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FAK regulates PTEN under the control of p110δ PI3 kinase

Master's Thesis

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Abstract

The tumor suppressor activity of PTEN is mostly attributed to its lipid phosphatase activity, antagonizing the PI3K signaling. Reduced or lost activity of PTEN is often detected in cancer cells, contributing to constitutive PI3K pathway and downstream promotion of cell survival and proliferation. Overall the regulation of PTEN activity by various mechanisms is not well understood. Posttranslational modification of PTEN by tyrosine phosphorylation has been reported, however, the mechanism remain elusive. The inhibitory effect of PTEN on PI3K signaling does not discriminate among the different PI3K isoforms, however, p110δ isoform was found to inactivate PTEN through a pathway involving RhoA with the latter to induce the tyrosine phosphorylation of PTEN. In the present study we have used genetic and pharmacological approaches and cell-free assays to dissect the mechanism of RhoA-induced tyrosine phosphorylation and activation of PTEN downstream of p110δ PI3K. FAK is a cytoplasmic tyrosine kinase that has been shown to play critical roles in development of human cancer. We now show that FAK phosphorylates and activates PTEN and that the regulation of FAK is under the negative control of p110δ PI3K and under the positive regulation of RhoA and ROCK. Indeed, the phosphorylation of FAK was unexpectedly increased in macrophages derived from mice expressing kinase-dead p110δ while the phosphorylation of FAK was decreased in macrophages derived from mice expressing constitutively active p110δ. Pharmacological inactivation of RhoA or ROCK reduced the phosphorylation of FAK to normal levels in cells with genetically inactivated p110δ. Likewise, pharmacological inactivation of FAK restored the functional defects of p110δ inactivation, including Akt phosphorylation and cell proliferation, and in parallel reduced the phosphorylation and activity of PTEN. Collectively, this work identifies FAK as a target of p110δ PI3K that links RhoA with PTEN and establishes for the first time that PTEN is a substrate of FAK for tyrosine phosphorylation. Our data show a novel mechanism of PTEN regulation and provide new information for the biological roles of FAK. The important role of PTEN activity in cell growth together with the fact that FAK inhibitors have been developed as anti-proliferative agents raise the question if the efficacy of FAK inhibition might be counterbalanced by the PTEN inhibition.

Contents

INTRODUCTION

1. PI3K/PTEN pathway

Phosphoinositide 3-kinases (PI3Ks) consist of a group of enzymes that can transmit signals inside cells by the production of intracellular second messenger lipid signals. They can be activated by various extracellular signals and are involved in a variety of important cellular processes including cell proliferation, survival, metabolism, and migration. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is the main antagonist of PI3K signaling. Both the activation of PI3K activity, though oncogenes or gene amplification, and inactivation of the suppressive activity of PTEN are commonly occur in human cancers. In following sections we will discuss the main aspects of PI3K/PTEN pathway and how its deregulation can lead to pathological conditions such as cancer development.

1.1 PI3K family members

PI3Ks have been divided into three major classes according to their structural features and substrate specificity (reviewed in Vanhaesebroeck et al., 2010). Independently of class, all PI3Ks share PI3K homology regions that contain a C2 domain, a helical domain and the catalytic lipid kinase domain (Figure I).

Class I PI3Ks use as substrates and phosphorylate inositol lipids specifically at the 3-position of the inositol ring, generating lipid products such as phosphatidyl-inositol-3,4,5-trisphosphate [PI(3,4,5)P3], which is the main second messenger to trigger signal transduction events and regulate many cellular responses including cell division, survival, metabolism, intracellular trafficking, differentiation, cytoskeleton re-organization and cell migration (Vanhaesebroeck et al., 2010; Hawkins et al., 2006; Low et al., 2010; Zwaenepoel et al., 2012). Class I PI3Ks are further divided into subclasses IA and IB, based on their preferential activation by tyrosine kinase receptors (RTKs) and G-protein coupled receptors (GPCRs) respectively. **Class IA** PI3Ks are constitutive heterodimers of a 110 kDa catalytic subunit (p110) and an adaptor protein that recruits the p110 subunit to the intracellular locations of tyrosine kinase activation. Mammals have three separate genes for class IA catalytic subunits, *PIK3CA*, *PIK3CB* and *PIK3CD* which encode p110α, p110β and p110δ catalytic isoforms respectively, and several class IA regulatory subunits (p85α, p85β, p55γ, p55α, and p50α) collectively called 'p85s'. The catalytic domain contains phosphoTyr-X-X-Met motifs that permit the association with the Src homology 2 (SH2) domains of the p85 regulatory subunits. **Class IB** comprises only one isoform, the p110γ, which exists in complex with the regulatory subunit p101 or p87. Both Class IA and IB catalytic subunits contain additionally a Ras-binding domain. Activated RTKs and their associated proteins activate class IA PI3Ks directly or via the protein Ras. Class IB p110γ, and as recently discovered, p110β isoform, are activated by Gβγ subunits of heterotrimeric G-proteins, downstream of GPCRs (Guillermet-Guibert et al., 2008).

Class II PI3Ks consist of the ubiquitously expressed PIK3C2α and PIK3C2β and one liver-specific isoform PIK3C2γ and use phosphatidylinositol phosphates (PIPs) or PI4P as substrates. These PI3Ks have no regulatory subunits but are characterized by the presence of N- and C-terminal extensions, which may mediate protein–protein interactions. A possible role of Class II PI3Ks in membrane trafficking and receptor internalization has been proposed (Cantley, 2002). **Class III PI3Ks** contain a catalytic subunit named PIK3C3 and an adaptor subunit, the PIK3R4 (or p150) and uses only phosphatidylinositol lipids as substrates. PIK3C3 can be activated by GPCRs and is implicated in the regulation of the mammalian target of rapamycin (mTOR) under the control of amino acid availability and the regulation of autophagy (Juhasz et al., 2008).

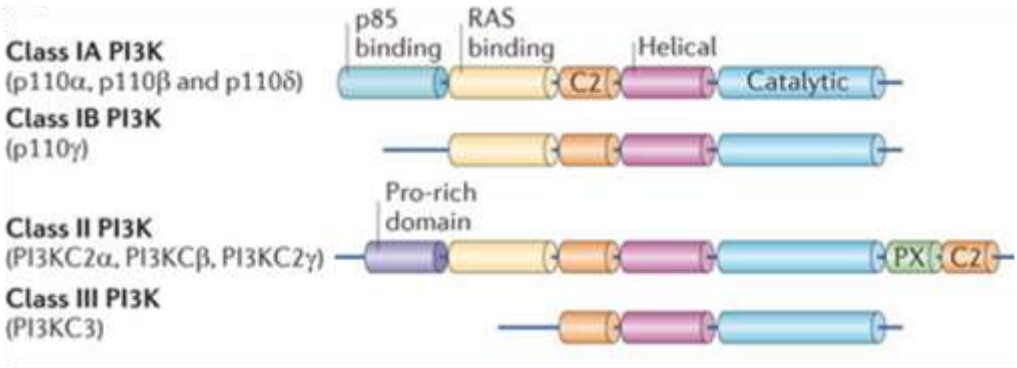


FIGURE I: Classification and domain structure of mammalian PI3Ks. PI3Ks are divided into three classes based on their structural and biochemical features. All PI3K catalytic subunits have a PI3K core structure consisting of a C2 domain, a helical domain and a catalytic domain. PX, phox domain (figure taken from Vanhaesebroeck et al., 2010).

1.2 Activation of Class I PI3K signaling

Class IA PI3Ks are activated by growth factor receptors or adaptor proteins. Binding of the ligand to its receptor leads to receptor dimerization and auto-phosphorylation of tyrosines which are located in pYxxM motifs. The p85 regulatory subunits have SH2 domains which bind to phosphorylated tyrosines in YxxM motifs recruiting thus the class IA PI3Ks to the plasma membrane where their lipid substrates are located. Class IB PI3K is activated by G protein-coupled receptors (GPCRs). Binding of the ligand (e.g., chemokine) to its cognate GPCR induces the dissociation of heterotrimeric G-proteins and the Gβγ subunits interact with the class IB PI3K.

