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**14. ABSTRACT** The PTEN/MMAC1/TEP1 is a tumor suppressor gene, targeted for biallelic somatic inactivation in a variety of cancers including advanced prostate adenocarcinomas among many others malignancies. PTEN is a phosphatase and has an important role in regulation of the PI3K/AKT signaling pathway, which plays a key role in regulating cellular functions including proliferation, apoptosis, glucose homeostasis, cell size, nutrient response and DNA damage. Furthermore, PTEN functions in the cell to restrict both growth and survival in absence of growth signals. Studies performed in our laboratory indicated that in addition to its 47Kda form, PTEN could be detected as a part of a >600Kda complex. Further, we have also shown that PTEN acts as an antagonist of the PI3K/AKT signaling, only when it is unphosphorylated and recruited into the large protein complex. We have identified a novel partner of the PAC. Our biochemical purification and immuno-precipitation experiments show that the p85 regulatory subunit of PI3K is a part of the PAC. The p85 subunit was co- immuno-precipitated with PTEN and migrates in parallel to PAC fractions both in the cytoplasm and the nucleus gel filtration, an observation which is consistent with our previous conclusion that there may be a single complex that can shuttle between the two compartments and that the same complex might migrate to the membrane where the PTEN substrate is localized.

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# Introduction

Prostate cancer is the second leading cause of cancer related deaths in American men resulting in nearly 30,000 deaths and 235,000 new cases per year [1]. Despite widespread adoption of early detection strategies based upon prostate specific antigen test screening, a significant number of men relapse after local therapy and progress to metastatic prostate cancer. Hormone-withdrawal therapy typically induces a response, however, nearly all patients treated in this fashion will eventually develop hormone-refractory disease.

The *PTEN/MMAC1/TEP1* tumor suppressor gene [2-4] maps to chromosome 10q23, a region frequently altered by loss-of-heterozygosity in a variety of tumors including advanced, metastatic prostate adenocarcinomas [2, 5-10]. Up to 70% of primary prostate tumors lose one PTEN allele [11-13]. Alterations in the remaining allele of PTEN leading to biallelic inactivation, including nonsense, frame-shift, deletion or insertion mutations, (reviewed in [14]) are observed in prostate cancer, as well as in endometrial cancer, glioblastoma and melanoma (reviewed in[15]). Previously, the Sellers lab and others have shown that loss of PTEN is associated with more aggressive forms of prostate cancer in man. This data is complimented by a number of genetic experiments in mice [10, 16-18]. Thus, PTEN is an important tumor suppressor gene whose loss of function contributes to tumorigenesis and the development of aggressive forms of prostate cancer.

The PI3K/AKT/PTEN pathway plays an important role in balancing cell proliferation and death. The function of this pathway is dependent upon regulation of the levels of PIP3. PI3K catalyzes the D3 hydroxyl subunit of PI-4,5-P to give PI-3,4,5-P (PIP3). In vitro, PI-4-P can also serve as a substrate for the class Ia PI3K (Reviewed in [19]. PIP3 is the major substrate of PTEN. PTEN interacts and dephosphorylates the D3 hydroxyl subunit of PI-3,4,5-P (PIP3) to give PI-4,5-P [20]. It is also capable of interacting and dephosphorylating other phosphoinositides, like PI-3-P, PI-3,4-$P_2$, Ins-1,3,4-$P_3$, but with a lower affinity (reviewed in [14]). PTEN likely functions as a tumor suppressor primarily through its lipid phosphatase activity and its ability to negatively regulate signaling through the PI3K/AKT pathway (reviewed in [21]), which is involved in the regulation of cell proliferation and survival. Experiments in cellular and murine models indicate that PTEN, in addition to its function as antagonist of the PI3K/AKT pathway, might be actively involved in an upstream feedback loop regulating the PI3K activity [22-24].

There are three classes of PI3Ks in the mammalian system, each has a distinct role in cellular signal transduction. Class Ia PI3K is composed of a regulatory subunit, p85, and a catalytic subunit, p110. There are five isoforms of the p85 regulatory subunit: p85α, p55α, p50α, p85β, p55γ. Three of the subunits (p85α, p55α, p50α) are the products of alternative splicing of the same gene. The regulatory subunits have common and well defined domains involved in protein-protein interactions. All the subunits have two SH2 domains, these bind to the TK receptors and regulatory proteins, and an Inter-SH2 domain, which binds to the p110 catalytic subunit. The p85α and p85β subunits also have an N'-SH3 domain and a Bcr homology (BH) domain (reviewed in [19]). The Catalytic subunit, p110 has three isoforms: p110α, p110β (found in all cell types) and p110δ (found specifically in leukocytes). All Have p85 and Ras binding domains, in addition to the conserved catalytic domain (reviewed in[19]).

PTEN, similar to PI3K, has several well-characterized domains and motifs. There is an N-terminal phosphatase domain with the canonical HCXXXXXR active site motif. This active site is larger in comparison to that of other known protein phosphatases to fit the size of the phosphoinositol lipid [25]. It is followed by a lipid-binding-C2 domain (C2D), a C-terminal 50-amino acid "Tail", two PEST homology regions and a PDZ binding domain (PDZbd), at the extreme

C-terminus (reviewed in [14]). Recent studies reveal that the N-terminal phosphatase domain is essential for membrane binding, whereas the C2D, although known to be involved in cell signaling and membrane trafficking [25], plays an indirect role. Moreover, it was shown that through the N-terminus, PTEN can bind to PI-3,4-$P_2$, which may also serve as a putative partner for membrane binding [26, 27]. Our group was the first to demonstrate that PTEN is modified by phosphorylation at the C-terminus by Casein Kinase-2 (CK2), a serine/threonine kinase [28].

