300µl Geltrex (Invitrogen) and allowed to gel in a 37°C incubator for 60 mins. U87MG cells transiently expressing GFP or PTEN proteins were trypsinised and  $1 \times 10^5$  cells were suspended in 500µl medium and placed on top of the

Geltrex gel. After 4 hours, the medium was aspirated from the culture and an additional 500 µl layer of Geltrex was overlaid and allowed to gel for 60 mins before 0.5ml of media was added. These 3D cultures were incubated for 16 hrs. The top media was then removed and total RNA was isolated using RNeasy kit from Qiagen using standard manufacturers' protocol. Expression microarray analysis was performed by CXR Biosciences, Dundee, UK. RNA integrity was verified using an Agilent Bioanalyzer. 1 colour hybridisation of Cy3 labelled duplicate samples was to Agilent 4x44K Whole Human Genome Oligo Microarray slides (G4112F). Initial data analysis used Rosetta Resolver 6 software to identify probes significantly different between samples in pairwise comparisons. A minimum probability threshold of  $p < 0.01$  was applied in these comparisons. For real time PCR, 2-5µg total RNA was converted to cDNA using Sprint RT random hexamer kit (Clontech). Quantitative PCR used the TaqMan® assay system (Applied Biosystems) and a Step One Plus real time PCR system (Applied Biosystems). The expressed PTEN coding sequences from NCI-H196, U343MG and HEK293T cells were sequenced by preparing total RNA from each cell type and converting to cDNA as described above. Full length PTEN cDNA was PCR amplified and the product was sequenced using internal primers ([www.dnaseq.co.uk](http://www.dnaseq.co.uk), University of Dundee).

### **In-vitro PTEN enzyme assays and cellular PtdInsP<sub>3</sub> measurements**

The preparation of 3-<sup>33</sup>P labelled phosphoinositide substrates, the purification of glutathione S-transferase (GST) tagged PTEN, the removal of the GST tag and further purification of untagged PTEN have been described previously (McConnachie et al., 2003). The phosphorylation of the peptide polymer 4:1 polyGluTyr (Sigma) with insulin

receptor kinase (Upstate) has also been previously described (Leslie et al., 2007; Swarup & Subrahmanyam, 1989). PtdInsP<sub>3</sub> assays were conducted with substrate vesicles prepared by sonication of 100 μM phosphatidylcholine, 1μM unlabeled PtdInsP<sub>3</sub>, and 100,000 dpm 3-[<sup>33</sup>P] PtdInsP<sub>3</sub>. These were incubated in 10mM HEPES pH 7.4, 125mM NaCl, 1mM EGTA, and 10mM DTT with 100ng enzyme at 37°C for the indicated time. PolyGlu-Tyr(P) phosphatase assays were conducted in 25mM HEPES pH 7.4, 1mM EGTA, and 10 mM DTT with 2μg of enzyme and 100,000 dpm (approximately 1 μg) of phosphorylated substrate per assay, also at 37°C for the indicated time. Reactions were terminated directly by the addition of 500 μl of ice-cold 1M perchloric acid and 100 μg/ml BSA, left on ice for 30 min, and spun at 14,000rpm at 4°C for 10 min. The supernatant was removed, and ammonium molybdate was added to a final concentration of 10 mg/ml. After extraction with 2 vol of toluene/isobutanol (1:1 vol/vol), the upper phase was removed and radioactivity was determined by scintillation counting. Cellular PtdInsP<sub>3</sub> measurements were made as described (48).

### **PTEN dephosphorylation in vitro**

A PTEN C-terminal tail substrate was purified by expressing in bacteria a fusion of this region of PTEN (351-403) with an N-terminal GST tag, following expression and purification conditions described for GST-PTEN unless specified. Expression was induced with 1mM IPTG at 30 degrees for 4 hrs. Glutathione sepharose beads (Amersham) with bound GST-PTEN tail were washed with wash buffer (50mM Tris (pH 7.4), 400mM NaCl, 1mM EDTA and protease inhibitors). Approximately 1mg immobilised GST-PTEN tail protein was phosphorylated with 1 unit of CK2 using 2mM

unlabelled ATP for 90 minutes in buffer 20mM HEPES (pH 7.4), 10mM MgCl<sub>2</sub> and 1mM DTT to phosphorylate S370 along with the S380-S385 cluster. The kinase was then extensively washed away using wash buffer before 1 unit of purified GSK3 beta and 20mM unlabelled ATP and radiolabelled  $\gamma^{32}\text{P}$ -ATP (adjusted to 20,000 counts/pmol of phosphate incorporated) were added for 90 minutes to incorporate radiolabelled phosphate specifically onto Thr366. The phosphorylated GST-PTEN tail protein was then extensively washed again in wash buffer (above), before eluting the protein with 20mM reduced glutathione. This purified substrate was then stored at -80°C in 25% glycerol and used in PTEN phosphatase assays as described above for the poly(GluTyr) substrate. Experiments were performed with two independent preparations of phosphorylated substrate. For experiments with lambda phosphatase, GFP-PTEN was expressed in 293T cells, immunoprecipitated using GFP-TRAP beads (ChromoTek) and washed three times in lysis buffer. The beads were incubated with 800-1000 units of GST-Lambda phosphatase (DSTT, University of Dundee) in a buffer containing 50mM Tris-HCl, 0.1mM Na<sub>2</sub>EDTA, 5mM dithiothreitol (DTT), 2mM MnCl<sub>2</sub> and 0.01% Brij-35 (pH 7.5 at 25°C) for 1hr at 30 degrees.

### **Statistical Analyses**

The significance of differences between sample groups was tested using either the students T-test or by one way analysis of variance (ANOVA) and Tukey test as detailed in each figure legend. Further statistical tests applied during the analysis of microarray data are dealt with in a supplementary methods section.

## **Supplementary Materials**

Figure S1. Regulation of AKT phosphorylation by wild-type PTEN and PTEN Y138L

Figure S2. Assigned gene sets associated with transcript changes induced by wild-type PTEN expression in U87MG cells

Figure S3. Autodephosphorylation analysis of PTEN mutant proteins

Figure S4. Effects of kinase inhibition and hyperosmotic stress on PTEN Thr366 phosphorylation

Figure S5. Further analysis of PTEN Thr366 phosphorylation

Figure S6. Effects of PTEN on U87MG cell morphology in 3D matrigel cultures

Figure S7. Quantitation of blotting data

Figure S8. Quantitation and statistical analysis of blotting data

Table S1. Wild type PTEN responsive gene list used for clinical data comparison

Supplementary Methods 1. 1. Details of Antibodies

Supplementary Methods 2. Additional analysis of microarray data

Supplementary Methods 3. Bioinformatic comparisons with available clinical data

## References

1. M. Keniry, R. Parsons, The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene* **27**, 5477-5485 (2008).
2. L. Salmena, A. Carracedo, P. P. Pandolfi, Tenets of PTEN tumor suppression. *Cell* **133**, 403-414 (2008).
3. N. R. Leslie, M. Foti, Non-genomic loss of PTEN function in cancer: not in my genes. *Trends Pharmacol Sci* **32**, 131-140 (2011).
4. C. B. Knobbe, V. Lapin, A. Suzuki, T. W. Mak, The roles of PTEN in development, physiology and tumorigenesis in mouse models: a tissue-by-tissue survey. *Oncogene* **27**, 5398-5415 (2008).
5. P. T. Hawkins, K. E. Anderson, K. Davidson, L. R. Stephens, Signalling through Class I PI3Ks in mammalian cells. *Biochem Soc Trans* **34**, 647-662 (2006).
6. P. G. Charest, R. A. Firtel, Feedback signaling controls leading-edge formation during chemotaxis. *Curr Opin Genet Dev* **16**, 339-347 (2006).
7. A. Gassama-Diagne, W. Yu, M. ter Beest, F. Martin-Belmonte, A. Kierbel, J. Engel, K. Mostov, Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nature cell biology* **8**, 963-970 (2006).
8. S. H. Shi, L. Y. Jan, Y. N. Jan, Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112**, 63-75 (2003).
9. O. D. Weiner, Regulation of cell polarity during eukaryotic chemotaxis: the chemotactic compass. *Current opinion in cell biology* **14**, 196-202 (2002).
10. S. K. Yoo, Q. Deng, P. J. Cavnar, Y. I. Wu, K. M. Hahn, A. Huttenlocher, Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. *Developmental cell* **18**, 226-236 (2010).
11. N. H. Chadborn, A. I. Ahmed, M. R. Holt, R. Prinjha, G. A. Dunn, G. E. Jones, B. J. Eickholt, PTEN couples Sema3A signalling to growth cone collapse. *J Cell Sci* **119**, 951-957 (2006).
12. B. Heit, S. M. Robbins, C. M. Downey, Z. Guan, P. Colarusso, B. J. Miller, F. R. Jirik, P. Kubes, PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils. *Nat Immunol* **9**, 743-752 (2008).
13. M. Iijima, P. Devreotes, Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* **109**, 599-610 (2002).
14. F. Martin-Belmonte, A. Gassama, A. Datta, W. Yu, U. Rescher, V. Gerke, K. Mostov, PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* **128**, 383-397 (2007).
15. D. J. Freeman, A. G. Li, G. Wei, H. H. Li, N. Kertesz, R. Lesche, A. D. Whale, H. Martinez-Diaz, N. Rozengurt, R. D. Cardiff, X. Liu, H. Wu, PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* **3**, 117-130 (2003).
16. N. R. Leslie, H. Maccario, L. Spinelli, L. Davidson, The significance of PTEN's protein phosphatase activity. *Advances in enzyme regulation* **49**, 190-196 (2009).