In vivo, activated PI3Ks phosphorylate primarily the phosphatidylinositol (PI)-4,5-bisphosphate [PI(4,5)P2] yielding the product PI(3,4,5)P3 (Vanhaesebroeck et al., 2001). The generation of PI(3,4,5)P3 leads to the recruitment of adaptor/effector proteins containing pleckstrin-homology (PH)-domains at the cell membrane. PH-domain containing proteins that are recruited downstream of PI3K activation include, among others, regulators of small GTPases and Ser/Thr kinases (such as PDK1 and Akt/PKB) (Klarlund et al., 1997; Krugmann et al., 2002; Welch et al., 2002; Marone et al., 2008) (Figure II). Regulation of small GTPases is required for the proper cell motility (Ridley et al., 2003) whereas Akt and PDK1 are crucial proteins for the activation of several tumor associated functions such cell growth, survival, proliferation and migration (Manning and Cantley, 2007).

1.3 Inactivation of Class I PI3K signaling by PTEN

PI3K signaling pathway is inactivated by lipid phosphatases with PTEN lipid phosphatase to be the most extensively investigated. PTEN is a phosphatase that can dephosphorylate both phosphoinositide and proteins

substrates. Importantly, PTEN dephosphorylates the PI(3,4,5)P3 lipid product of Class I PI3Ks, with specific affinity for the phosphate group at the D3 position of the inositol ring (yielding PI(4,5)P2) (Maehama and Dixon, 1998) (Figure IIIA). PTEN also contains a catalytic signature motif, HCXXGXXR, which is characteristic for the family of protein tyrosine phosphatases (PTPs) (Denu et al., 1996). Indeed, PTEN can interact with other proteins by dephosphorylating highly acidic substrates on Tyr, Ser and Thr- phosphorylated peptides (Myers et al., 1997). However, the antagonizing effect of PTEN on PI3K signaling, though the unique PI(3,4,5)P3 lipid phosphatase activity, is what makes PTEN one of the most important tumour suppressors. Following PTEN activity loss, excessive PI(3,4,5)P3 products of PI3K are accumulated at the plasma membrane recruiting and activating constitutively PH-domain-containing proteins, and thus leading to abnormal cell growth, survival and migration (Ali et al., 1999; Vivanco and Sawyers, 2002; Luo et al., 2003). The importance of the physiological function of PTEN is illustrated by its frequent inactivation in cancer, leading to the constitutive activation of PI3K pathway (Cully et al., 2006; Leslie and Downes, 2004; Parsons, 2004; Sansal and Sellers, 2004). Although PTEN imparts many of its cellular roles though its lipid phosphatase activity and repression of the PI3K pathway, PTEN also may has PI3K-independent functions though its phosphatase activity towards other proteins such as focal adhesion kinase (FAK) protein (Raftopoulou et al., 2004; Tamura et al., 1999a).

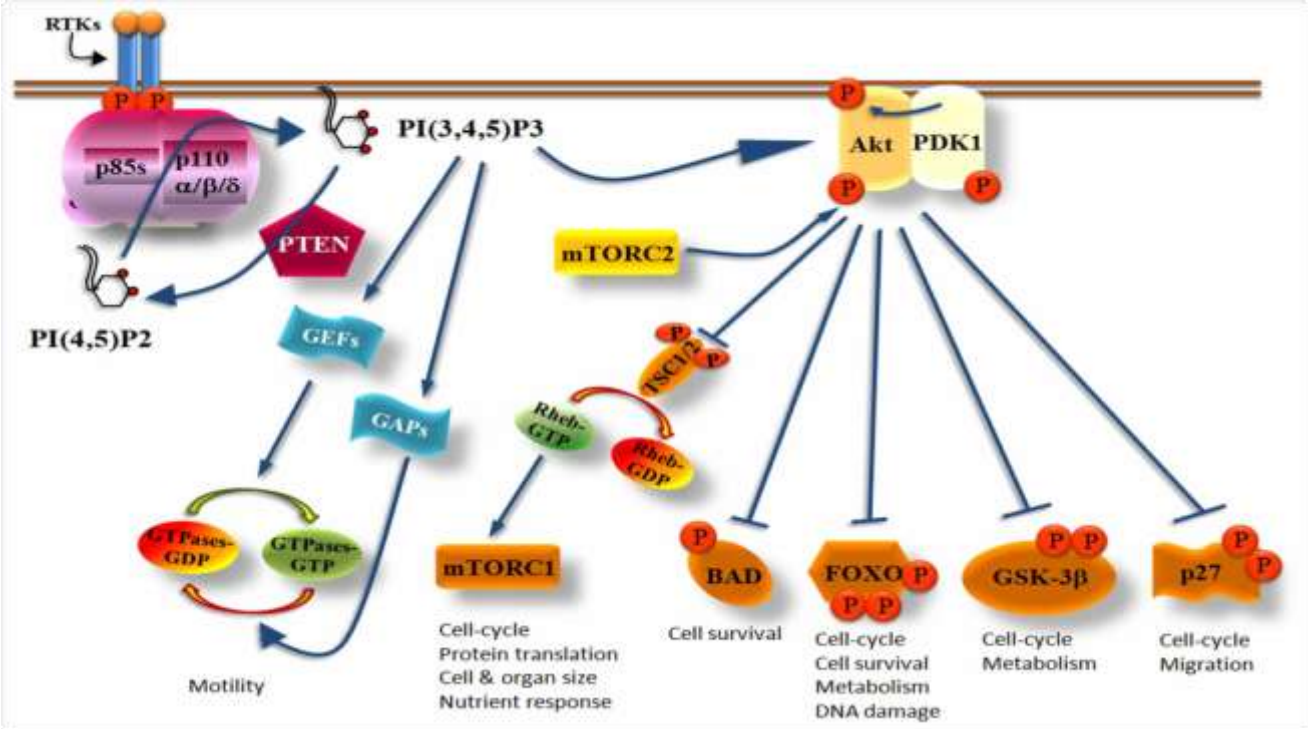


FIGURE II: Simplified the critical role of Class IA PI3Ks in multiple cellular functions. Upon PI3Ks activation, PI(3,4,5)P3 is produced and recruits Akt at the cell membrane where it becomes phosphorylated at Thr308 by PDK1 and at Ser473 by mTORC2. Fully activated Akt phosphorylates a variety of effector molecules including the TSC1/2, BAD, FOXO, GSK-3β, and p27 which then control cell growth, metabolism, survival, cell cycle, or migration. PI(3,4,5)P3 also activates GEFs or GAPs which then regulate the activity of small GTPases controlling cell motility.The PI(3,4,5)P3 levels produced by PI3Ks are regulated by the PTEN phosphatase which counteracts the PI3K reaction by dephosphorylating the 3-position of the inositol ring of PI(3,4,5)P3 yielding back PI(4,5)P2 (Figure taken from Tzenaki & Papakontsanti, 2013).

Crystallographic analysis gave evidence about the structure of PTEN and the importance of different domains in its physiological functions (Lee et al., 1999) (Figure IIIB). Inside the catalytic phosphatase domain, the HCKAGKGR catalytic signature motif forms the phosphate binding loop (P loop, residues 123-130) that is located at the bottom of the active site pocket. The formation of a wide and deep substrate-binding pocket of PTEN with the positive charge is important for the accommodation of phosphoinositide substrates. This PTEN-specific feature is evolutionarily conserved and is also the target of many cancer-associated PTEN mutations (Maehama and Dixon, 1998; Stambolic et al., 1998). PTEN also contains a C-terminal C2 domain that can bind phospholipids membranes *in vitro* (Raftopoulou, 2004), a N-terminal PI(4,5)P2-binding domain (PBD), C-terminal tail containing PEST (Pro, Glu, Ser, Thr) sequences and a PDZ domain-interaction motif (Figure IIIB).

Tumour-associated mutations occur in all PTEN domains implying that each distinct region is physiologically important for the PTEN tumour-suppressive activity. In addition to genomic disruptions that inactivate PTEN, other pathological mechanisms that repress PTEN expression levels are associated with cancer. Many of those PTEN-regulating mechanisms are discussed more extensively in a below chapter. Following sections describe briefly the downstream PI3K signaling and how its constitutive transduction via PTEN deregulation can affect the magnitude of various cancer-associated cellular functions.