Gel-filtration experiments over Sephacryl S-300 column using rat liver extracts revealed two major peaks of endogenous PTEN protein. The first peak eluted with a molecular weight greater than 600 kDa while the second eluted with a molecular weight of 44-100 kDa. In this experiment, while unphosphorylated PTEN migrated as both a monomeric and higher molecular weight form (>600 kDa), the phosphorylated form of PTEN was found only in the monomeric peak (44-100 kDa) [29]. Additional data from our lab showed that the non-phosphorylated form of PTEN strongly cooperated with MAGI-2 to block Akt activation, in addition to PTEN interaction with other PDZ domain containing proteins [29]. This data suggested that PTEN phosphorylation might regulate its ability to form associations with a larger protein complex, and that PTEN activity is regulated through this association [29].

In past years, additional evidence has accumulated in support of the notion that PTEN forms a multi protein complex. A number of new putative PTEN interactors have been suggested: including LKB1, a nuclear serine/threonine kinase and a known tumor suppressor protein [30]; and MSP58/MCRS1, another PDZ domain containing oncoprotein [31]. By using a yeast two-hybrid system it was also suggested that PTEN might indirectly interact with β-catenin though direct binding to MAGI-1b [32]. PTEN interacts directly with the NHERF1 and NHERF2 ($Na^+/H^+$ exchanger regulatory factors) homologous adaptor proteins through the PDZ motif of PTEN and the PDZ1 domain of NHERF1 or both PDZ domains of NHERF2 [24].

In aggregate, these data provide additional evidence that PTEN forms a complex that contributes to its function as a tumor suppressor.

# Objectives

The specific aims of my ongoing research are:

1. Identification of the PTEN associated complex (PAC) by biochemical purification.
2. Identification of the PTEN associated proteins by Tandem Affinity Purification.
3. To determine whether the interactions of PTEN with components of the PAC are necessary for PTEN function as tumor suppressor gene.

# Body

## ***Specific Aim 1: Identification of the PTEN associated complex (PAC) isolated from distinct cellular compartments by biochemical purification.***

In keeping with previously published data [29], we detected PTEN PAC in subcellular fractions of HeLa and HEK293cells. In both nuclear and cytoplasmic extracts separated by gel filtration on a Superose 6 column, PTEN monomer was detected in a low molecular weight fraction (44-150 kDa) and in high molecular weight fractions adjacent to the 670kDa fraction (Fig 1, original

report). Recently, Takahashi et al, have shown a similar gel filtration pattern in cell lines for PAC and monomer separation [24].

In recent years, additional information has accrued that PTEN, in addition to its function as antagonist of the PI3K/AKT pathway, might be actively involved in an upstream feedback loop regulating PI3K activity [22-24]. Raftopoulou and colleagues have shown that PTEN might be involved in regulation of cell migration. Moreover, their evidence suggests an important role for the unphosphorylated C-terminal tail in this process [33]. In parallel, evidence from additional studies shows that the regulatory subunit of the PI3K, the p85, has an important function on its own. Like PTEN, p85 in addition to its function as the regulatory subunit of PI3K might have a role in cell migration [34], and regulate a feedback loop of the PI3K [35].

Based on this evidence we set to investigate if p85 might be a part of the PTEN complex.

Gel filtration of 293 cells

In order to determine whether p85 is a part of the PAC, whole cell extract (WCE), cytoplasmic and nuclear fractions (fallowing the protocol described in the original report) from 293-T were subjected to gel filtration using either SE 1000/17 column (WCE and nuclear extract) or Superose 6 column (Cytoplasmic extract). 293-T express both the wild type (wt) PTEN and wt class Ia p85α and p85β proteins, while HeLa is a p85 α-null cell line (Figure 2A).

Our pilot purification of HeLa cytoplasmic and nuclear subcellular fractions suggested the presence of a uniform complex in both cytoplasmic and nuclear fractions, in addition to the monomer form of PTEN. In agreement with our results in HeLa cells, in 293-T we detected the monomer form of PTEN in gel filtration fractions corresponding to low molecular weight proteins (~ 44 kDa -100 kDa) and an addition peak harboring PTEN in fractions corresponding to high molecular weigh proteins (~ 670 kDa) (Figure 1). The same pattern of migration was observed in the WCE and in cytoplasmic and nuclear subcellular fractions. The p85 subunits form several complexes in the cell that differ in their molecular weight: a heteredimer with p110; a complex with IRS1 with and without p110 [19]. We were therefore not surprised to observe that p85 was detectable in several peaks. The fractions of particular interest to us included the monomer, corresponding to its predicted molecular weight of p85kDa (adjacent to ~ 100kDa fractions), and a high molecular weight peak that migrates in parallel to the high molecular fractions of PTEN, indicating that it may be a part of the PAC (Figure 1). As before, these results emphasize that there may be a single complex that can shuttle between the two compartments and that the same complex might migrate to the membrane where the PTEN substrate is localized.