17. M. P. Myers, J. P. Stolarov, C. Eng, J. Li, S. I. Wang, W. M.H., R. Parsons, N. K. Tonks, PTEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci U S A* **94**, 9052-9057 (1997).
18. K. Okumura, M. Zhao, R. A. Depinho, F. B. Furnari, W. K. Cavenee, Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor. *Proc Natl Acad Sci U S A* **102**, 2703-2706 (2005).
19. M. Raftopoulou, S. Etienne-Manneville, A. Self, S. Nicholls, A. Hall, Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science (New York, N.Y)* **303**, 1179-1181 (2004).
20. W. H. Shen, A. S. Balajee, J. Wang, H. Wu, C. Eng, P. P. Pandolfi, Y. Yin, Essential Role for Nuclear PTEN in Maintaining Chromosomal Integrity. *Cell* **128**, 157-170 (2007).
21. L. Davidson, H. Maccario, N. M. Perera, X. Yang, L. Spinelli, P. Tibarewal, B. Glancy, A. Gray, C. J. Weijer, C. P. Downes, N. R. Leslie, Suppression of cellular proliferation and invasion by the concerted lipid and protein phosphatase activities of PTEN. *Oncogene* **29**, 687-697 (2010).
22. F. B. Furnari, H. J. Huang, W. K. Cavenee, The phosphoinositol phosphatase activity of PTEN mediates a serum- sensitive G1 growth arrest in glioma cells. *Cancer Res* **58**, 5002-5008 (1998).
23. M. P. Myers, I. Pass, I. H. Batty, J. Van der Kaay, J. P. Stolarov, B. A. Hemmings, M. H. Wigler, C. P. Downes, N. K. Tonks, The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc Natl Acad Sci U S A* **95**, 13513-13518 (1998).
24. D. Birle, N. Bottini, S. Williams, H. Huynh, I. deBelle, E. Adamson, T. Mustelin, Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation. *J Immunol* **169**, 286-291 (2002).
25. A. M. Al-Khouri, Y. Ma, S. H. Togo, S. Williams, T. Mustelin, Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. *J Biol Chem* **280**, 35195-35202 (2005).
26. H. Maccario, N. M. Perera, L. Davidson, C. P. Downes, N. R. Leslie, PTEN is destabilized by phosphorylation on Thr366. *Biochem J* **405**, 439-444 (2007).
27. J. Torres, R. Pulido, The Tumor Suppressor PTEN Is Phosphorylated by the Protein Kinase CK2 at Its C Terminus. IMPLICATIONS FOR PTEN STABILITY TO PROTEASOME- MEDIATED DEGRADATION. *J Biol Chem* **276**, 993-998. (2001).
28. F. Vazquez, S. Ramaswamy, N. Nakamura, W. R. Sellers, Phosphorylation of the PTEN tail regulates protein stability and function. *Molecular and cellular biology* **20**, 5010-5018 (2000).
29. J. T. Murray, D. G. Campbell, N. Morrice, G. C. Auld, N. Shpiro, R. Marquez, M. Pegg, J. Bain, G. B. Bloomberg, F. Grahmmer, F. Lang, P. Wulff, D. Kuhl, P. Cohen, Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem J* **384**, 477-488 (2004).

30. N. R. Leslie, D. Bennett, Y. E. Lindsay, H. Stewart, A. Gray, C. P. Downes, Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *The EMBO journal* **22**, 5501-5510 (2003).
31. H. Maccario, N. M. Perera, A. Gray, C. P. Downes, N. R. Leslie, Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress. *J Biol Chem* **285**, 12620-12628 (2010).
32. L. Odriezola, G. Singh, T. Hoang, A. M. Chan, Regulation of PTEN activity by its carboxyl-terminal autoinhibitory domain. *J Biol Chem* **282**, 23306-23315 (2007).
33. M. Rahdar, T. Inoue, T. Meyer, J. Zhang, F. Vazquez, P. N. Devreotes, A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. *Proc Natl Acad Sci U S A* **106**, 480-485 (2009).
34. S. Y. Han, H. Kato, S. Kato, T. Suzuki, H. Shibata, S. Ishii, K. Shiiba, S. Matsuno, R. Kanamaru, C. Ishioka, Functional evaluation of PTEN missense mutations using in vitro phosphoinositide phosphatase assay. *Cancer Res* **60**, 3147-3151 (2000).
35. N. R. Leslie, D. Bennett, A. Gray, I. Pass, K. Hoang-Xuan, C. P. Downes, Targeting mutants of PTEN reveal distinct subsets of tumour suppressor functions. *Biochem J* **357**, 427-435. (2001).
36. E. Forgacs, E. J. Biesterveld, Y. Sekido, K. Fong, S. Muneer, Wistuba, II, S. Milchgrub, R. Brezinschek, A. Virmani, A. F. Gazdar, J. D. Minna, Mutation analysis of the PTEN/MMAC1 gene in lung cancer. *Oncogene* **17**, 1557-1565 (1998).
37. M. L. Chen, P. Z. Xu, X. D. Peng, W. S. Chen, G. Guzman, X. Yang, A. Di Cristofano, P. P. Pandolfi, N. Hay, The deficiency of Akt1 is sufficient to suppress tumor development in Pten<sup>+/-</sup> mice. *Genes Dev* **20**, 1569-1574 (2006).
38. S. Wullschlegel, K. Sakamoto, L. Johnstone, S. Duce, S. Fleming, D. R. Alessi, How moderate changes in Akt T-loop phosphorylation impact on tumorigenesis and insulin resistance. *Dis Model Mech*, (2010).
39. D. F. Restuccia, B. A. Hemmings, From man to mouse and back again: advances in defining tumor AKTivities in vivo. *Dis Model Mech* **3**, 705-720 (2010).
40. N. Rhodes, D. A. Heerding, D. R. Duckett, D. J. Eberwein, V. B. Knick, T. J. Lansing, R. T. McConnell, T. M. Gilmer, S. Y. Zhang, K. Robell, J. A. Kahana, R. S. Geske, E. V. Kleymenova, A. E. Choudhry, Z. Lai, J. D. Leber, E. A. Minthorn, S. L. Strum, E. R. Wood, P. S. Huang, R. A. Copeland, R. Kumar, Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity. *Cancer Res* **68**, 2366-2374 (2008).
41. J. O. Lee, H. Yang, M. M. Georgescu, A. Di Cristofano, T. Maehama, Y. Shi, J. E. Dixon, P. Pandolfi, N. P. Pavletich, Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323-334 (1999).
42. N. Pinal, D. C. Goberdhan, L. Collinson, Y. Fujita, I. M. Cox, C. Wilson, F. Pichaud, Regulated and polarized PtdIns(3,4,5)P3 accumulation is essential for

- apical membrane morphogenesis in photoreceptor epithelial cells. *Curr Biol* **16**, 140-149 (2006).
43. M. T. van Diepen, M. Parsons, C. P. Downes, N. R. Leslie, R. Hindges, B. J. Eickholt, MyosinV controls PTEN function and neuronal cell size. *Nature cell biology* **11**, 1191-1196 (2009).
44. F. I. Comer, C. A. Parent, Phosphoinositides specify polarity during epithelial organ development. *Cell* **128**, 239-240 (2007).
45. S. Merlot, R. A. Firtel, Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J Cell Sci* **116**, 3471-3478 (2003).
46. N. R. Leslie, A. Gray, I. Pass, E. A. Orchiston, C. P. Downes, Analysis of the cellular functions of PTEN using catalytic domain and C- terminal mutations: differential effects of C-terminal deletion on signalling pathways downstream of phosphoinositide 3-kinase. *Biochem J* **346**, 827-833 (2000).
47. N. R. Leslie, X. Yang, C. P. Downes, C. J. Weijer, PtdIns(3,4,5)P3-dependent and -independent roles for PTEN in the control of cell migration. *Curr Biol* **17**, 115-125 (2007).
48. A. Gray, H. Olsson, I. H. Batty, L. Priganica, C. Peter Downes, Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts. *Anal Biochem* **313**, 234-245 (2003).
49. We would like to thank Hilary McLauchlan, James Hastie and the staff in the DSTT (University of Dundee) for provision of purified antibodies and purified lambda phosphatase. We also thank Dario Alessi for providing cDNAs encoding AKT1, 2 and 3 and Peter Downes for helpful discussions and critical reading of the manuscript. GZ was funded by a Wellcome Trust Clinical PhD Fellowship. Work in the Inositol Lipid Signalling laboratory is funded by the Medical Research Council, the Association for International Cancer Research and the pharmaceutical companies of the DSTT consortium (Astra Zeneca, Boehringer Ingelheim, GlaxoSmithKline, Merck Serono and Pfizer).

## Figure legends

**Fig. 1.** PTEN requires both lipid and protein phosphatase activity to inhibit invasion. **(A-D)** U87MG cells were transduced with lentiviruses encoding GFP, untagged wild-type (WT) PTEN or PTEN point mutants before **(A-C)** being assayed for invasion over 16 hours. One set of samples was co-transduced with both untagged PTEN Y138L and GFP-PTEN G129E. Representative of three experiments **(A)** Invading cells were fixed, stained and photographed. **(B)** Quantification represents the mean number of cells from ten randomly selected fields  $\pm$  S.E.M. \*\*\* shows  $p < 0.001$  t-test **(C)** PTEN expression and AKT phosphorylation were verified by immunoblotting of adherent cell lysates, including untransduced cells and GFP expressing cells treated with PI3K inhibitor ( $1\mu\text{M}$  PI103, 30 mins). HEK293 cell lysates were included to ensure PTEN expression is close to physiological. **(D)** Transduced U87MG cells grown in matrigel for 3 days, fixed and stained for F-actin and DNA. **(E)** Invasion assays were performed in DBTRG-05MG PTEN null glioma cells. Quantitation of invasion is as performed in panel (B) and immunoblotting data is as panel (C) \*\*\* denotes  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$  t-test. DBTRG-05MG experiments were performed twice with similar results. **(F)** Table representing activities of PTEN wild type and mutants.

**Fig. 2.** PTEN requires both lipid and protein phosphatase activities for many of its effects on gene expression. U87MG cells were transduced with lentiviruses encoding GFP or the indicated PTEN proteins and seeded into 3D matrigel cultures for 16h. RNA was prepared and gene expression analysed by microarray. **(A)** Mean transcript changes from

duplicate samples relative to sham transduced controls. Blue denotes upregulation, yellow downregulation. **(B and C)** Numbers of probes changed relative to 'No virus' sample at  $p < 0.01$  and 5-fold change threshold. Significance levels are output from Rosetta Resolver software. Immunoblotting shows PTEN expression and AKT phosphorylation in transduced adherent cells **(D)** Plot of fold changes for all probes in response to wild-type (WT) and Y138L PTEN expression. Probes are coloured according to the sum probability of their expression change. Note most probes respond either similarly to both WT and Y138L (clustering on diagonal) or only to WT PTEN (clustering near X axis). **(E)** Overlap analysis between a gene set most strongly regulated selectively by wild-type PTEN (169 probes with largest fold change in (C)) and publicly available microarray data from human gliomas (84% with a recognised loss of PTEN expression or gene dose). **(F)** PTEN expression and AKT phosphorylation by immunoblotting from matrigel cultures.