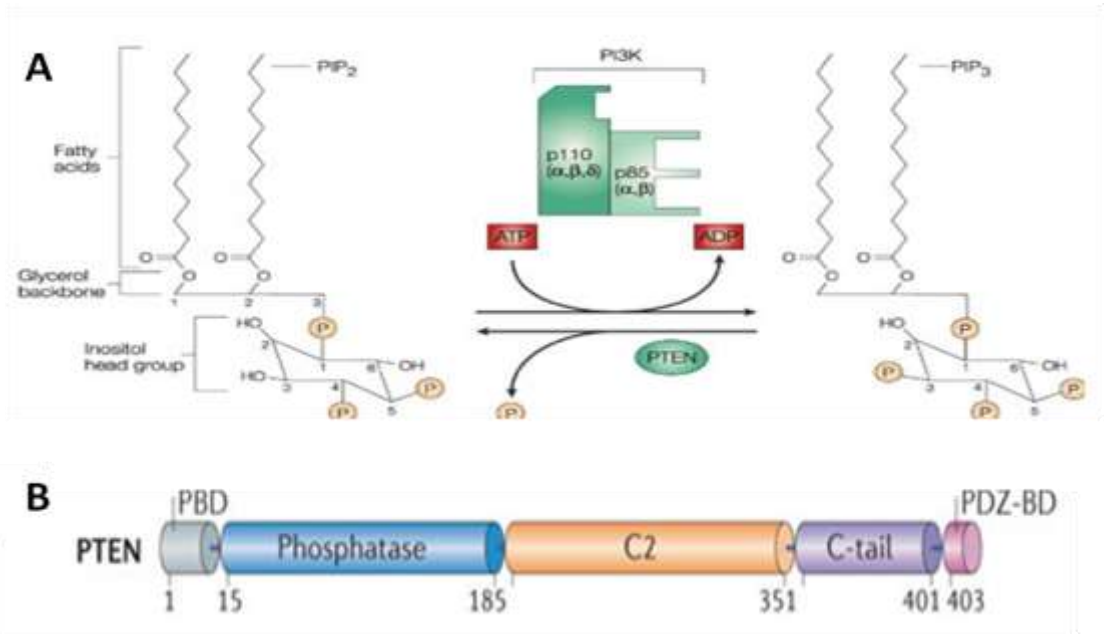


FIGURE III: (A) Function of PTEN tumor suppressor_ PI(4,5)P_2-PI(3,4,5)P_3 cycle. Phosphatidylinositol phosphates (PIP) are composed of a membrane-associated phosphatidic acid group and a glycerol moiety that is linked to a cytosolic phosphorylated inositol head group. PI3K can phosphorylate PI(4,5)P2 at the D3 position to form the second messenger PI(3,4,5)PI3, whereas dephosporylation of PI(3,4,5) is accomplished by PTEN. **(B) Structure of PTEN.** The domain structure of structure of phosphatase and tensin homologue (PTEN) is composed of five functional domains: a PI(4,5)P2-binding domain (PBD), a phosphatase domain, a C2 domain, a C-terminal tail and PDZ-binding domain. (figures taken from Song et al., 2012 (B) and Vivanco and Sawers, 2002 (A)).

1.4 Downstream of PI3K signaling: Akt

Akt Ser/Thr protein kinases comprise three mammalian isoforms: Akt1, Akt2 and Akt3 (reviewed in Manning and Cantley, 2007). These isoforms are homologues more than 80% and include an N-terminal PH domain, a central kinase domain and a C-terminal regulatory domain. PDK1, which is constitutively active becomes additionally activated upon cell stimulation (Alessi et al., 1997a; Pullen et al., 1998; Currie et al., 1999) and phosphorylates Akt on Thr308 residue inside the activation loop of the catalytic domain (Alessi et al., 1997a; Alessi et al., 1997b; Stokoe et al., 1997; Stephens et al., 1998). Fully activation of Akt activity requires the additional phosphorylation on Ser473 into the regulatory domain by the mTOR complex 2 (mTORC2) [composed of mTOR, DEP domain-containing mTOR-interacting protein (DEPTOR), mammalian lethal with SEC13 protein 8 (mLST8), stress-activated MAP kinase interacting protein 1 (mSIN1), Pro-rich protein 5 (PROTOR) and rapamycin insensitive companion of mTOR (RICTOR)] (Alessi et al., 1996, Sarbassov et al., 2005). Activation of Akt drive the stimulation of several cellular functions including cell growth, survival, proliferation and migration by phosphorylating a variety of downstream effector proteins (Figure II). Constitutive Akt activation by either increased PI3K activity or loss of PTEN activity can contribute to cancer development. Bellow we summarize the most important functions triggered by Akt which are also correlated with oncogenic transformation.

Survival: Constitutive activation of Akt by PI3K leads to the prolonged cell survival of cancer cells, by directly affecting several components of the cell-death machinery. Firstly, Akt phosphorylates the death promoter BAD, leading to the release of the anti-apoptotic proteins B cell lymphoma-2 (Bcl-2) and Bcl-XL (Datta et al., 1997; Peso et al., 1997).Secondly, Akt phosphorylates the pro-apoptotic protease caspase-9, inhibiting its activity (Cardone et al., 1998). Other crucial substrates of Akt are the members of the forkhead box O (FOXO) family of transcription factors. Phosphorylated FOXOs bind to the 14-3-3 proteins resulting in their exclusion from the nucleus. Cytosolic localization of FOXOs prevents the transcription of FOXO gene targets, which include several pro-apoptotic proteins such as Fas ligand (Brunet et al., 1999). Akt can also influence cell survival indirectly by affecting the activity of two central regulators of cell death, the nuclear factor of NF-κB (NF-κB) and the p53. NF-κB promotes survival in response to several apoptotic stimuli and Akt positively affect NF-κB function (Romashkova et al., 1999). From the other hand, Akt negatively influence the activity of the pro-apoptotic tumour suppressor p53, through phosphorylation and activation of its E3 ubiquitin protein ligase, MDM2 (Mayo et al., 2001).

Proliferation: Akt affect proliferation through several signals to the cell-cycle machinery. The cell cycle is regulated by the coordinated action of cyclin-dependent kinase (CDK) complexes and CDK inhibitors (CKIs). Akt prevents cyclin D1 degradation by regulating the activity of the cyclin D1 kinase glycogen synthase kinase-3(GSK3) (Jope and Johnson, 2004). Phosphorylation of GSK-3β by Akt prevents its activity and leads to the accumulation of cyclin D1 and the consequent transition of cells from G1 to the S phase of the cell cycle (Liang and Slingerland, 2003). Other important regulators of the cell cycle are the FOXO transcription factors. Phosphorylation of FOXOs by Akt and exclusion from the nucleus leads to the increased transcription of cyclin D1 and the reduced transcription of the p27 and p21cyclin CKIs (Alvarez et al., 2001; Burgering and Medema, 2003). Additionally, Akt also regulates the p21 and p27 CKIs post-translationally resulting in their exclusion from the nucleus (Zhou, 2001; Fujita et al., 2002; Liang et al., 2002), leading to increased cell proliferation due to decreased inhibition of cyclins. p27 also acts as an oncoprotein in the cytoplasm where binds to and inhibits RhoA thus promoting cell migration (Besson et al., 2004). Indeed, the high cytoplasmic localization of p21 and p27 is

associated with high tumor grade, tumor cell invasiveness and metastasis (Sáez, 1999; Slingerland, 2000; Philipp-Staheli et al., 2001).

Cell growth: Increased Akt activity is correlated with the activation of the central regulator of cell growth, mTORC1 complex [(composed of mTOR, DEPTOR, mLST8, PRAS40 and regulatory associated protein of mTOR (RAPTOR)]. mTOR is a serine/threonine kinase which serves as a molecular sensor regulating protein synthesis on the basis of nutrient availability. Active mTORC1 complex regulates biogenesis and activate protein translation by phosphorylating the p70 ribosomal protein S6 kinase (S6K) and the inhibitory 4E‑binding protein 1 (4EBP1). The mechanism by which Akt leads to increased mTORC1 activity is: Akt directly phosphorylates and inactivates the tuberous sclerosis complex 1/2 (TSC1/2) which acts as a GAP protein on Ras homologue enriched in brain (RHEB), thus resulting in its activation (Garami et al., 2003; Li et al., 2004); Active (GTP-bound) RHEB stimulate the phosphotransferase activity of mTOR and consequently its downstream effector proteins (Inoki et al., 2003). Constitutive activation of mTORC1 as a consequence of PTEN inactivation, leads to the enhanced translation of specific mRNAs that are crucial for cell growth and proliferation (Salmena et al., 2008). The multiple roles of mTORC1 and especially those correlated with mRNA translation and cell cycle has made the PI3K/Akt/mTORC1 axis an attractive target for the development of dual PI3K/mTOR inhibitors, mTOR-selective inhibitors and Akt inhibitors as anti-cancer drugs (Marinov et al., 2007; Sabbah et al., 2011; Castillo et al., 2012; Sheppard et al., 2012; Weigelt and Downward, 2012; Willems et al., 2012).