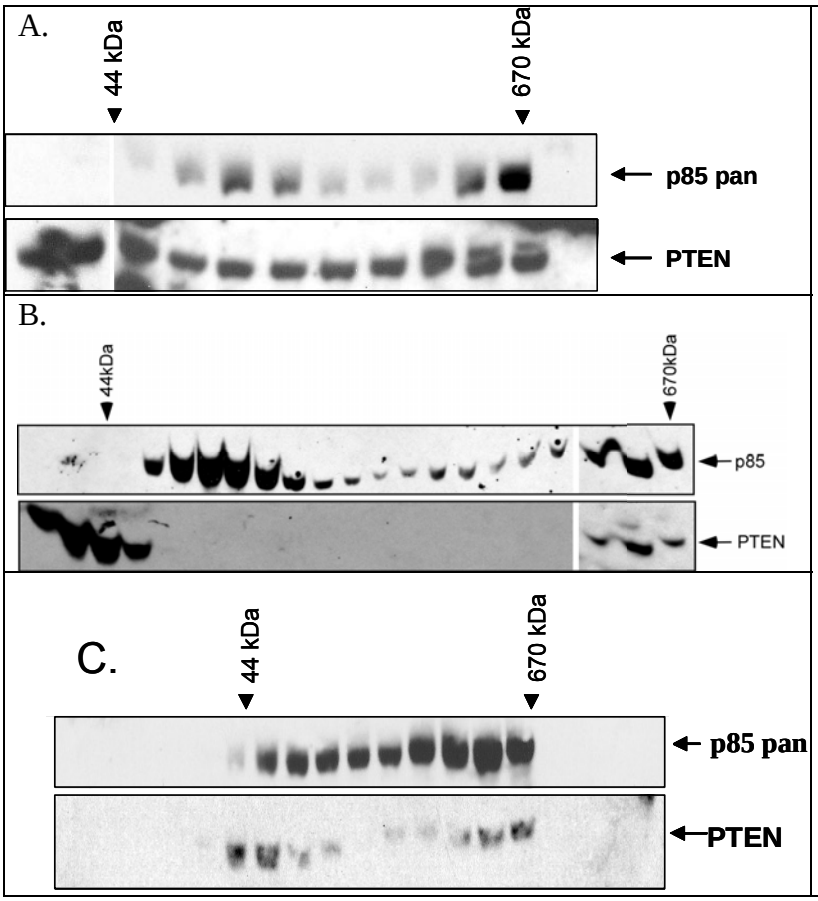


Figure 1: p85 migrates parallel to the high molecular peak of PTEN. 293-T WCE, cytoplasmic and nuclear fractions were separated on gel filtration columns.
A. 293-T WCE was separated on SE 1000/17 gel filtration column; B. 293-T cytoplasmic fraction was separated on Superose 6 gel filtration column; C. 293-T nuclear fraction was separated on SE 1000/17 gel filtration column. PTEN was detected in a low molecular weight fractions and in high molecular weight fractions in all 3 runs. p85 was detected in fractions corresponding to monomer size of the protein and in high molecular weight fraction, running in parallel to the PAC.

Co-Immunoprecipitation of p85 and PTEN

To confirm the gel filtration results, we immunoprecipitated (IPed) the endogenous PTEN by using both monoclonal (6H2.1) (Figure 2A, B, C) and polyclonal anti PTEN antibodies (Abs) (C54) (Figure 2D), PTEN was detected by immunobloting with C54 and 6H2.1 anti PTEN Abs, respectably. The endogenous p85 was co-immunoprecipitated (co-IP) together with the endogenous PTEN and detected by p85 pan Ab (Figure 2), able to detect all p85 subunits. The co-IP was preformed on different human cell lines, including prostate adenocarcimoma cell lines and on mouse embryonic fibroblasts (MEFs). p85 was co-IPed from 293-T and ACHN cell lines (Figure 2A, D), but not from DU145 (Figure 2B), although all are PTEN, p85α and p85β wt. The cell lines 786-O, LNCaP, and PC3, which are PTEN-null but p85α and p85β wt, were used as negative controls (Figure 2 A, B). The p85β subunit runs slightly above the p85α and although it is hard to see the separation between the subunits on the WCE panel (Figure2 B), in the co-IP panel we can clearly detect both subunits with a distinctive preference for p85α (Figure 2 A, B). Moreover, the co-IP failed in HeLa cells which are PTEN wt but p85α – null, as demonstrated by specific immunostaining against p85α (Figure 2 A).

The endogenous p85 was also co-IPed together with the endogenous PTEN from MEFs derived from transgenic p85 mice [34]. All the MEF lines were PTEN positive and differ in their expression of p85α and p85β. As expected, IP of PTEN in p85α and p85β wt cells and p85 α+/- and p85β-/- cells resulted in co-IP of p85α, and probably p85β also in the wt. Contradictory to our results

with HeLa cells, IP of PTEN, co-IPed the p85β from the p85α-/- and p85β+/- MEF cell line (Figure 2C).