**Fig. 3.** Evidence that PTEN autodephosphorylates Thr366. U87MG cells transiently expressing wild-type PTEN or PTEN point mutants were lysed, total PTEN was immunoprecipitated and phosphorylation investigated by replicate phospho-specific immuno-blotting. In **(A)** and **(B)**, unphosphorylated bacterially expressed PTEN or GST-PTEN was used as a control. Representative of 3 experiments. **(C)** U87MG cells transiently expressing PTEN or catalytically dead PTEN C124S were treated with the GSK3 inhibitor CT99021 ( $5\mu\text{M}$ ) for up to 4 hours as shown. PTEN Thr366 phosphorylation was then investigated by lysis, immunoprecipitation and immunoblotting. In order to directly compare the rate of dephosphorylation of these

proteins, a longer film exposure is presented for the more weakly phosphorylated wild-type protein. Representative of 3 experiments. **(D)**. GST-PTEN tail (351-403) phosphorylated with  $^{32}\text{P}$  on T366 (see Fig S3C) was used as a substrate for PTEN protein phosphatase activity. Data are presented as mean picomoles phosphate from triplicate samples  $\pm$  S.E.M. Two way comparisons were made between PTEN WT and individual PTEN mutants. \*\*\* shows  $p < 0.001$  t-test. This experiment was repeated three times using two independent substrate preparations. Quantitation of blotting data from (A) and (B) is presented in Fig. S7.

**Fig. 4.** PTEN Thr366 phosphorylation inversely correlates with PTEN activity in a protein molecule autonomous manner. **(A)** U87MG cells transiently expressing wild-type PTEN were treated for one hour with either 0.5M sorbitol, 1mM hydrogen peroxide, 100 $\mu\text{M}$  zinc sulphate or washed into HEPES buffered Krebs Ringer, also for one hour. **(B)** U87MG cells were simultaneously co-transduced to high expression levels with viruses encoding catalytically inactive GFP-PTEN C124S and untagged PTEN that was either catalytically active (WT) or also catalytically dead (C124S). Cells were lysed, total PTEN was immunoprecipitated and phosphorylation investigated by replicate phospho-specific immuno-blotting. **(C)** GFP PTEN WT and phosphatase dead (C124S) were expressed in 293T cells and immunoprecipitated from cell lysates. These immune complexes were divided and one half dephosphorylated with  $\lambda$ -phosphatase before western blotting. All data are representative of at least 3 experiments. Quantitation of blotting data from (A) and (B) is presented in Fig. S7.

**Fig. 5.** Non-phosphorylatable mutation of Thr366 rescues the ability of PTEN Y138L to inhibit invasion. U87MG cells were transduced with recombinant lentiviruses encoding GFP, wild-type PTEN or PTEN point mutants. **(A)** Cells were then serum starved for 1hr in the presence or absence of PI3K inhibitor (PI103, 1 $\mu$ M) or Akt inhibitor (Akti1/2, 1 $\mu$ M), and assayed for matrigel transwell invasion over 16 hrs. The cells were fixed, stained and photographed in random fields. **(B)** Quantification represents the mean number of cells from ten randomly selected fields  $\pm$  S.E.M. \*\*\* shows  $p < 0.001$ , t test. Expression of PTEN and the effects of PTEN and the inhibitors on AKT phosphorylation were verified by western blotting. **(C)** U87MG cells were transduced with GFP, wild-type PTEN or both wild-type PTEN and oncogenic active E17K mutants of AKT1, AKT2 or AKT3. Transduced cells were then assayed for invasion and parallel samples analysed by immunoblotting. All cells transduced with PTEN displayed significantly reduced invasion, but there was no significant effect of mutant AKT expression (\*\* shows  $P < 0.01$ , ns not significant, t test). The experiments in (A and B) and (C) are representative of three and two experiments respectively.

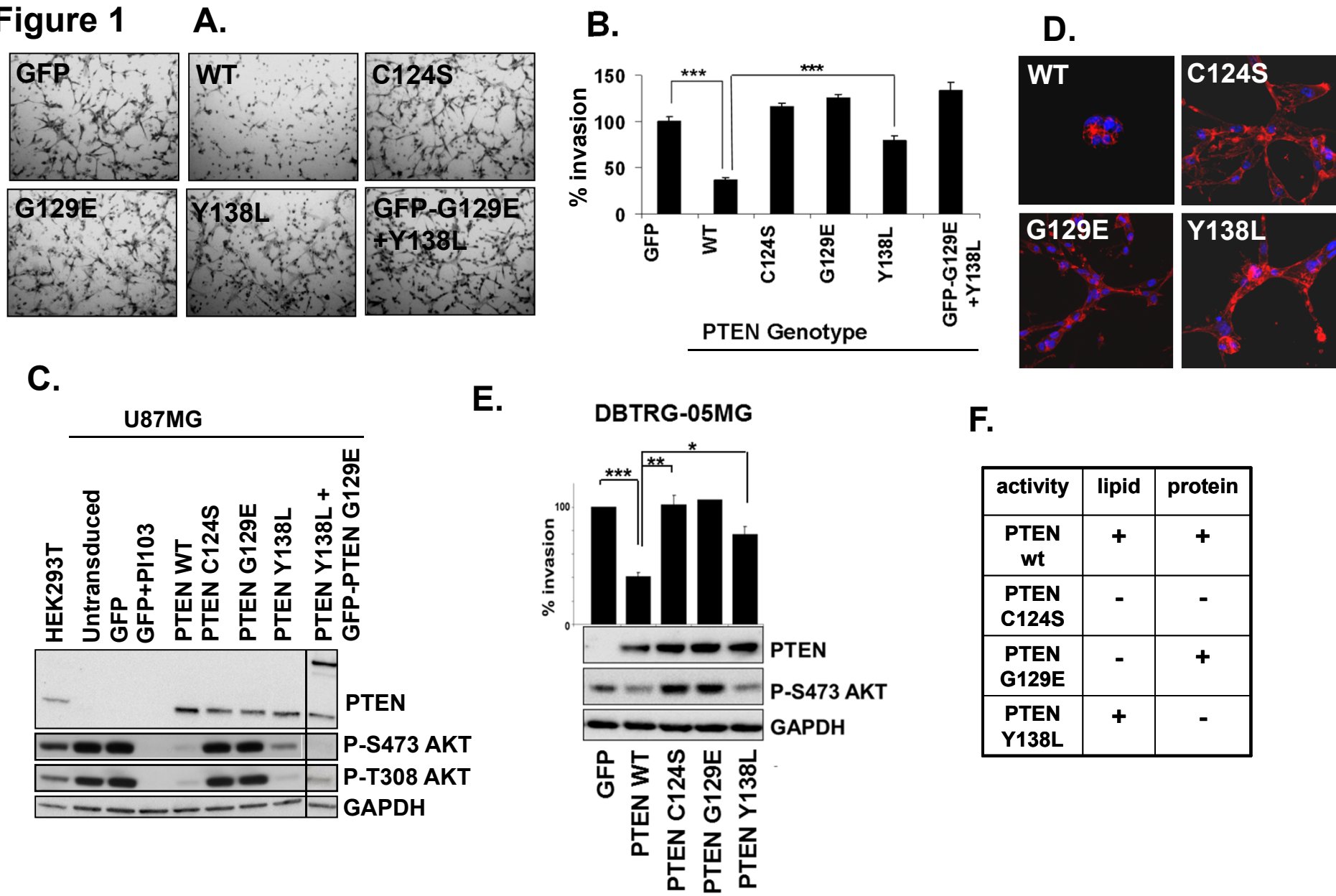
**Fig. 6.** PTEN Y138C selectively retains lipid phosphatase activity and regulates PtdInsP<sub>3</sub> and AKT, but not invasion. **(A)** Purified PTEN wild type (WT), Y138L and Y138C. **(B and C)** Timecourse activity of the indicated proteins assayed using **(B)** radiolabelled PtdInsP<sub>3</sub> in vesicles and **(C)** radiolabelled phosphorylated PolyGluTyr substrates. Data are mean activity  $\pm$  range/2 from duplicate assays. Representative of  $n=5$ . **(D, E, F and G)** U87MG cells were transduced with lentiviruses encoding GFP, PTEN or PTEN mutants or treated with PI3K inhibitor wortmannin (100nM, 20mins). **(D)** PTEN

expression and AKT signalling analysed by immunoblotting with PTEN, P-Thr308 AKT, P-Ser473 AKT, P-Ser 21/9 GSK3 and AKT-substrate phosphorylation consensus antibodies. Representative of n=4 **(E)** Total cellular PtdInsP<sub>3</sub> measured and presented as mean of triplicate samples ± S.E.M.. Wort represents a wortmannin treated control. Experiment performed once with duplicate analyses of three cell samples for each condition **(F)** Transduced cells were assayed for invasion over 16hrs. The cells were fixed, stained, photographed and **(G)** counted. Quantification represents the mean cell number from ten randomly selected fields ± S.E.M. \*\*\* p<0.001, t test. Representative of three experiments. Quantitation and statistical analysis of blotting data from **(D)** is presented in Fig. S8.

**Fig. 7.** PTEN Y138C endogenously expressed in NCI-H196 small cell lung carcinoma cells suppresses AKT activity. **(A and B)** Total RNA was isolated from HEK293T cells, U343MG cells and NCI-H196 cells, reverse transcribed and the expressed full length PTEN cDNA population sequenced. **(C)** Endogenous PTEN protein was immunoprecipitated from HEK293T, U343MG and NCI-H196 cells. Immune complexes were assayed against radiolabelled PtdInsP<sub>3</sub> vesicles and phosphorylated PolyGluTyr. The activity is shown as a mean activity +/- range/2 from duplicate assays normalised to the activity of HEK293T cells. Representative of 3 experiments. **(D)** PTEN was immunoprecipitated from HEK293T, U343MG and NCI-H196 cells and phosphorylation of PTEN investigated by replicate phospho-specific immunoblotting. Representative of 3 experiments. **(E)** PTEN expression was knocked down using lentiviruses encoding PTEN shRNA in HEK293T cells expressing wild type PTEN, U343MG cells expressing

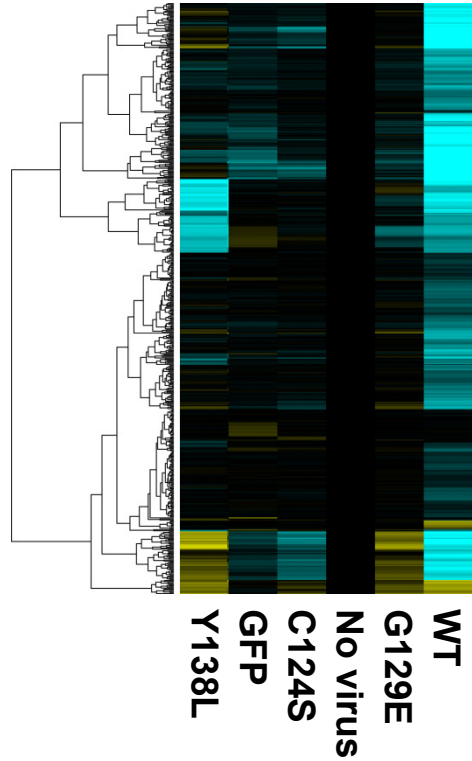
catalytically dead PTEN C124S mutant and NCI-H196 cells expressing protein phosphatase dead PTEN Y138C mutant. Effects on AKT phosphorylation were determined by western blotting. Representative of 4 experiments. Quantitation and statistical analysis of blotting data from (E) is presented in Fig. S8.