As it is clear, PTEN exerts many of its cellular roles though the PI3K/Akt pathway. However, PTEN also operates though PI3K/Akt independent functions. Indeed, there are some differences between mouse models of PTEN loss and Akt overexpression (Blanco-Aparicio et al., 2007; Kharas et al., 2010). For instance, JNK pathway seems to be activated following PTEN loss in an Akt-independent manner (Vivanco et al., 2007). In addition, the eukaryotic translation initiation factor 2 α kinase 2 (eLF2αK2)-elF2α phosphorylation and microspherule protein 1 (MSP58)-mediated cellular transformation are regulated by PTEN independently of its phosphatase activity (Okumura et al., 2005; Mounir et al., 2009). Moreover, as we have already discussed, PTEN also show activity toward highly acidic substrates (Myers et al., 1997) and protein substrates such as FAK (Tamura et al., 1998) and cAMP responsive-element-binding protein (CREB) (Gu et al., 2011).

1.5 Downstream of PI3K signaling: Rho small GTPases

PI3K activity and PTEN are crucial for the determination of cell polarity as has been demonstrated mainly in fast migrating cells, such as *Dictyostelium discoideum* and neutrophils (Fenteany et al., 2004; Merlot et al., 2003). Similar to these cells, tumors cells during cancer progression, loss their intracellular and stromal adhesions and acquire an amoeboid-leukocyte-like individual mode of migration. Except from PI3K/PTEN, Rho family of small GTPases are also key signaling molecules in the regulation of cell polarity and migration. Proper PI3K activity defines the leading edge of the cell, whereas Rho GTPases regulate the cytoskeletal remodeling during polarization and migration (Fenteany et al., 2004; Merlot et al., 2003; Ridley et al., 2003).

The asymmetrical localized activation of PI3K at the cell membrane near the chemoattractant source, leads to the localized synthesis and accumulation of PI(3,4.5)P3 which define the leading edge of the cell (Servant et al., 2000; Procko et al., 2005) (Figure IV); and this early response is important for the proper polarization and chemotaxis of leukocytes (Niggli, 2000; Weiner et al., 2002). The specific PI(3,4,5)P3 localization is directed by the stimulation of PI3K activity by growth factor receptors and GPCRs and the concomitant suppression of PTEN at same cellular regions. Pharmacological or genetic inhibition of whole PI3K or different PI3K isoforms confirm the vital roles of

these enzymes in the directed cell polarization and migration in almost all eukaryotic cell types (Merlot et al., 2003; Wang et al., 2002; Niggli et al., 1997; Chung et al., 2001; Papakonstanti et al., 2007; Sawers et al., 2003; Reif et al, 2004; Papakonstanti, unpublished data). PI3K isoforms p110γ and p110δ seems to be responsible for the PI(3,4,5)P3-generation associated with migration in most of the cases.

PI3K activity promotes cell motility and cell spreading through the regulation of key downstream effectors, such as the Rho family of small GTPases members, Rho, Rac and Cdc42 (Barber and Welch, 2006). oreover, the asymmetric PI3K activation and PI(3,4,5)P3 distribution needs to be maintained in chemotaxing cells though spatially and temporally controlled positive feedback and negative regulation, processes that also involve Rho GTPases. Activation of PI3Ks leads to the recruitment PH-domain containing regulators of small GTPases (such as guanosine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Welch et al., 2002; Marone et al., 2008) (Figure II). Recruitment of GEFs at the plasma membrane leads to the activation of small GTPases (become GTP-bound) whereas the return from their active state to an inactive state (GDP-bound) is catalyzed by GAPs. This cyclic activation-inactivation of the small GTPases is required for cell body to move properly (Ridley et al., 2003). Rac seems to be the Rho GTPase responsible for mediating the positive feedback of PI3K activity at the leading edge (Weiner et al, 2002; Srinivasan et al., 2003). Accordingly, PI3K activation leads to the activation of Rac at the leading edge, though the activation of Rac-GEFs. Rac also catalyses the remodeling of the actin cytoskeleton at the leading edge required for the formation of novel cell protrusions. In addition to positive feedback on PI3K activity, asymmetric PI(3,4,5)P3 accumulation is maintained by excluding it from the trailing edge; and this is succeeded by the action of PTEN which is indirectly activated by the Rho GTPase RhoA at the trailing edge (Li et al., 2005). RhoA is localized and activated at the back of the cell, wherein it activates its target protein ROCK which then activates PTEN (Li et al., 2005). Active PTEN at the back of the cell further strengthens the asymmetrical distribution of PI(3,4,5)P3 at the leading edge, thus enhancing the cell polarization according to the chemoattractant gradient (figure IV).

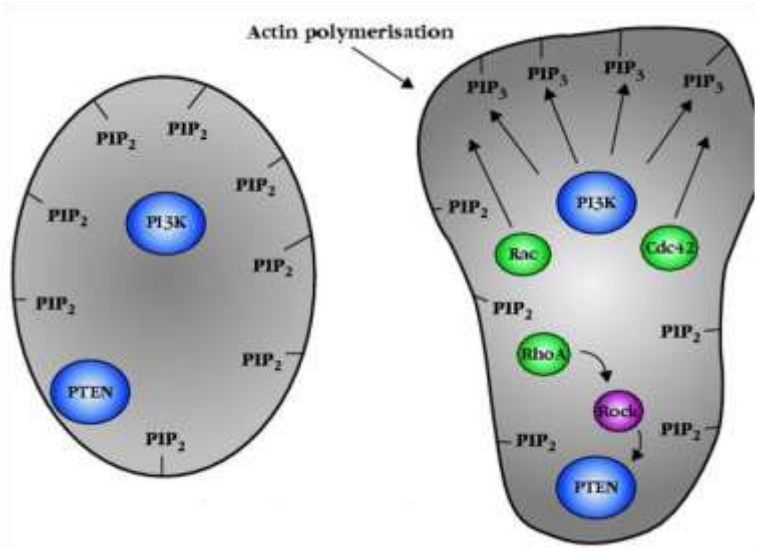


FIGURE IV: Role of PI3K and Rho-family GTPases in leukocyte polarization (simplified model). Unstimulated, resting leukocytes have a spherical shape_PI3K is localised in the cytoplasm and PTEN at the plasma membrane. Chemattractant binding to cell surface receptors stimulates PI(3,4,5)P3 production by PI3K in the membrane closest to the chemoattractant source. The external chemoattractant gradient is amplified internally by positive feedback of PIP3 production at the leading edge and by dephosphorylation of PIP3 at the trailing edge through PTEN. The localized accumulation of PIP3 is the signal that leads to activation of the Rho-family GTPase Rac at the leading edge. Active Rac regulates the remodeling of the actin cytoskeleton to allow cell protrusions to form at the leading edge. In parallel, RhoA activation at the trailing edge promotes PTEN activation and uropod formation (figure taken from Barbel and Welch, 2006).

Data from our laboratory demonstrated that RhoA/PTEN pathway can be regulated by PI3K itself and more specifically to be under the negative control of the p110δ PI3K isoform (Papakonstanti et al., 2007). Activation of

p110δ PI3K positively regulate the p190RhoGAP activity which is known to catalyze the inactivation of RhoA (return to RhoA-GDP inactive state) (Bernards and Settleman, 2005) and consequently to decreased PTEN activity. This isoform-selective role of p110δ in the inhibition of RhoA/PTEN activity is not currently understood but it may be related with differential activation at certain membrane and cellular compartment. This feedback mechanism was originally found in primary and transformed macrophages (Papakonstanti et al., 2007; Papakonstanti et al., 2008), wherein the negative regulation of RhoA/PTEN activity by p110δ PI3K has apparently physiological consequences in both chemotaxis and proliferation of these cells.

2. PTEN regulation

PTEN gene is one of the most commonly mutated of all tumour suppressor genes, and if not mutated, it is often suppressed or downregulated. Given the central role of PTEN function in suppressing PI3K signaling, it is clear why *PTEN* gene is commonly mutated in cancer cells. In addition to genomic disruptions that inactivate PTEN, other pathological mechanisms that repress PTEN expression have been described. PTEN expression can be disrupted by epigenetic silencing, transcriptional repression and microRNA (miRNA) regulation. Moreover, PTEN-interacting proteins and post-translational modifications such as phosphorylation in different sites seem to have important roles in the regulation of PTEN activity and subsequently in cancer predisposition. Our increasing knowledge of PTEN regulation and pathologies in which its function is altered will undoubtedly help to the designation of various novel therapies. In this chapter we summarize briefly the diverse regulatory mechanisms of PTEN in cell biology.