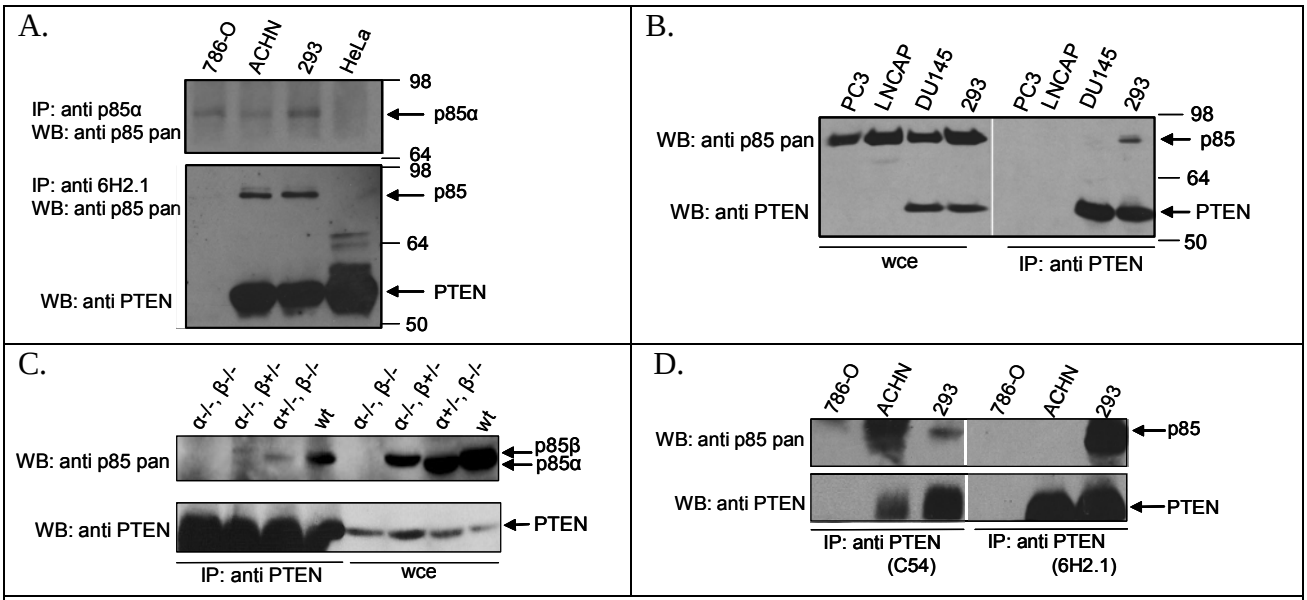


Figure 2: Co-IP of p85 and PTEN. A. 1mg of 293-T, ACHN, 786-O, HeLa WCE lysates were IPed with 1 µl of monoclonal anti PTEN (6H2.1) Ab. Under similar conditions co-IP was also preformed on a panel of prostate adenocarcinoma cell lines: DU145, LNCaP, and PC3, with 293-T cell line as a positive control (B) and on PTEN-positive MEFs that differ in their expression of p85α and p85β subunits (C). For additional conformation, 1mg of 293-T, ACHN, 786-O, WCE lysets were IPed with 1 µl of monoclonal anti PTEN (6H2.1) Ab and 1 µl of polyclonal anti PTEN (C54) Ab in parallel (D).

GST- pull down

A number of GST-PTEN constructs are available in our laboratory and can be used for *in vitro* purification of proteins capable of interacting with PTEN. Based on our biochemical purification strategy, unphosphorylated PTEN is expected to be integrated into the PAC. As this is produced in bacteria, the PTEN products are unphosphorylated. We used this approach to confirm the interaction between the unphosphorylated GST-PTEN and p85α. The GST proteins were expressed in bacteria and purified over anti GST-antibodies conjugated to agarose beads. For this experiment protein lysets from 293, HeLa and 786-O cells were prepared by using hypotonic lyses buffer. The WCE were incubated overnight at 4˚C with full length GST-PTEN, and 2T an empty GST vector that was used as a negative control (Figure 3). Because GST-PTEN runs approximately in the same range as the p85 we were concerned

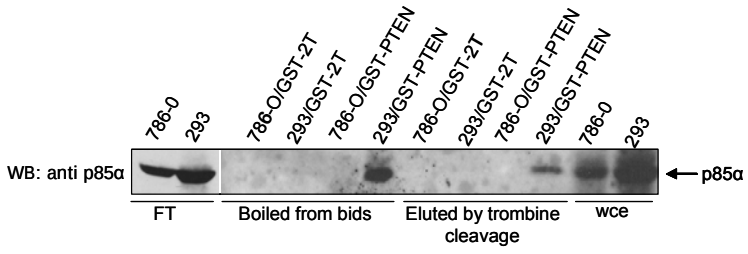


Figure 3: GST pull down. p85α is pulled down by GST-PTEN. The unphosphorylated GST-PTEN pull down p85α from 293 cells, p85α was detected by immunobloting with anti p85α monoclonal Ab.

that it might interfere with the immunoblot staining to overcome this interference the samples were divided in two and one was treated with thrombin for 2 hrs at room temperature, the second one was boiled to release the bound proteins from the agarose beads. p85α was detected by immunobloting with anti p85α monoclonal Ab. The unphosphorylated GST-PTEN pulled down p85α from 293-T cells but not from 786-O. The total expression of p85α in 786-O was much lower in comparison to 293-T cells. The flow through (FT) samples indicated that not all of the available p85α binds to PTEN.