**Figure 1**

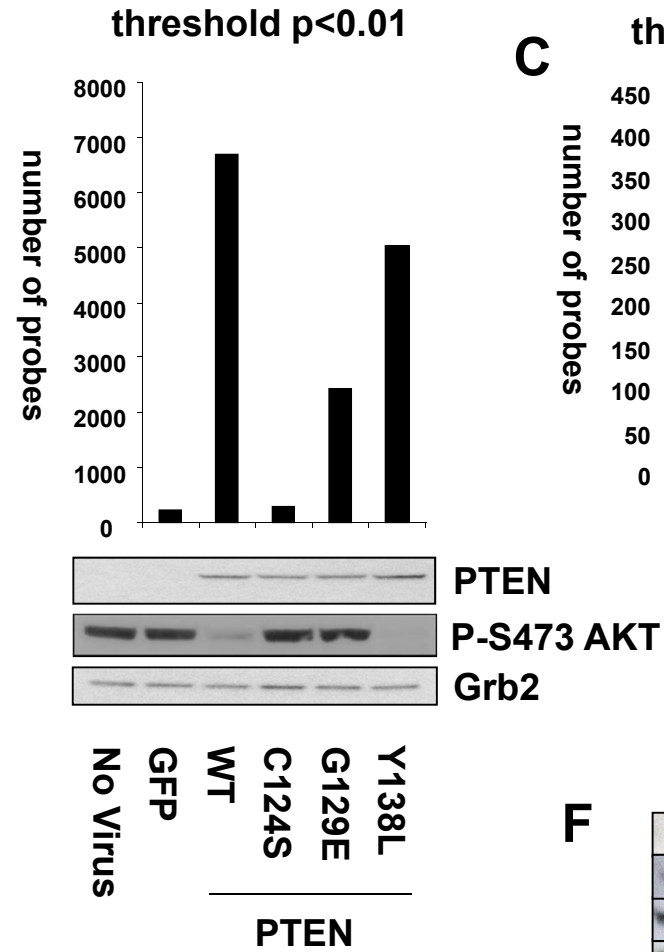


**Figure 2**

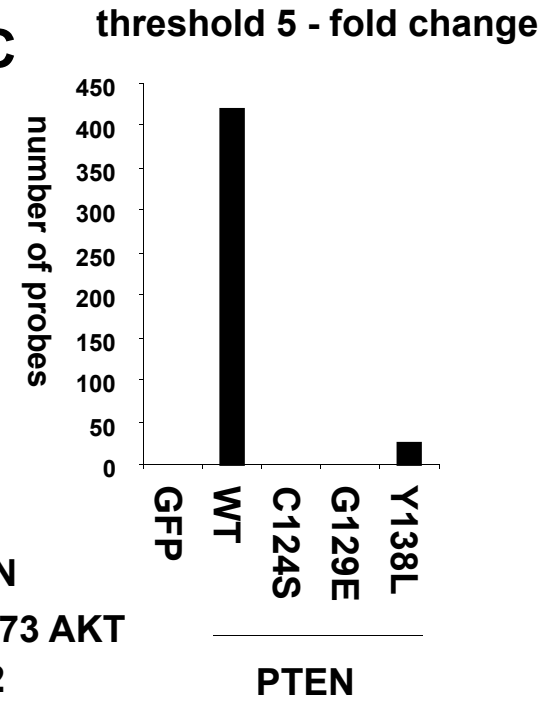
**A**



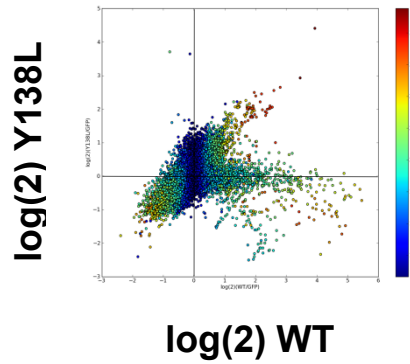
**B**



**C**



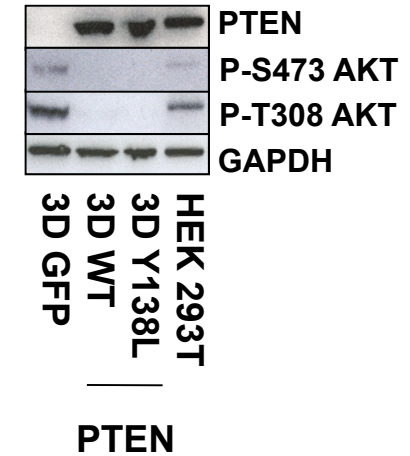
**D**



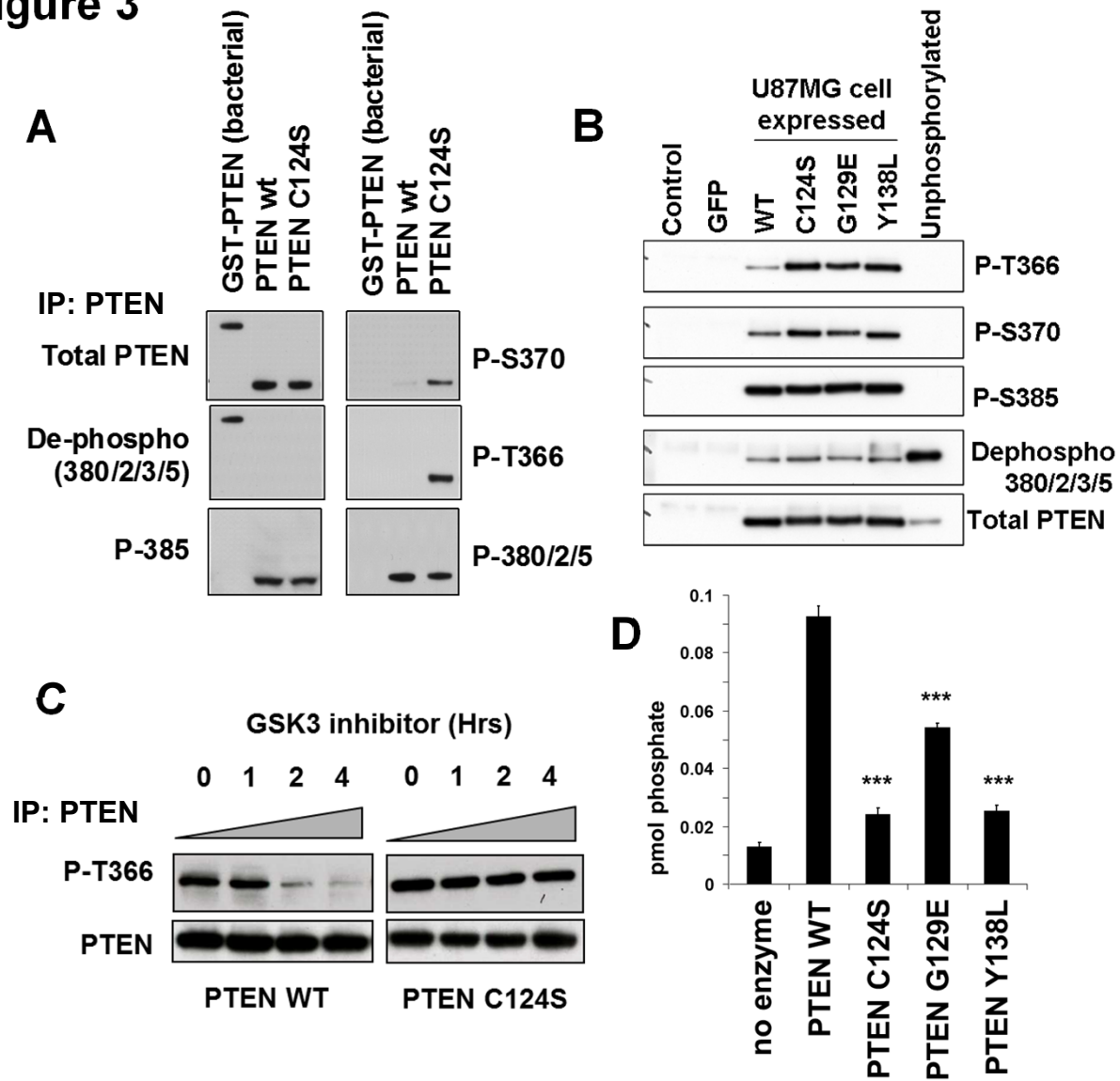
**E**

TCGA glioma data	Probability of overlap with PTEN WT specific signature
Upregulated genes	$p = 0.96 \pm 0.01$
Downregulated genes	$p = 0 + 0.01$