Genetic alterations of PTEN: *PTEN* gene is located on human chromosome 10q23, a locus that is highly susceptible to mutation in human cancers. Allelic losses result in complete deletion of *PTEN* locus, whereas point mutations can produce PTEN truncated mutants which are functional inactivated (Hollander et al., 2011). Germline mutations of *PTEN* gene are found in most cases of PTEN hamartoma tumour syndromes (PHTSs), including Cowden disease (Hollander et al., 2011). About two-thirds of *PTEN* mutations in Cowden disease are found in the region of phosphatase catalytic domain (Eng, 2003). However, many tumour-associated PTEN mutants retain partial catalytic function, suggesting that alternative mechanisms can lead to the inactivation of PTEN. For example mutation at Lys289 alters the localization of the PTEN protein (Trotman et al., 2007). Somatic mutations of *PTEN*, including insertions and deletions, occur in the whole gene, although there are some hotspot mutations at specific amino acids such as Arg130, Arg173 and Arg233 (Hollander et al., 2011).

Epigenetic silencing and transcriptional regulation*:* Epigenetic silencing through methylation of the *PTEN* promoter is observed in many cancer types (Hollander et al., 2011). In addition, several proteins regulate PTEN transcription negatively (SALL4, SNAIL, ID1, BMI1, c-JUN, EVI1 and MYC) and positively (PPARγ, EGR1, p53 and CBF1) (reviewed in Song et al., 2012). Especially for p53, experimental evidence and sequence analysis of the *PTEN* promoter have confirmed that the p53-mediated apoptosis is highly connected to the activation of *PTEN* transcription (Lee et al., 2009; Stambolic et al., 2001). In line with this, transcriptional regulation of PTEN expression is inhibited by the *trans*-acting transcription factor SNAIL and the oncogenic factor inhibitor of DNA binding 1

(ID1), which compete the binding-site of p53 on the *PTEN* promoter (Escriva et al., 2008). Overall, the transcriptional control of PTEN is subject to a complex network of tumour suppressors and oncoproteins.

Post-transcriptional regulation of PTEN by non-coding RNAs: Recently, numerous miRNAs have been demonstrated to promote tumorigenesis or other diseases by downregulating PTEN expression. These include miR-17~92 cluster in lymphoproliferative disease and autoimmunity, miR-19 in leukaemia and Cowden disease and miR-21 in multiple cancers, inflammation and metabolic diseases (Xiao et al., 2008; Olive et al., 2009; Mavrakis et al., 2010; Meng et al., 2007; Ma et al., 2011). Some of the molecular mechanisms regulating the control of PTEN-targeting miRNAs are now being elucidated. For example, MYC downregulate PTEN expression, upregulating the expression of miR-19 in B-cell lymphomas (Mu et al., 2009). Surprisingly, a PTEN pseudogene, *PTENP1*, was found to regulate PTEN expression through an unexpected coding-independent function. Actually, the *PTENP1* transcript (which shares high sequence homology with PTEN mRNA) acts as a decoy for PTEN-targeting miRNAs acting as a trans-regulator of PTEN (Poliseno et al., 2010). This model of transcriptional regulation was termed as ceRNA hypothesis (Salmena et al., 2011). Further bioinformatic and experimental approaches identified a large regulatory network of ceRNA transcripts that controls PTEN expression in prostate cancer, glioblastoma and melanoma (Karreth et al., 2011; Sumazin et al., 2011; Tay et al., 2011). ceRNA hypothesis of regulation of protein expression is a totally new area of investigation and further studies are needed to unravel the complexity and the impact of tumour suppressor and oncogenic ceRNA networks in normal and pathological conditions.

Regulation of PTEN by post-translational modifications (Figure V)**:**

Phosphorylation of PTEN on the C-terminal tail at Ser380, Thr382, Thr383 or Ser385 residues is thought to inhibit its phosphatase activity while favoring its stabilization: C-terminal tail phosphorylation promotes a more closed and stable PTEN conformation, which reduces its plasma membrane localization and lipid phosphatase activity (Rabinovsky et al., 2009; Vazquez et al., 2001). Proteins such as Casein kinase 2 (CK2) downstream of Src phosphorylate PTEN at Ser370 and Ser385 and glycogen synthase kinase 3α (GSK3α) phosphorylates Ser362 and Thr366 residues (Al-Khouri et al., 2005; Miller et al., 2002; Torres and Pulido, 2001). Unlikely, Glioma tumor suppressor candidate region 2 (GLTSCR2 or PICT-1) upregulate the levels of PTEN activity by promoting its phosphorylation on Ser380 (Okahara et al., 2006; Yim et al., 2007). Thus the consequences of the phosphorylation of certain residues are still under debate. RhoA-associated kinase (ROCK) activates also PTEN by phosphorylating its C2 domain on Ser229, Thr232, Thr319 and Thr321 (Li et al., 2005). As was previously shown C2 domain phosphorylation can target PTEN to bind directly phospholipid membranes *in vitro* (Raftopoulou et al., 2004). Phosphorylation of PTEN on Tyr residues by Tyr protein kinases, such as RAK which lead to increased PTEN activity, has been reported on several studies (Koul et al., 2002; Papakonstanti et al., 2007; Sanchez et al., 2005; Yim et al., 2009).

Acetylation of PTEN inhibits its catalytic activity and enhances its interaction with PDZ domain-containing proteins. PTEN seems to be acetylated at Lys125–Lys128 by the p300/CREB-binding protein (CBP)-associated factor (PCAF) and at Lys402 by CBP, whereas Sirtuin 1 (SIRT1) can act as a deacetylase of PTEN (Okumura et al., 2006; Chae et al., 2011). **Oxidation** can regulate PTEN like other PTPs. Indeed, the catalytic activity of PTEN is modulated by reactive oxygen species (ROS) through oxidative stress-induced formation of a disulphide bond between the Cys71 and Cys124 (Lee et al., 2002). Finally, **ubiquitylation** of PTEN at conserved Lys13 and Lys289 residues, which are often mutated in somatic cancers and Cowden disease respectively, is necessary for the nuclear import of PTEN (Trotman et al., 2007). Moreover, NEDD4-1 is an E3 ligase that ubiquitylates PTEN,

resulting in different activity outcomes (Yim et al., 2009; Wang et al, 2007). Overall, modulation of PTEN activity by posttranslational modifications represents an attractive subject for novel therapeutics.

PTEN regulation by protein–protein interactions: Protein–protein interactions can affect the tumour-suppressive activity of PTEN by modifying its conformation, stability, lipid membrane-binding potential and subcellular distribution (Takahashi et al., 2006; Molina et al; 2012; van Diepen et al., 2009). Interestingly, recent studies identified an interaction between p85 and PTEN which downregulate the downstream PI3K/Akt pathway (Rabinovsky et al., 2009; Chagpar et al., 2010). Indeed, hepatic carcinomas that arose from *p85*-deficient mice exhibited increased PI(3,4,5)P3 levels and Akt activation combined with decreased PTEN expression (Taniguchi at al., 2010). Numerous proteins have also been found to bind with PTEN and to inhibit its tumour-suppressive activity. One of those seems to be the Parkinson protein 7 (PARK7or DJ1) (Cotter et al., 2010; Kim et al., 2005). DJ1 expression is associated with increased levels of activated Akt and poor clinical outcome in various cancer types (Kim et al., 2009). Other interesting regulators that bind PTEN inhibiting its lipid phosphatase activity include the proteins PI(3,4,5)P3-dependent RAC exchanger factor 2a (PREX2a), shank-interacting protein-like 1 (SIPL1 or SHARPIN) and α-mannosidase 2C1 (MAN2C1) (Fine et al., 2009; He et al., 2010; He et al., 2011). Analysis of clinical samples reveals that PTEN expression correlates with the expression of PREX2a, SIPL1 and MAN2C1 in breast, cervical and prostate cancers, respectively. To better understand the regulation of PTEN phosphorylation, stability and function, we need to identify the various PTEN-interacting proteins.