## ***Specific Aim 2: Identification of the PTEN associated proteins by Tandem Affinity Purification (TAP) purification.***

The TAP system is based on a retroviral pZome vectors (generated from pBabe), that enables near endogenous levels of fusion proteins to be expressed in cell cultures. In pZome there is a leader sequence that contains the Protein A gene, a sequence for the TVE protease digestion and the gene for calmodulin binding protein (CBP). Although The TAP system has proved itself to be a useful tool in large-scale identification of protein complexes in yeast and mammalian systems [36-38], in our hands we found that this system is not applicable for tumor suppressor proteins.

We thought to use the TAP system as a tool to introduce and evaluate the function of PTEN mutants. In particular, the non-phosphorylated form PTEN;A4, in which S380, T382, T383 and S385 residues were substituted with alanine, was expected to be integrated into the PAC, based on our biochemical purification strategy for the unphosphorylated form of PTEN. However, although we have generated the appropriate plasmids that expresses TAP-PTEN, as described in last year's report, we were unable to detect the expression of the TAP-PTEN fusion protein in the cells, despite achieving antibiotic resistance. Upon introduction of the TAP-PTEN fusion protein into 786-O and PC3, both PTEN null cell lines resulted in cell growth arrest (particularly in the case of PC3 cells), and loss of the PTEN expression. It is well documented that ectopic expression of wild-type PTEN in PTEN-null tumor cell lines results either in G1 growth arrest, anoikis, or apoptosis, depending on the cell type [39-42]. We were able to establish and expend polyclonal antibiotic resistant 786-O clones, but after a week in culture, PTEN expression was lost, implying that the fusion protein was degraded. Our attempt to establish monoclonal clones from infected 786-O cells, by performing limiting dilutions based on 0.5 cell/well calculation in 96 well plates, resulted in death of all the cells. Furthermore, attempts to express the fusion protein product in 293-T, a PTEN wt cell line also failed, implying again that the Protein A-PTEN fusion protein is degraded.

As described in Aim 1 we were able to identify p85 as a potential PAC protein. At this point we decided not to proceed further with this aim and concentrate our efforts on understanding the interaction between PTEN and p85.

Alternative approach: As we suggested in our previous report, if our attempts to express the Protein A-PTEN fusion protein in PTEN wt cell line will fail, as they have, we will re-clone the Protein A-CBP-PTEN fusion protein in an inducible system using a Tetracycline conditional expression "Gateway" cloning system (Invitrogen). To purify further the whole PAC, once induced with Tetracycline, TAP-PTEN extracts will be pooled from multiple plates of cells transfected with the relevant plasmid or control vector only and purified according to the TAP protocol.

Alternative approach: GST pull down of PAC

In our original report we suggested the GST pull down as an alternative approach to TAP purification. We used this technique to investigate and confirm the interaction between the unphosphorylated PTEN and p85α (see Specific Aim 1).

## ***Specific Aim 3: To determine whether the interactions of PTEN with components of the PAC are necessary for PTEN function as tumor suppressor gene.***

The central question of this aim is whether the identified associated proteins influence PTEN tumor suppressor activity. To determine whether there is a requirement for the interaction between PTEN and p85 for PTEN function, we investigated if loss of expression of p85 is sufficient to alter PTEN function as tumor suppressor.

Our laboratory is a part of the Broad Institute community, which includes the the RNAi Consortium, or TRC (http://www.broad.mit.edu/genome_bio/trc/rnai.html). The Consortium provided us with shRNA hairpins against both the human and murine p85α, p85β and PTEN genes.

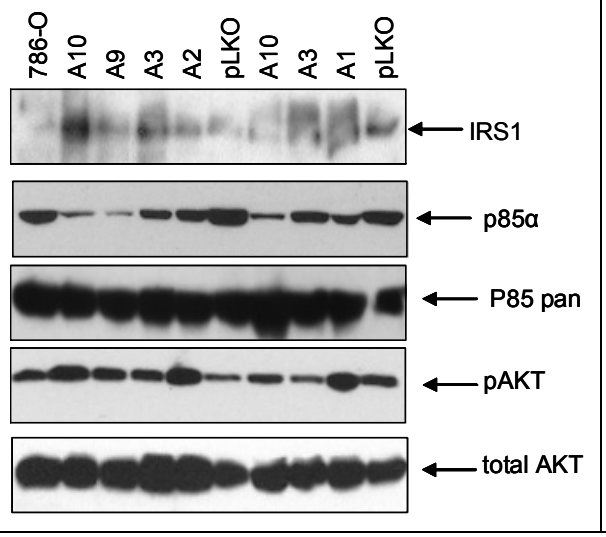


Figure 4: shRNA against p85α screening and the affect of K.D. on AKT/PI3K pathway proteins. The shRNAs were introduced into 786-O cells by lentiviral infection and screened for antibiotic resistance. The different clones, each infected with a different shRNA, were immunobloted for p85α, with anti p85α monoclonal Ab, to determine the level of inhibition. The level of p85β was evaluated by staining with polyclonal p85 pan Ab. pAKT and total AKT were stained with monoclonal and polyclonal Abs, respectably. IRS1 was stained with polyclonal anti IRS1 Ab.