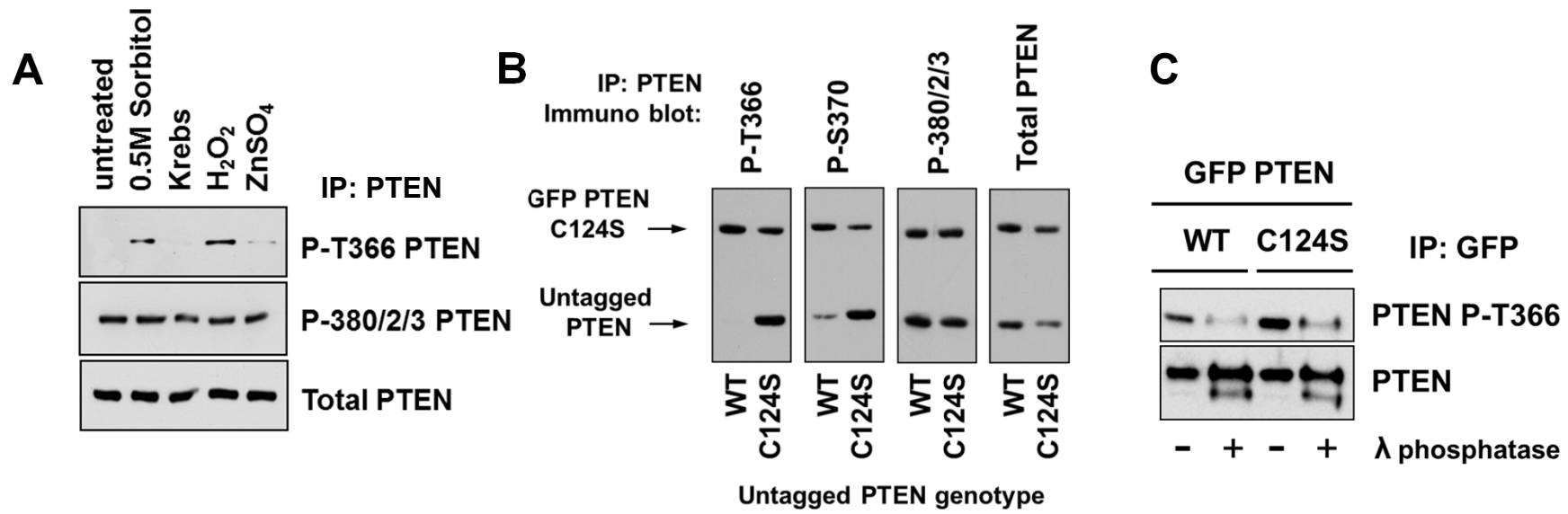
**F**



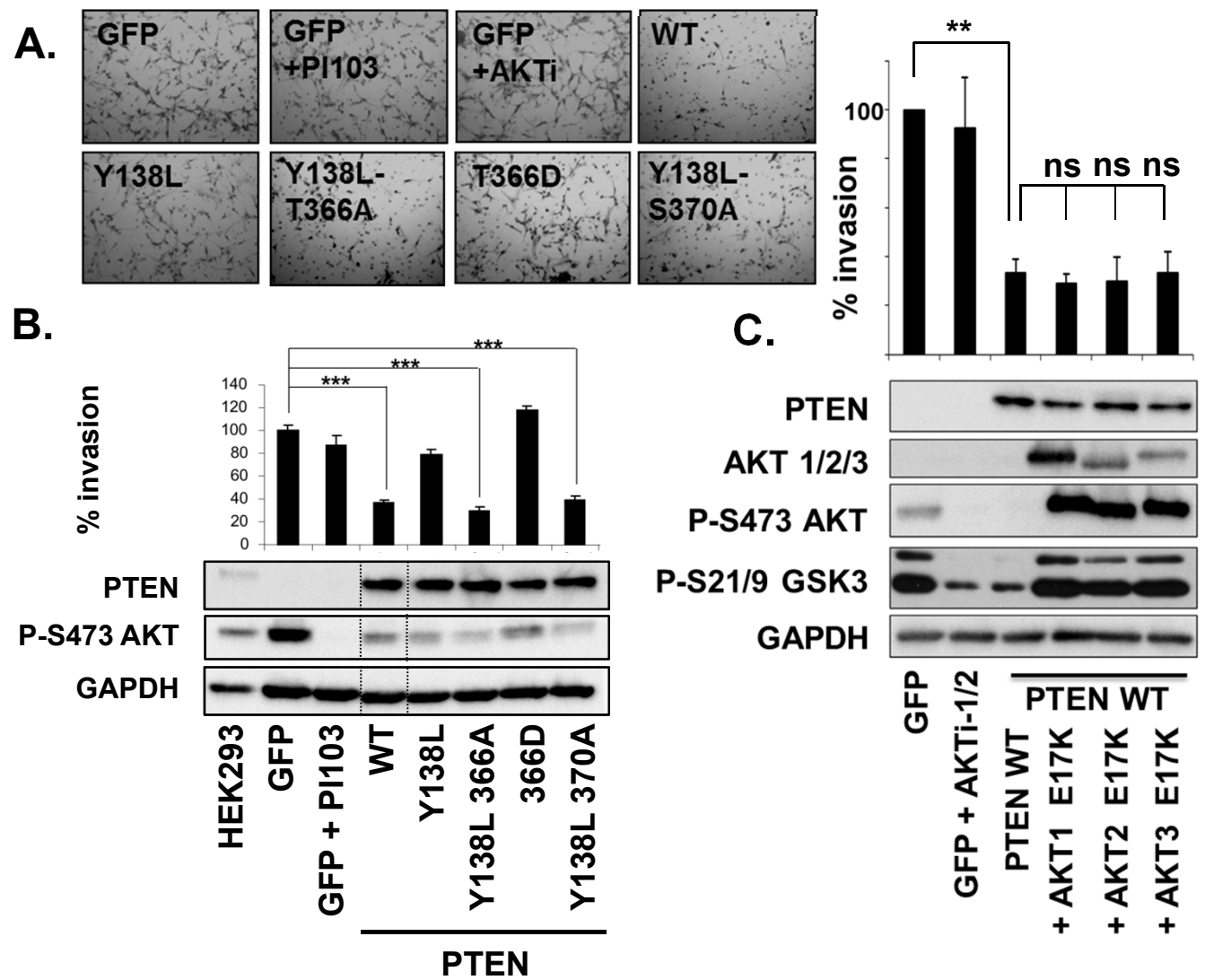
**Figure 3**



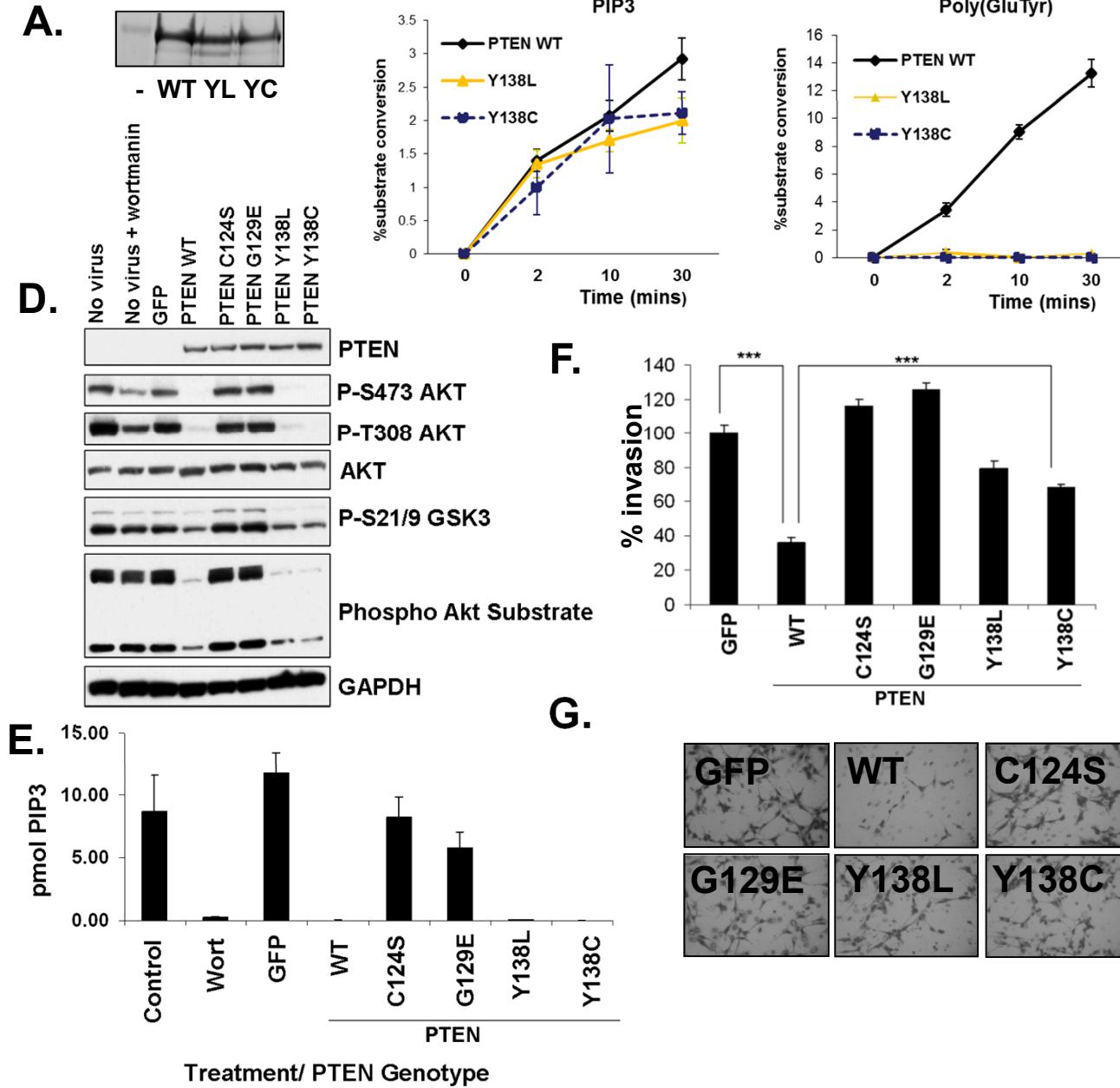
**Figure 4**



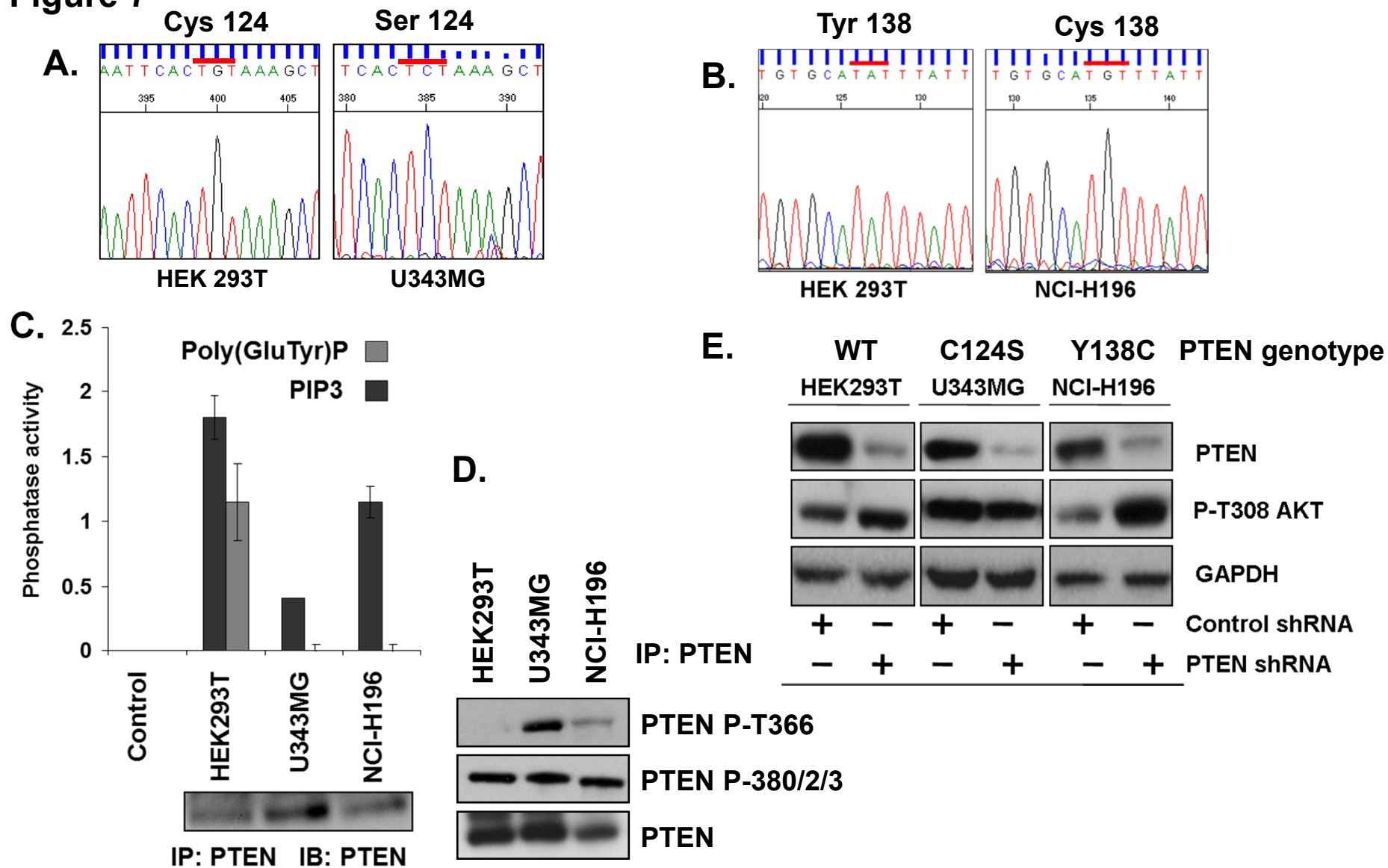
**Figure 5**



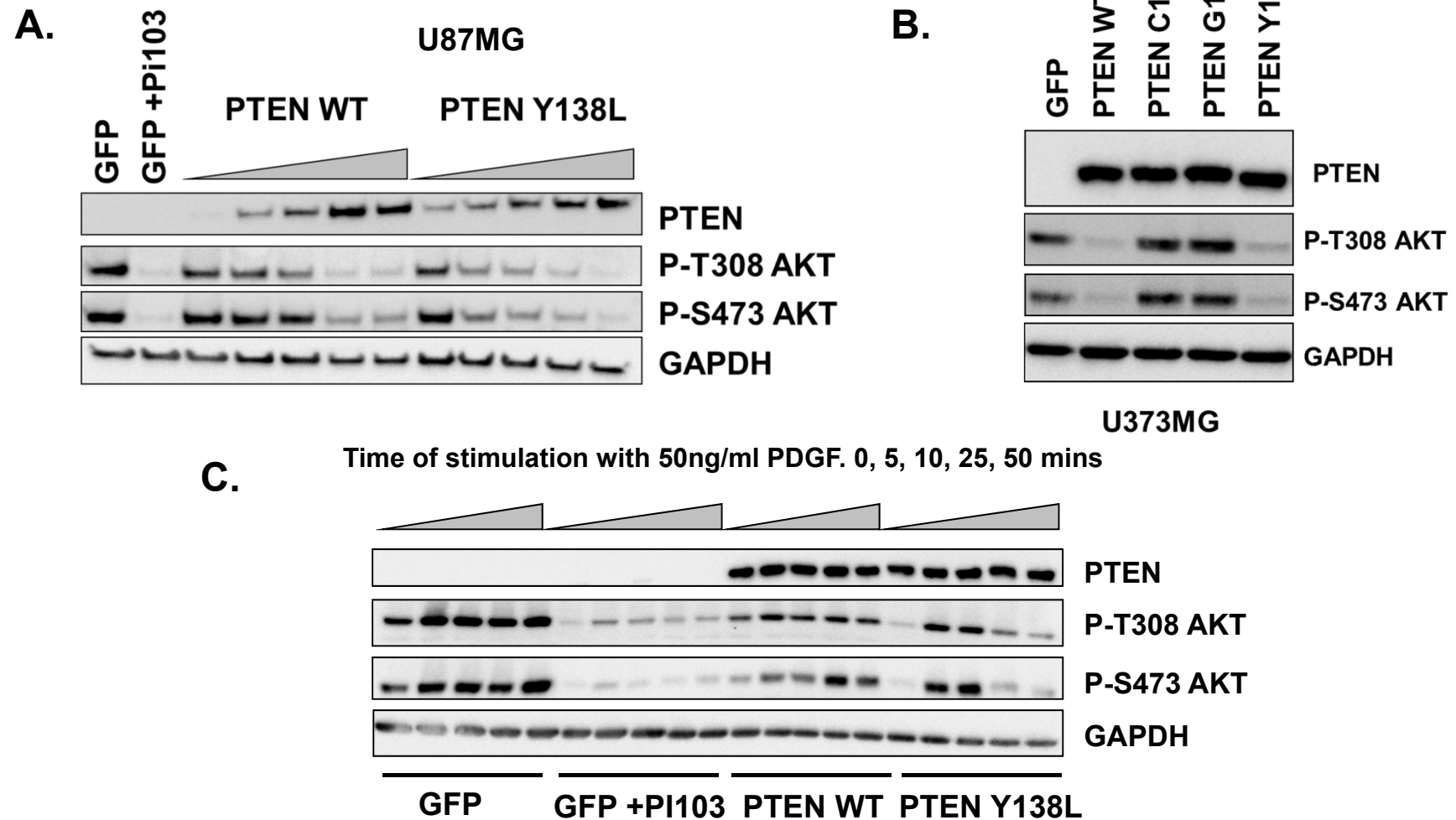
**Figure 6**



**Figure 7**

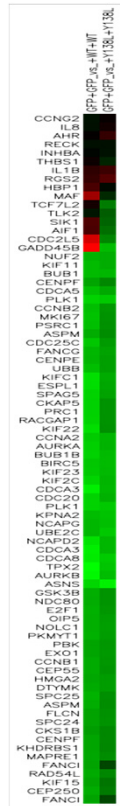


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Supplementary Figures

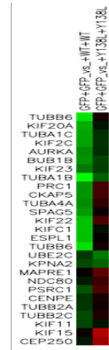


**Figure S1.** Regulation of AKT phosphorylation by wild-type PTEN and PTEN Y138L. The PTEN null glioma cell lines U87MG cells (**A** and **C**) and U373MG cells (**B**) were transduced as shown with lentiviruses encoding GFP, PTEN or mutant enzymes. 48 hours after transduction, cells were lysed and AKT phosphorylation assessed by phospho-specific immunoblotting. In (**C**) cells were stimulated with 50ng/ml PDGF for the indicated times before lysis. Data are representative of 2 experiments. Analysis of homogeneous GFP expression profiles in lentivirally transduced U87MG cell populations and selectable marker analysis indicates that even the lower lentiviral titres used in (**A**) successfully transduce almost all (90%+) of the cell population and therefore that increasing virus delivery increases PTEN expression per cell.