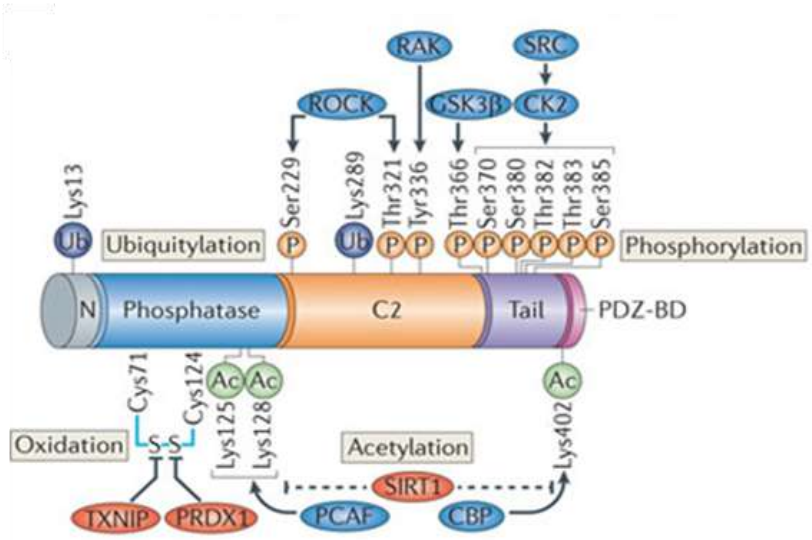


FIGURE V: PTEN regulation by post-transcriptional modifications. Phosphorylation of PTEN at Ser229, Thr321, Tyr336, Thr366, Ser 370, Ser380, Thr382 and Ser385 by the indicated kinases is implicated in modulating PTEN tumour suppressive function, cell membrane association and stability. P300/CBP-associated factor (PCAF) promotes PTEN acetylation at Lys125 and Lys128, whereas CBP acetylates Lys402. The formation of a disulphide bond between Cys124 and Cys71 by oxidation also reduces the catalytic activity of PTEN, but this may be averted by the deacetylase sirtuin 1 (SIRT1). Ubiquitylation of PTEN at Lys13 and Lys289 regulates PTEN tumour suppressive function, subcellular distribution and stability. (figure taken from Song et al., 2012).

The implication of PTEN in many crucial anticancer mechanisms makes it as an important therapeutic target. However, little progress has been made until now in developing agents to therapeutically enhance the tumour suppressive function of PTEN in cancer. Nevertheless, *PTEN* gene mutational status is commonly used as a diagnostic and prognostic factor in cancer-predisposition. Hopingly, the accumulating knowledge of PTEN function and regulation may give more information about its pharmacological manipulation

3. Class IA PI3K isoform-specific functions

3.1 Class IA PI3K isoform distribution

In contrast to Class IA PI3K isoforms p110α and p110β, which are constitutively expressed in all cell types and tissues, p110δ was found to be predominantly expressed in leukocytes (Hu et al., 1993; Bi et al., 1999; Bi et al., 2002; Geering et al., 2007; Chantry et al., 1997; Vanhaesebroeck et al., 1997b). However, previous studies combined with recent data from our laboratory revealed that p110δ PI3K isoform is also expressed at high levels in some cancer cell lines and human tissues of non-leukocyte origin such as breast and prostate cancer cells (Sawyer et al., 2003; Tzenaki et al., 2012) and at moderate levels in neurons (Eickholt et al., 2007). The regulation of the leukocyte-specific p110δ PI3K expression was recently explored. The identification of a highly conserved transcription factor binding cluster in the *PI3KD* gene, displaying higher promoter activity in leukocyte compared to non-leukocyte cells, provide an explanation for this selective high expression of p110δ levels in leukocytes (Kok et al., 2009b; Whitehead et al., 2012). Transcriptional regulation of *PIK3CD* by RUNX1 (Edwards et al., 2009) and leukocyte-dependent promoter DNA hypomethylation (Calvanese et al., 2012) were also proposed to be responsible for the high p110δ expression in leukocytes. It is possible that p110δ expression is transcriptionally regulated also in non-leukocyte cells, such as breast cancer cells that express high levels of p110δ. Indeed, several leukocyte-related transcription factors have been found to be activated in breast cancers (Teschendorff et al., 2007).

3.2 Class IA PI3K isoform functions

Functions of the three class IA PI3K isoforms are non-redundant besides their identical enzymatic activity. Indeed, the three isoforms are differentially implicated in various cellular responses such as metabolism and tumorigenesis (Roche et al., 1994; Roche et al., 1998; Vanhaesebroeck and Waterfield, 1999; Hill et al., 2000; Hooshmand-Rad et al., 2000; Leverrier et al., 2003; Vanhaesebroeck et al., 2005; Foukas et al., 2006; Ali et al., 2008; Graupera et al., 2008; Papakonstanti et al., 2008). The identification of many cancer-correlated gain-of-function mutations in *PIK3CA* gene placed p110α isoform in the centre of cancer research (Campbell et al., 2004; Samuels and Velculescu, 2004). In contrast to *PIK3CA* gene, no somatic mutations of genes encoding p110β or p110δ have been reported (Samuels and Velculescu, 2004; Thomas et al., 2007; Wood, 2007; Parsons et al., 2008). Nevertheless, p110β has a key role in platelet biology and thrombosis (Jackson et al., 2005) whereas recent studies have also shown a role of p110β in certain cancers and especially in tumor cells lacking PTEN (Ciraolo et al., 2008; Jia et al., 2008; Torbett et al., 2008; Wee et al., 2008; Zhu et al., 2008).

The p110δ isoform-specific functions were demonstrated by both genetic and pharmacological inhibition, using mice with inactivated p110δ (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Ali et al., 2004; Aksoy et al., 2012) and p110δ-selective inhibitors such as the IC87114 384 compound, which was the first isoform-selective inhibitor published (Sadhu et al., 2003). The fact that p110δ is preferentially expressed in leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997b) together with the absence of somatic mutations in *PIK3CD* gene turn researchers to early connect p110δ PI3K isoform with immune system and hematologic cancers. The functional role of p110δ in immune system has been extensively studied and p110δ proved to have many important isoform specific functions (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Ali et al., 2004; Aksoy et al., 2012). Targeting of p110δ is now considered very useful in pathological conditions of immunity and inflammation (Rommel et al., 2007; Rommel, 2010; Soond et al., 2010). Furthermore, accumulated findings have demonstrated a seminal role of p110δ PI3K in

lymphoid and myeloid malignancies (Okkenhaug and Fruman, 2010; Fruman and Rommel, 2011). Actually, a p110δ-selective inhibitor, CAL101, has almost entered clinical studies showing effective clinical outcomes in hematologic malignancies (Fruman and Rommel, 2011; Castillo et al., 2012).

3.3 A promising role of p110δ in non-hematologic cancer via the regulation of PTEN

Additional data have also suggested a role of p110δ PI3K in the promotion of cancers of non-hematopoietic origin. The role of p110δ in oncogenesis of non-hematopoietic cells was firstly indicated by the finding that genetically induced p110δ overexpression in avian fibroblasts is able to induce oncogenic transformation, comparable to this detected in cells expressing oncogenic p110α mutants (Kang et al., 2006). This p110δ oncogenic activity was independent from Ras and resistant to inhibitors of the MAPK pathway (Zhao and Vogt, 2008a; Vogt et al., 2009). Further data illustrating increase p110δ expression also support a role of p110δ in non-hematologic human cancers: overexpression of p110δ mRNA and increased copy number of the *PIK3CD* gene in cases of glioblastoma (Knobbe and Reifenberger, 2003; Mizoguchi et al., 2004), increased p110δ mRNA in prostate carcinomas compared with normal prostate (Jiang et al., 2010), abnormally high p110δ expression levels in primary neuroblastoma tissue compared with the normal adrenal gland tissue (Boller et al., 2008). Moreover, suppression of p110δ expression in neuroblastoma cells led to impaired cell growth and survival (Boller et al., 2008). These evidence suggest that the oncogenic potential of wild-type p110δ might be correlated with its expression level in cancers of non-hematopoietic origin.