Our co-IP experiments (Figure 2) indicated a preference for p85α. Therefore we decided to concentrate first on the effect of loss of p85α expression on PTEN function as a tumor suppressor. We have screened 6 different shRNAs against p85α: A1, A2, A3, A9, A10 (A3 and A10 were screened twice) (Figure 4). The shRNA targeted different sites/sequences of the p85α mRNA or the 5` UTR, and were cloned into pLKO1, a lentiviral expression vector. The lentivirus was generated in 293-L cells, specially adapted to produce optimum lentiviral titer. We have screened six different shRNAs against p85α: A1, A2, A3, A9, A10 (A3 and A10 were screened twice) in 786-O, a PTEN-null cell line (Figure 4). The shRNAs had different knock-down efficacy against p85α. The p85α knock–down (K.D.) levels were compared to its protein expression in 786-O wt cells and in 786-O cells infected with pLKO1, control vector. A9 and A10 had the highest knock-down effect (Figure

4). The A9 and A10 hairpins were used to prepare stable cell lines, to be used in soft agar experiments.

In addition, we checked the effect of the K.D. on the AKT/PI3K pathway. We used the p85 pan Ab to evaluate expression of p85β. All the cell lines regardless of the level of p85α K.D. expressed similar levels of total p85 in the cell, indicating that p85β was overexpressed to compensate the loss of p85α. 786-O, by being a PTEN-null cell line has already elevated levels of pAKT. Knock-down of p85α induced further up-regulation of pAkt (Figure 4); although surprisingly, the highest up-regulation of pAkt did not correlate with the highest knock-down. It was challenging to detect the endogenous levels of IRS1 protein either in the wt or infected cells. In cells where IRS1 was detected we did not see a definite pattern (Figure 4).

To determine if p85α has an effect on PTEN function as a tumor suppressor, we introduced PTEN into pLKO/786-O, 786-O/p85α K.D. clones and into 786-O wt. PTEN cDNA sequence was cloned into the pBabe-neo retroviral vector. The retrovirus was produced in 293-T. The target cells were infected sequentially with virus collected 3 days in a row, to increase the infection. Antibiotic selection was used to isolate positively infected cells. The fallowing cell lines were infected: wt 786-O, A9/786-O, A10/786-O (p85α K.D. clones) and pLKO/786-O (empty vector (e.v.) control for the K.D. clones). After a week in selection the cells were used to perform soft agar experiments. Part of the cells were left in culture, as previously with the TAP vector infection. Although the cells continued to be resistant to antibiotics, protein levels of PTEN were undetectable by immunostaining after only a few passages.

The soft agar experiments were performed in duplicate. We plated $5x10^3$ cells/well, of the target cells in semi-liquid conditions and analysed for their ability to form colonies on soft agar. Introduction of PTEN into 786-O wt and 786-O/pLKO cell lines and comparison to the parental cell line provided us with the necessary controls for this experiment. 786-O, a renal adenocarcinoma cell line, and pLKO/786-O cells, form large size colonies in soft agar, indicating its tumorogenisity. As expected, PTEN expression induced inhibition of the formation of colonies on soft agar. Cells expressing PTEN formed less and/or smaller size colonies in comparison with wt and the empty vector.

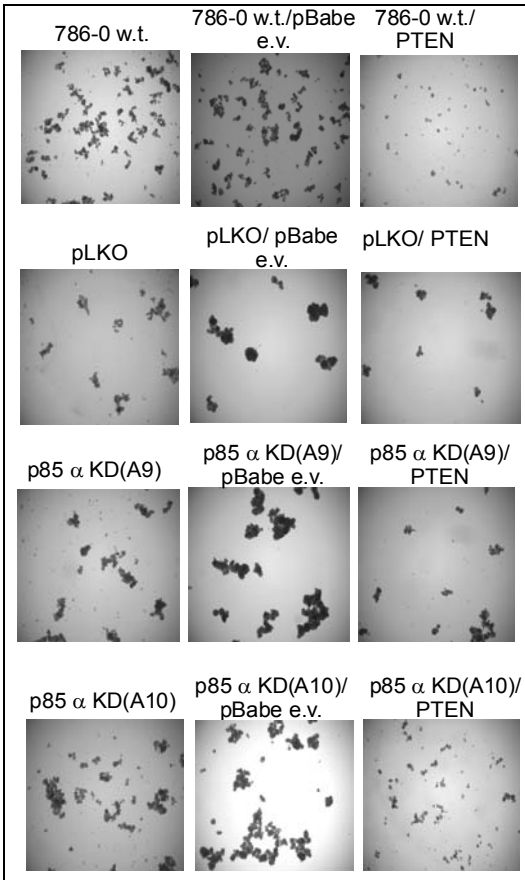


Figure 5: The effect of p85α K.D. on PTEN function as tumor suppressor was analysed by cell growth on soft agar. We plated $5x10^3$ cells/well, of the target cells in semi-liquid conditions and analysed for their ability to form colonies on soft agar. 786-O, pLKO/786-O, A9/786-O and A10/786-O cells were infected with pBabe or pBabe/PTEN. 786-O wt and pLKO/786-O cells were used as controls both for the p85α K.D. cells and PTEN infected cells. A9/786-O and A10/786-O (p85α K.D. stable cell lines) were analysed for their ability to form colonies and used as controls for PTEN infected cells.