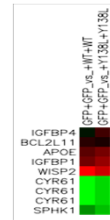
**Cell cycle**  
p<0.001



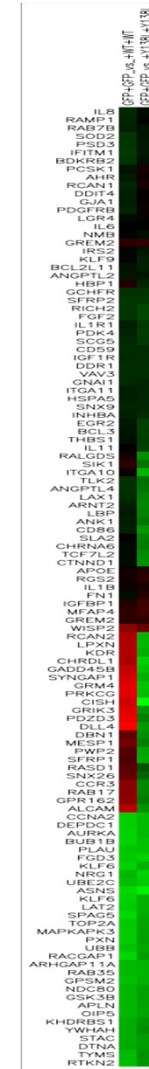
**Microtubule regulation**  
p<0.001



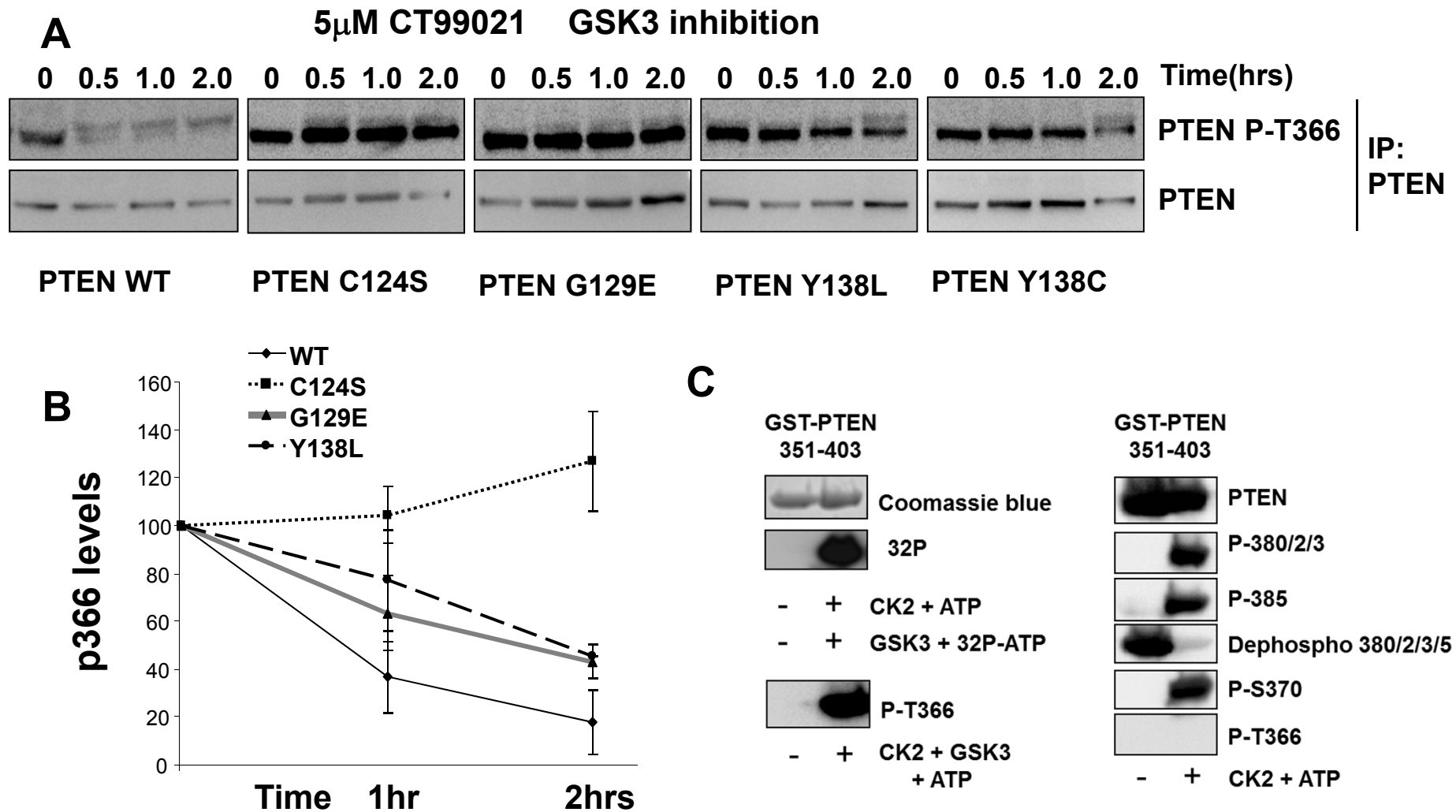
**Cell growth**  
p=0.004



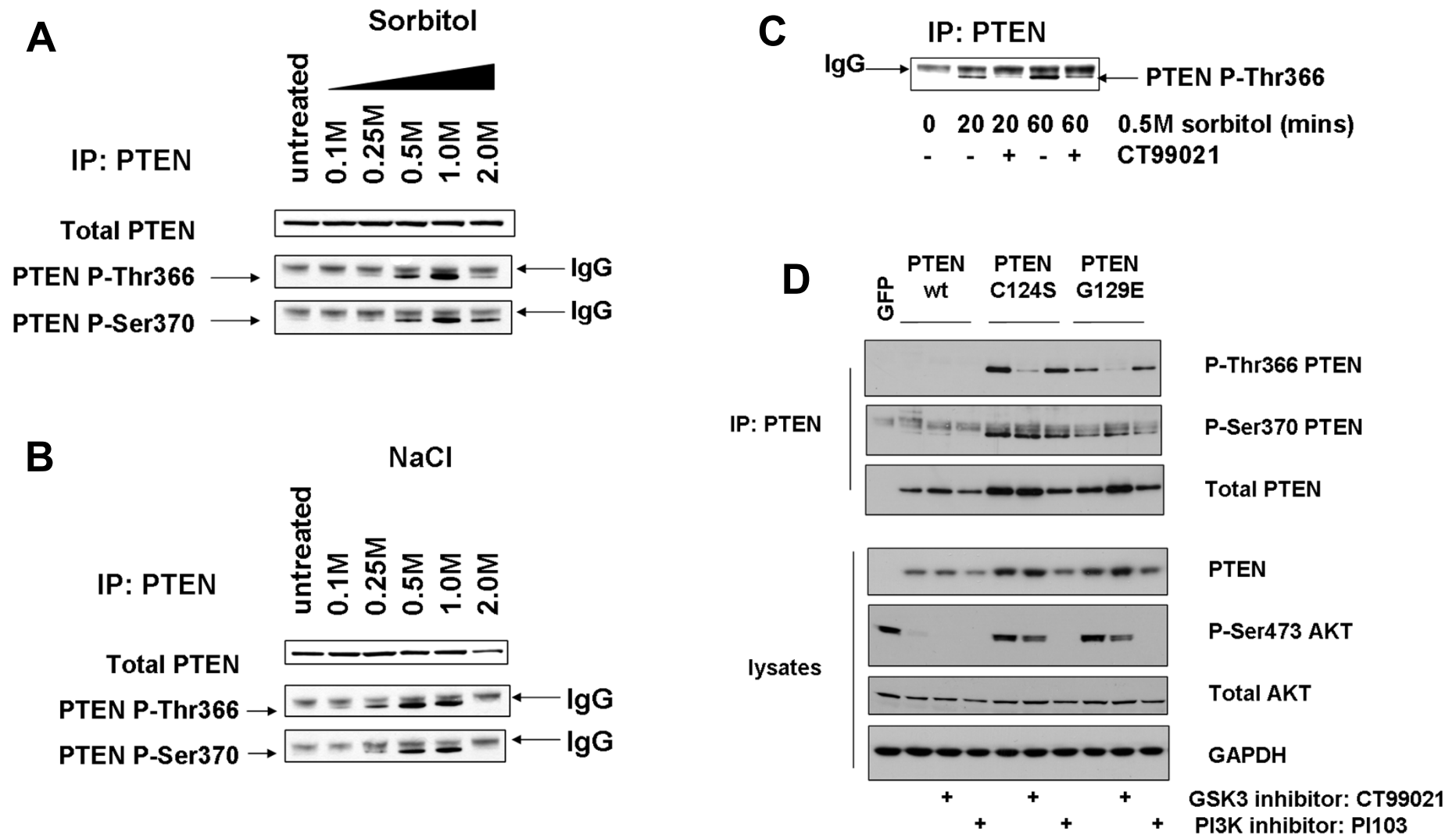
**Cell signalling**  
p=0.012



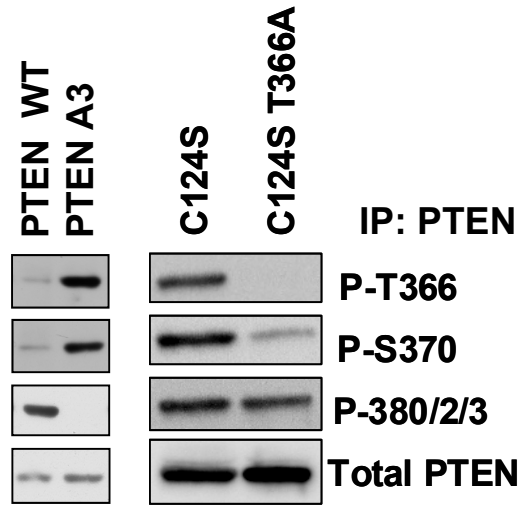
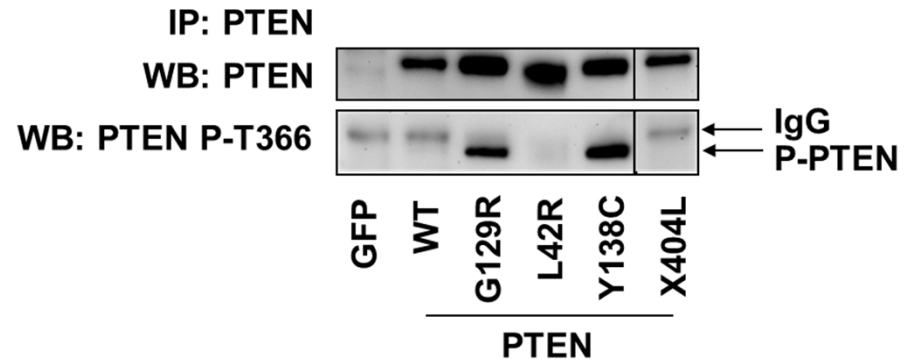
**Figure S2.** Assigned gene sets associated with transcript changes induced by wild-type PTEN expression in U87MG cells. Analysis used annotated gene sets defined by the Gene Ontology consortium. For each gene set, differential gene expression between GFP control and WT PTEN expressing samples is shown, along with data comparing GFP expression with PTEN Y138L. Presented sets were selected based upon probability value, number of associating transcripts and specificity of the gene set definition. Red denotes upregulation and green denotes downregulation.



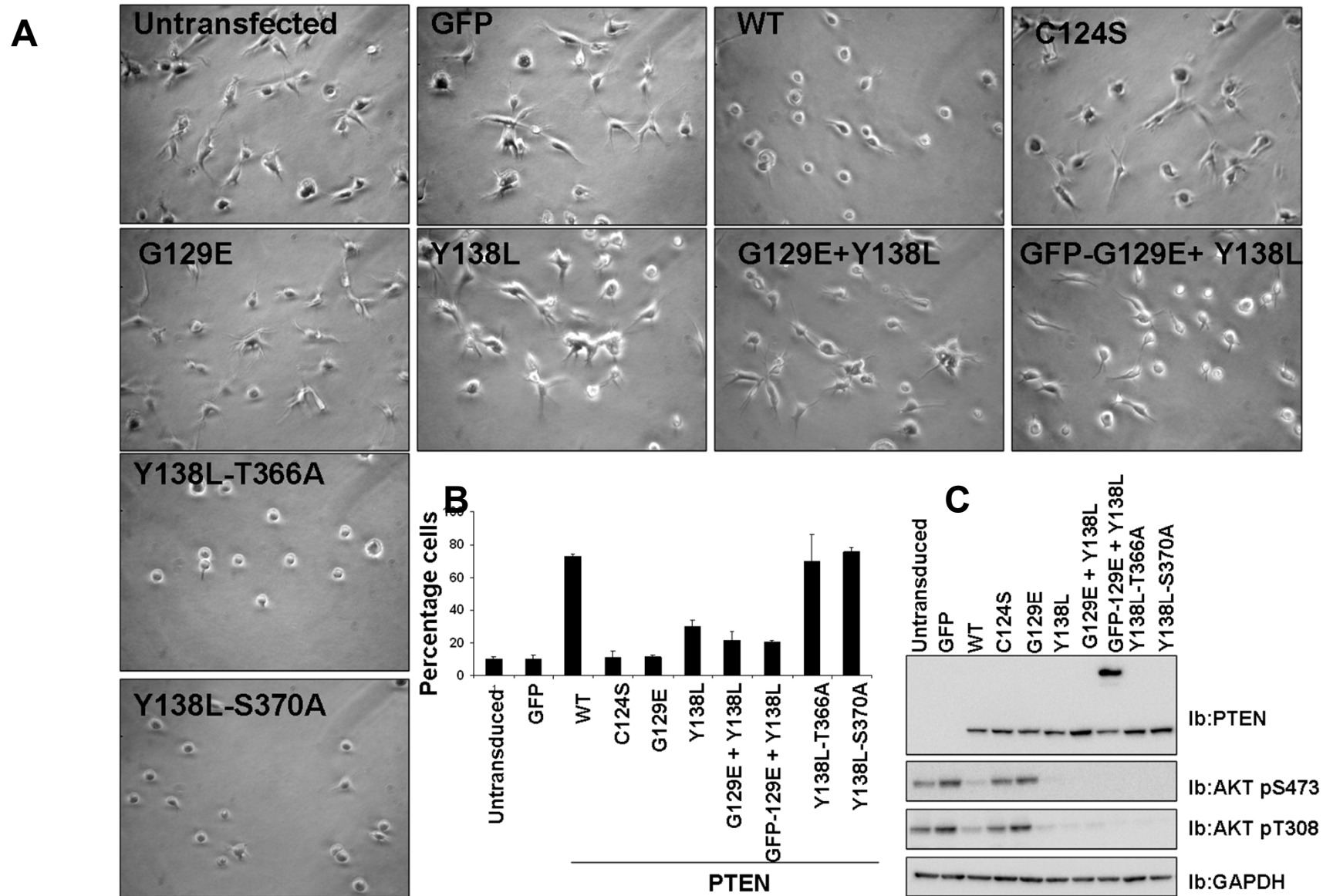
**Figure S3.** Autodephosphorylation analysis of PTEN mutant proteins (A) U87MG cells transiently expressing wild-type (WT) PTEN or PTEN mutants were treated with the GSK3 inhibitor CT99021 (5 $\mu$ M) for up to 2 hours as shown. The phosphorylation of PTEN Thr366 was then investigated by lysis, immunoprecipitation and immunoblotting. (B) Quantification of the blots was performed for n=3 normalised to total PTEN level. (C) Preparation of phosphorylated GST-PTEN tail phosphatase substrate (for Fig 3D). Bacterially expressed GST-PTEN tail (351-403) was immobilised and phosphorylated with CK2 and unlabelled ATP before being extensively washed and phosphorylated with GSK3 and radiolabelled ATP. It was then further washed, eluted and used as a phosphatase substrate. Data here show the analysis of the substrate by phospho-specific immunoblotting to verify the loss of de-phospho PTEN signal.