A molecular mechanism that could explain the oncogenic potential of p110δ was firstly indicated in studies performed in macrophages (Papakonstanti et al., 2007; Papakonstanti et al., 2008) and in mouse brain tissue (Eickholt et al., 2007). PI3K p110δ activity was found to inhibit the PTEN tumor suppressor via a negative signaling pathway, involving the inhibition of RhoA/ROCK pathway (Papakonstanti et al., 2007) (Figure VI). Most precisely, p110δ was found to positively regulate the p190RhoGAP activity and to result in the accumulation of p27 in the cytoplasm (Papakonstanti et al., 2007). Given that p190RhoGAP is a negative regulator of RhoA activity (catalyzes the return of RhoA-GTP to RhoA-GDP) (Bernards and Settleman, 2005) and p27 prevents the return of RhoA-GDP to RhoA-GTP (Besson et al., 2004), the activation of p110δ leads to decreased RhoA activity and consequently to decreased PTEN activity. Accordingly, both genetic and pharmacological inactivation of p110δ leads to increased PTEN activation and decreased Akt activation and cell proliferation. More recently we showed that this mechanism is also the case in those cancer contexts where p110δ is expressed at high levels (Tzenaki et al., 2012). The p110δ protein was found to be expressed, although at different levels, in different cancer types: the p110δ PI3K is the predominant isoform expressed in human primary breast carcinoma, whereas ovarian and cervical human carcinomas mainly express p110α and p110β (Tzenaki et al., 2012). The activity of wild-type PTEN was found to be suppressed in breast and prostate cancer cells that express high levels of p110δ suggesting that the elevated expression of p110δ might provide these cells with a competitive advantage by keeping their wild-type PTEN inactive (Tzenaki et al., 2012). Breast and prostate cancer cells expressing functional PTEN were also sensitive to the anti-proliferative effect of p110δ inhibitors through PTEN activation (Tzenaki et al., 2012).

Together these data suggest that the level of p110δ expression might represent one of the parameters that correlate with the cancer type-specific response to PI3K pathway inhibitors. This hypothesis is also collaborated by other contracting results. For example, breast cancer cells without mutations in *PTEN* or *PIK3CA* genes, were found to be sensitive to growth inhibition by PI3K inhibitors, whereas breast cancer cells with PTEN deficiency were found to be resistant to PI3K- , mTOR- and combined PI3K/mTOR-inhibitors (O'Brien et al., 2010; Tanaka et al., 2011; Brachmann et al., 2009; Weigelt et al., 2011). Unlikely, in ovarian cancer cells, *PIK3CA* gain-of-function mutations

and PTEN deficiency are strictly correlated with the response to PI3K pathway inhibitors (Ihle et al., 2009; Di Nicolantonio et al., 2010; Meuillet et al., 2010; Santiskulvong et al., 2011;Tanaka et al., 2011; Meric-Bernstam et al., 2012). Other findings also confirm that in breast cancer cells there is no good correlation between the presence of *PIK3CA* gain-of-function mutations and the PI3K and Akt activity (Stemke-Hale et al., 2008) suggesting that other regulatory mechanism may affect the status of PI3K activity. Better understanding of p110δ regulation and function in different cancer types may reveal whether the efficacy of p110δ-selective inhibitors could be beneficial. Given that *PTEN* gene is often wild-type in human breast cancers (Stemke-Hale et al., 2008;Chalhoub and Baker, 2009), p110δ-selective inhibitors alone or combined with other inhibitors of PI3K pathway could be useful in this cancer type.

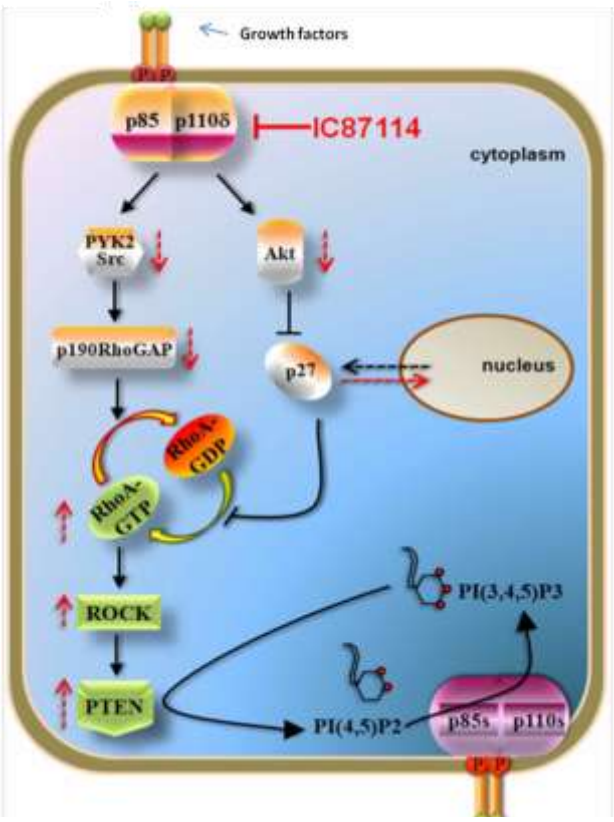


FIGURE VI: p110δ PI3K mediates the effects of growth factors (CSF-1, EGF) via RhoA and PTEN in macrophages and solid tumor cells. Activation of p110δ leads to increased activity of p190RhoGAP (through PYK2/Src activation) and to cytoplasmic accumulation of p27 (which is mediated by the increased Akt activity). p190RhoGAP induces the inactivation of RhoA whereas p27 prevents the activation of RhoA by both leading to reduced RhoA activity and consequently to decreased PTEN activity. Inactivationof p110δ by IC87114 reverses these pathways leading to PTEN activation which then opposes the PI3K reaction of the remaining active p110 isoforms. The p190RhoGAP-driven mechanism almost solely keeps RhoA activity level lower than basic whereas that of p27 contributes upon stimulated conditions. Red arrows represent alterations in activity or location in the presence of IC87114 (figure taken from Tzenaki & Papakonstanti 2013).

4. FAK and its interaction with PTEN

FAK is a cytoplasmic tyrosine kinase protein constitutively expressed in almost all tissues. Its sequence is highly conserved among species and its structure contains: an N-terminal FERM domain, a central catalytic tyrosine kinase domain, two proline-rich motifs and a C-terminal focal-adhesion targeting (FAT) domain (Girault et al., 1999). The FERM domain interacts with the catalytic domain in order to autoinhibit the kinase activity, whereas the proline-rich regions serve as docking sites for binding downstream effector proteins. FAK expression is necessary for the early embryonic development and for postnatal angiogenesis (Braven et al., 2006; Shen et al., 2005; Tavora et al., 2010). Importantly, FAK overexpression is often connected with the development of various human diseases, including human cancer (Golubovskaya et al., 2009; Schaller, 2010).

FAK was firstly identified as a protein associated with focal adhesions downstream of intergrin signaling (Schaller et al., 1992). Except of integrins, FAK responds and activated by several extracellular stimuli including growth factors, chemokines and regulates important cellular processes such as proliferation and cell migration (reviewed in Scaller, 2010). The first and determinant event upon FAK activation is its autophosphorylation on Tyr397 residue, which eventually promotes the recruitment/activation of many SH2 domain-containing proteins including the Src-family of kinases and the p85 subunit of PI3K (Schaller et al., 1994; Xing et al., 1994; Chen et al., 1994; Chen et al., 1996) (Figure VII). The proximity of Src to FAK results in its additional phosphorylation at other sites. Other crucial phosphorylation sites of FAK are the residues Tyr861 and Tyr925 at the C-terminal domain which leads to the activation of downstream protein effectors at adhesion sites including the Ras-MAPK pathway and Akt (Calalb et al., 1996; Qi et al., 2001; Abu-Ghazalef et al., 2001; Schlaepfer et al., 1994; Mitra et al., 2005). RhoA and ROCK were also suggested to be positive regulators of FAK activity, as their inhibition resulted in the inhibition of FAK phosphorylation in a variety of cell systems (Chaturvedi et al., 2011; Goundiam et al., 2012; Sinnett-Smith et al., 2001; Torsoni et al., 2005; Yagi et al., 2006), whereas PI3K activation was suggested to downregulate the CSF-1-induced FAK phosphorylation in macrophages (Rovida et al., 2005). No explanation for these observations has been put forward so far.