The knock down of p85α did not affect the tumorigenicity of 786-O cells. A9/786-O and A10/786-O formed large colonies, comparable in size and phenotype to pLKO/786-O and 786-O (wt) cell lines (Figure 5). As noted from the immuno-staining with p85 pan Ab in Figure 4, knock-down of p85α induced overexpression of the p85β subunit in our cells. It is therefore possible that p85β compensates in function for the down regulation of the K.D. subunit. It was shown previously that in the absence of p85α, p85β is the one to interact with the catalytic p110 subunit of the PI3K[43]. Introduction of PTEN into the A9/786-O or A10/786-O inhibits the formation of colonies. PTEN induces the dominant phenotype on the PTEN/PI3K/AKT pathway.

To approach the problem of p85β compensation we decided to K.D. the p85β in addition to the already knocked down p85α. To our surprise we did not select any double K.D. clones. Although, several shRNAs successfully knocked down p85β. The A9/786-O, A10/786-O (p85α K.D. clones) infected with shRNAs against p85β, re-expressed p85α in comparison to the parental clones (Figure 6).

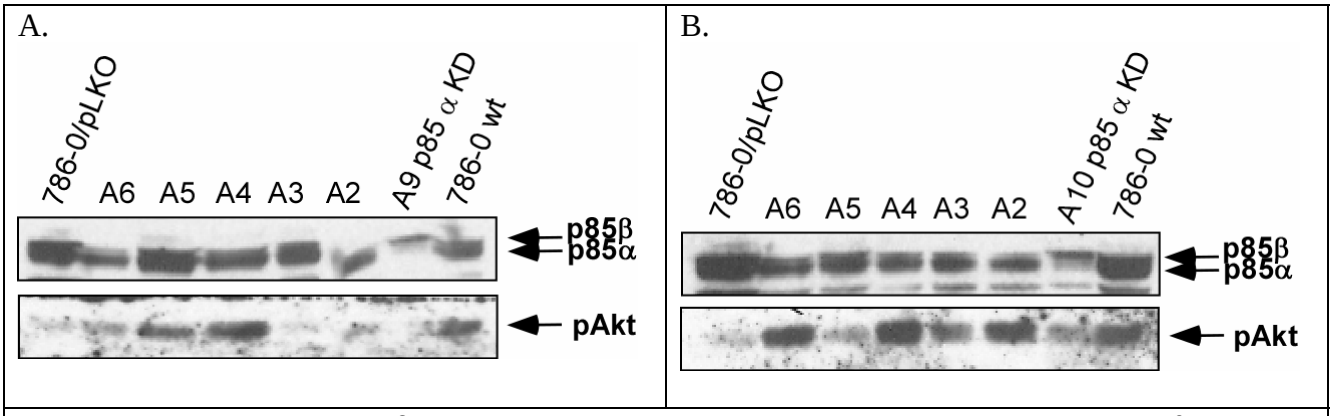


Figure 6: shRNA against p85β screening. A9/786-O (A) and A10/786-O (B) cells were infected with lentivirus expressing the different hairpins against p85β. Positively infected clones were screened for antibiotic resistance. The different clones were immunobloted with anti p85 pan Ab, to determine the level of inhibition.

Alternative approach: To answer the question of whether p85α can alter or regulate PTEN function we decided to use HeLa cells which, although p85α-null, have a similar pattern of PAC and monomer distribution to 293-T cells. In this cell line we could knock-down p85β and introduce p85α. In addition we could generate PTEN K.D. and PTEN/p85β K.D. cells as control. shRNA against PTEN (A11, A12, B1, B2, B5, and B6) were screened in ACHN, a renal adenocarcinoma cell line. The level of K.D. was evaluated by immuno-staining with anti PTEN Ab. Two hairpins with the highest inhibitory effect were chosen for further use. We have generated stable K.D. clones for PTEN, in ACHN and HeLa cell lines. Currently we are in the process of establishing p85β K.D. and PTEN/p85β double K.D. HeLa cells.