**Figure S4.** Effects of kinase inhibition and hyperosmotic stress on PTEN Thr366 phosphorylation. **(A, B and C)** U87MG cells transiently expressing wild-type PTEN were treated with Sorbitol or NaCl at indicated concentrations and GSK3 inhibitor CT99021 (5 $\mu$ M) for 1hr or as shown. The phosphorylation of PTEN Thr366 and Ser370 was then investigated by lysis, immunoprecipitation and phospho-specific immunoblotting. **(D)** U87MG cells transiently expressing wild-type (WT) PTEN or PTEN mutants were treated with GSK3 inhibitor CT99021 (5 $\mu$ M) or PI3K inhibitor PI103 (1 $\mu$ M) for 24hrs. The phosphorylation of PTEN Thr366 and Ser370 was then investigated by lysis, immunoprecipitation and immunoblotting. Expression of WT and mutant PTEN and effects on AKT phosphorylation were verified by western blotting of total cell lysates. **(A)** and **(B)** are representative of 2 experiments. **(C)** and **(D)** are representative of 3 experiments.

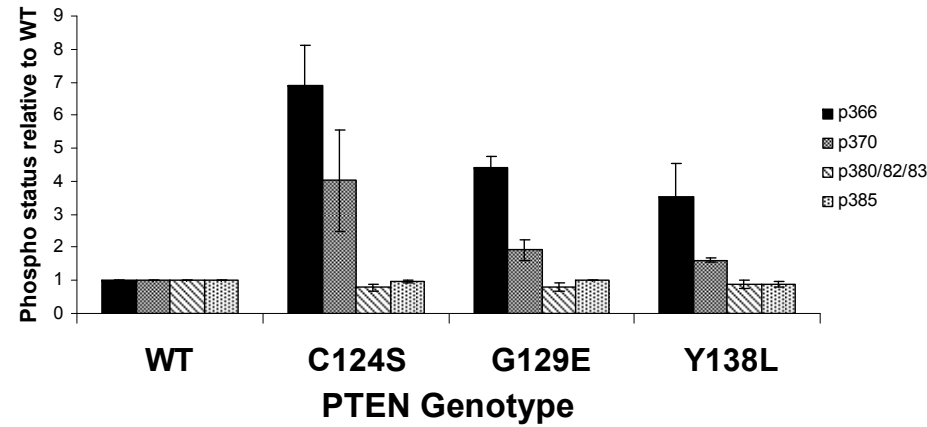
**A****B**

**Figure S5. (A and B).** Further analysis of PTEN Thr366 phosphorylation. U87MG cells transiently expressing wild-type PTEN or PTEN point mutants were lysed, total PTEN was immunoprecipitated and phosphorylation of PTEN investigated by replicate phospho-specific immuno-blotting. (A) left and right panel sets are from separate unrelated experiments. All data are representative of 2 experiments.

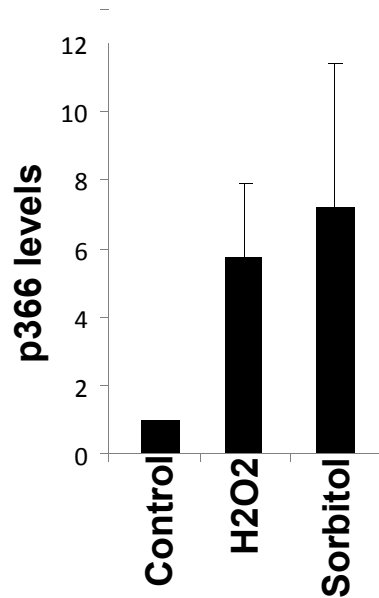


**Figure S6.** Effects of PTEN on U87MG cell morphology in 3D matrigel cultures. U87MG cells were transduced for 24h with lentiviruses encoding wild-type PTEN and PTEN point mutants. **(A)** Cells were seeded at low density between two layers of matrigel, fixed after 8hr, and photographed by phase contrast microscopy. **(B)** Cells were counted in 10 random fields. Quantitation represents percentage of rounded cells. **(C)** Expression of WT and mutant PTEN and effects on AKT phosphorylation were verified by western blotting of total cell lysates of initial adherent cultures. Data are representative of 2 experiments.

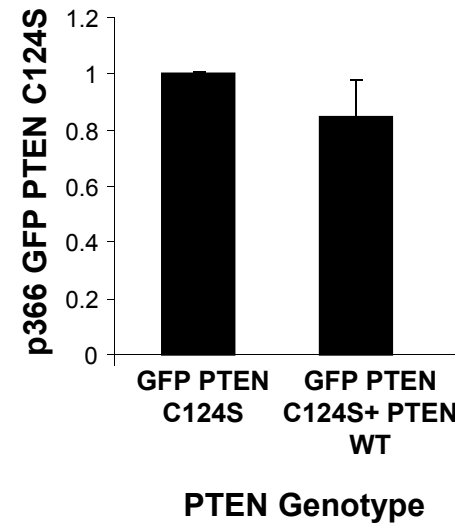
**A**  
Fig 3A, B



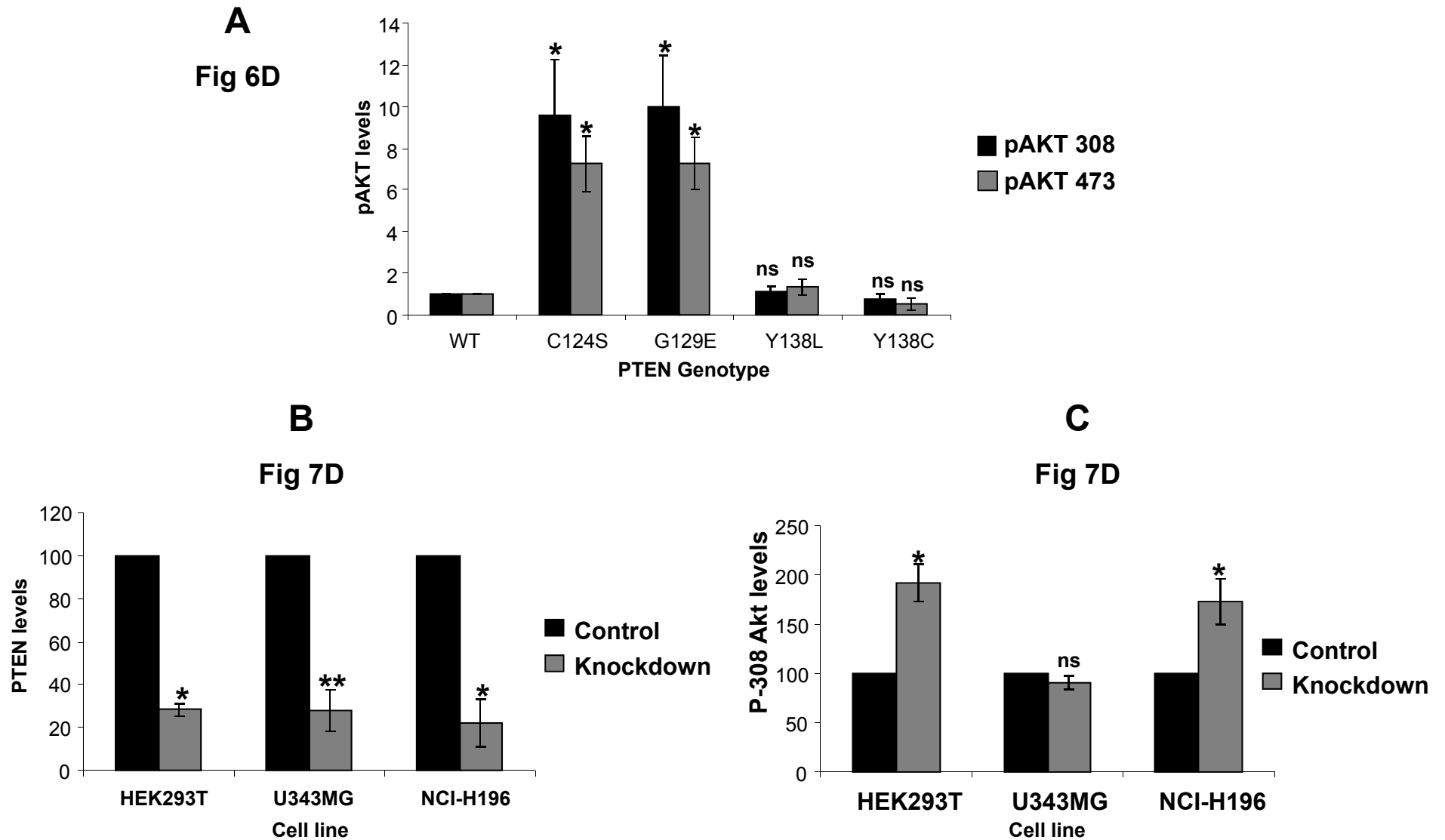
**B**  
Fig 4A



**C**  
Fig 4B



**Figure S7.** Quantitation of blotting data. **(A)** represents quantitation of blots from Fig.3A and 3B for n=3. Pair-wise comparisons were made between PTEN wild type and individual PTEN mutants for individual phospho-sites. **(B)** U87MG cells expressing PTEN wild type were treated for one hour with either 0.5M sorbitol or 1mM hydrogen peroxide (blots in Fig.4A). Quantitation of blots from n=4. Pair-wise comparisons were made between control and treated samples. **(C)** represents quantitation of blots from Fig.4B for n=2. Analyses here in **(A)** are derived from the data in Fig 3B from which a robust value for the T366 phosphorylation of wild-type PTEN can be taken. Although this result is representative of at least seven experiments, in many of these, the T366 phosphorylation of PTEN WT was below the detection threshold of the experiment and thus valid quantitation and statistical analysis are not possible.



**Figure S8.** Quantitation and statistical analysis of blotting data. **(A)** represents quantitation of blots from Fig.6d for n=4. Two way comparisons were made between PTEN WT and individual PTEN mutants. **(B and C)** represents quantitation of blots from Fig.7d for n=3. In both cases, two way comparisons were made between control and knockdown for individual cell lines. Asterisk represent P values (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001) and 'ns' denotes non significant.