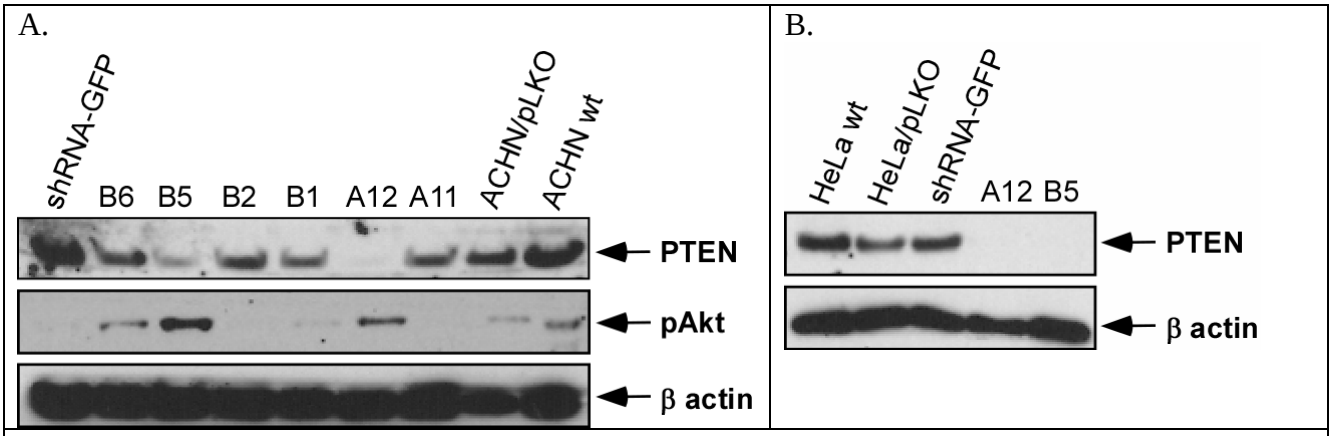


Figure 7: shRNA against PTEN screening. A. ACHN cells were infected with lentivirus expressing the different hair pins against PTEN. Positive infected clones were screened for antibiotic resistance. The different clones were immunobloted with anti PTEN Ab, to determine the level of inhibition. B. HeLa cells were infected with two shRNAs (A12, B5) that had the highest inhibitory effect on ACHN cells.

## Key Research Accomplishments:

1. In the past year, we were successful in identifying a novel partner of PAC. We have identified the p85 subunit of PI3K as an important member of the PAC.

2. During the first year of funding, we generated several important biochemical tools that will contribute to our success in future analyses of the PAC, including: a. a functional high affinity immuno-column. The C54-DSS-Protein A is a stable column that maintains the ability to bind and precipitate the endogenous PTEN. We anticipate that this column will be a useful tool as an additional step in our biochemical purification strategy; b. We have been successful in generating an antiserum that selectively recognizes the unphosphorylated form of PTEN. Furthermore, we have optimized the conditions for scale up of our biochemical purification of additional members of the PAC.

## Reportable outcomes

1. Rabinovsky R, Levi H, Dripkin R, Duke-Cohen J, Sellers WR. Functional analysis of the PTEN tumor suppressor gene.* DF/HCC prostate/Renal Cancer SPORE Retreat, 2004 October. * The Poster was awarded the certificate of "Poster session Honorable Mention" of the retreat.
2. Rabinovsky R, Levi H, Dripkin R, Duke-Cohen J, Sellers WR. Functional analysis of the PTEN tumor suppressor gene. Broad Institute First annual Retreat, 2005, October.
3. Anti unphosphorylated PTEN antibodies: T382/383; AD2; AD3; AD4.

## Conclusions

We have identified the p85 regulatory subunit of PI3K is a part of the PAC. The p85 subunit migrates in parallel to PAC fractions both in the cytoplasm and the nucleus, an observation which is consistent with our previous conclusion that there may be a single complex that can shuttle between the two compartments, and that the same complex might migrate to the membrane where the PTEN substrate is localized. Our IP results in human malignant cells indicate that there is a preference for p85α. In p85 wt cell lines, like 293-T or ACHN, when co-IPed with PTEN we were able to detect both p85 subunits, were in Hela, a p85α-null cell line, the co-IP was negative. In contrary to the last observation, in p85α -/- / p85β +/- MEFs, p85β is coming down with IP of PTEN in absence of p85α. Moreover, our knock down experiments emphasizes that p85β substitutes for p85α.

More experiments are needed to understand the involvement and the role of the p85 regulatory subunits in the PAC. Alanine mutations of T382 or T383, as representatives of the unphosphorylated PTEN, have been associated with increased efficacy in cell-cycle arrest assays, accelerated degradation, increased membrane affinity, and increased activity in regulating migration [26, 28, 33, 44-47], and studies of p85α -/- / p85β +/- and p85α +/- /p85β -/- transgenic mice imply, that PTEN function as phosphatase of PIP3 might be regulated by p85α [22, 34, 35].

To date, we have identified a novel partner of the PAC and we have generated unique tools that will contribute to our success to purify additional partners. Contrary to many studies in the field, we concentrated our efforts on endogenous proteins. We generated an immunoaffinity column, based on highly potent C54 Ab covalently coupled to Protein A agarose beads. C54 Ab is an antibody with high affinity and specificity for both phosphorylated and unphosphorylated endogenous forms of PTEN. The C54-DSS-Protein A is a stable column that maintains all the qualities of the C54 Ab, i.e. the high ability to bind and precipitate the endogenous PTEN, allowing us to search for additional PAC partners.

Our T3832/383, AD1, AD2, AD3, AD4 Abs, that selectively recognize the unphosphorylated form of PTEN is an important tool that not only will allow us to further purify the less abundant unphosphorylated form of PTEN present in the PAC but also will allow us to study the role of p85 as part of the unphosphorylated PAC.

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# ABBREVIATIONS

Ab; antibody

Abs; antibodies

C2D; C2 domain

CBP; calmodulin binding protein

CK2; Casein Kinase-2

FT; flow through

IP; immunoprecipitation

MS; mass spectrometry

NES; nuclear export sequence

NLS; nuclear localization sequence

PAC; PTEN associated complex

PDZbd; PDZ binding domain

PI3K; Phosphatidylinositol-3-kinase

PI3,4,5P3/PIP3; phophoinositide l-3,4,5-triphosphate

PI3,4P2; phophoinositide l-3,4,-bisphosphate

PTEN; Phosphatase with homology to Tensin located on chromosome 10

TAP; Tandem affinity purification system

WCE; whole cell extract

WT; wild type