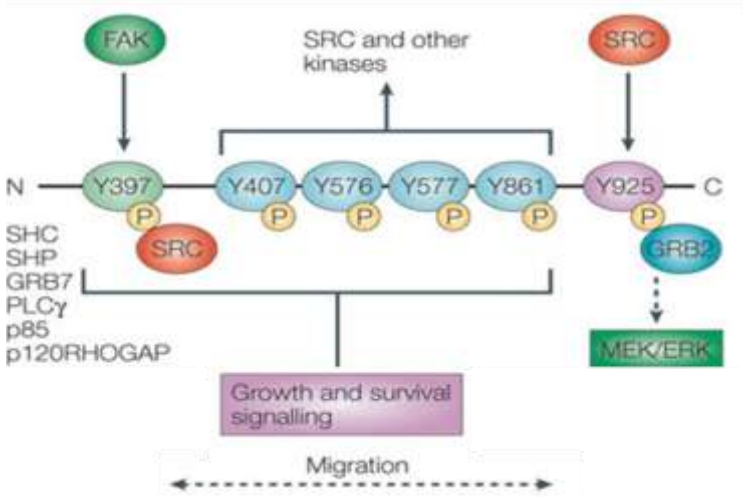


Figure VII: FAK tyrosine phosphorylation regulates downstream signaling events. Phosphorylation of Tyr397 is mainly due to autophosphorylation or transphosphorylation by growth-factor receptors. This creates a high-affinity binding site for the SH2-domain of SRC (and other SH2-domain-containing proteins), which can then phosphorylate focal-adhesion kinase (FAK) on additional tyrosine residues, of which Y925, in particular, appears to be a SRC-specific site. Phosphorylation of Y397 or Y925 cause increased complex formation between FAK and other SH2-containing proteins (p85, PLCg, GRB7, GRB2, p120RHOGAP). Other phosphorylation sites are likely to mediate growth and survival signalling, probably by creating binding sites for partner proteins. The SRC-specific FAK-Y925 phospho-acceptor site is also proposed to link FAK to the RAS–MAPK pathway. In this way, altered tyrosine phosphorylation of FAK in tumour cells could control subcellular localization, adhesion type predominance, growth and survival signalling, and cancer cell behavior (figure taken from McLean et al., 2005).

Src binding to FAK promotes the cell spreading though its recruitment and activation at the cell-extracellular matrix adhesion sites (Yeo et al., 2006) and FAK-Src complex is frequently implicated in the promotion of migration of breast cancer cells (Chan et al., 2009). Many other studies have confirmed the involvement of FAK in cell migration (Erickson, 1990; Sieg et al., 2000; Braven et al., 2006, Mitra et al., 2006). Activation of FAK is also crucial for protecting cells from apoptosis, though the attachment to cell matrix (Frisch et al., 1996; Hungerford et al., 1996; Xu et al., 2000; Sonoda et al., 2000; Garces et al., 2006). Moreover, a lot of studies have linked the inhibition of FAK with reduced proliferative responses (Zhao and Guan, 2009). Unlikely, other published data have correlated reduced levels of FAK with elevated survival signals and growth (Nakamura et al., 2008; Sweeney et al., 2003; Wang and Basson, 2011).

The role of FAK in diverse signal transduction events suggests that abnormal FAK signaling may lead to cancer development. Indeed, several reports have indicated that increased FAK expression within tumors correlates with tumor progression, however high levels of FAK do not always predict the disease outcome (Golubovskaya et al., 2009; Zhao et al., 2009; Slack-Davis et al., 2009; Luo et al., 2010; Schmitz et al., 2005; Furuyama et al., 2006; Theocharis et al., 2003; Ozkal et al., 2009; Carelli et al., 2006; Lahlou et al., 2007). A very recent study illustrated that reduction of tumor stromal-FAK expression in FAK-heterozygous mice leads to enhanced tumour growth and angiogenesis and this effect is correlated with enhanced endothelial cell proliferation and Akt activity (Kostourou et al., 2013). The mechanism by which stromal cells in FAK-heterozygous mice induced tumor growth is not clear. Moreover, low doses of the FAK-inhibitor PF-228 (that mimicked the FAK-heterozygosity) induced tumor growth and angiogenesis (Kostourou et al., 2013). Treatment also of cancer cell lines with PF-228 failed to block cell growth and instead, at least in some cancer cell lines, increased the cells growth (Slack-Davis et al., 2007). Such contradicting results suggest that FAK may have nonlinear dose-dependent roles, supposing that different FAK expression levels and activity may differentially regulate cellular functions within the same or other cell contexts. Indeed, although FAK is expressed ubiquitously, its expression and activity levels can vary between cells and even within the same cell type (Cai et al., 2008). In some cancer cases, the FAK expression may be upregulated by different transcription factors. Analysis of human *fak* gene promoter identified p53 and NFκB proteins as potential repressor and activator respectively of the promoter (Golubovskaya et al., 2004; Golubovskaya et al., 2007). The development of FAK inhibitors as potential anticancer drugs (Brunton et al., 2008) and the fact that complete pharmacological inhibition of FAK is difficult to maintain in vivo (Reynolds et al., 2009) make the need of elucidation of the effect of FAK activity in cancer progression of major priority.

FAK-PTEN interaction (previous data)**:** PTEN has previously been shown to interact and dephosphorylate FAK leading to the regulation of various processes such as cell spreading and formation of focal adhesions (Tamura et al., 1999a; Tamura et al., 1998; Tamura et al., 1999b).The effect of FAK-PTEN interaction was firstly investigated in order to elucidate the mechanisms by which the PTEN overexpression leads to inhibited cell migration, whereas PTEN inhibition enhance migration (Tamura et al., 1998). Indeed, PTEN was found to interact with FAK and to inhibit its activity though tyrosine dephosphorylation (Tamura et al., 1998; Tamura et al., 1999b). Subsequently, a study from the same group using a PTEN trapping mutant, demonstrated that PTEN interact and binds directly the wild type FAK protein, a possess that is dependent on the phosphorylation status of FAK at Tyr397 residue (Tamura et al., 1999a). This study testified that PTEN directly dephosphorylates tyrosine-phosphorylated FAK, but failed to prove the direct binding of wild-type PTEN with FAK (Tamura et al., 1999a). These scientific results suggest that the important roles of PTEN in the tumour progression may relay partly at its interaction with FAK and so the downregulation of FAK downstream pathways.

5. Purpose and conclusion of the present study

- **In the present study we have used genetic and pharmacological approaches and cell-free assays to dissect the mechanism of RhoA-induced activation of PTEN downstream of p110δ PI3K. We show the unexpected finding that FAK protein interacts with and activate PTEN, though phosphorylation on Tyr residues, and that the upstream regulation of FAK is under the negative control of p110δ PI3K.**

- **These are the first data documenting that the activity and tyrosine phosphorylation of PTEN are controlled by FAK protein. We firstly show that the relationship of PTEN with FAK is actually bidirectional whereby FAK phosphorylates and thus regulates PTEN activity. We also show that this FAK-mediated regulation of PTEN has a significant role in the regulation of the biological functions of PTEN, including Akt activation and cell proliferartion/survival.**

Materials and methods

Cell culture and treatments

Culture of cancer cell lines: Cells were cultured in DMEM (Life Technologies; Invitrogen) supplemented with 10% heat-inactivated FBS (Life Technologies). Cells were detached using trypsin/EDTA, centrifuged, resuspended in growth medium, and seeded for experiments. In all experiments described below (unless otherwise specified), the medium was changed to starvation medium (growth medium without FBS) 24 h prior to the actual experiments. The concentration of EGF (Sigma) for cell stimulation was 50 ng/ml. Cancer cell lines used in this study were: MDA-MB-231 (breast), OVCAR-3 (Ovary) and HeLa (Cervix).

Isolation and culture of bone marrow-derived macrophages (BMMs): BMMs were derived from at least three 6- to 8-week-old mice per experiment and pooled. Cells were seeded on bacteriological plastic plates at 10^6 cells/ml in macrophage growth medium consisting of RPMI 1640 (Life Technologies; Invitrogen), 1 mM sodium pyruvate (Life Technologies; Invitogen), 1X nonessential amino acids (Life Technologies), 0.029 mM β-mercaptoethanol (Sigma), and 10% heat-inactivated FBS (Life Technologies) supplemented with 10% L-cell-conditioned medium as a source of CSF-1. After 3 days, nonadherent cells were collected and either cryogenically stored in FBS containing 10% DMSO or seeded at 6–8 X 10^5cells/ml on bacteriological Petri dishes and cultured for 4 d before use. Cells were detached using EDTA, centrifuged at 1000 *g*, resuspended in macrophage growth medium, and seeded for the experiments. All results were obtained from cells that had been cultured for no longer than 10 d after dissection. In all experiments described below (unless otherwise specified), the medium was changed to macrophage starvation medium (macrophage growth medium without L-cell-conditioned medium) 16–20 hours prior to the actual experiments. Unless otherwise indicated, the concentration of CSF-1 (PeproTech) for cell stimulation was 30 ng/ml.

Inactivation of class IA isoforms in macrophages and cancer cells: BMMs were derived from wild-type (WT) mice, or from mice in which both p110δ alleles are replaced by a kinase-dead version of p110δ ($\delta^{D910A/D910A}$), as a result of knock-in mouse gene targeting, or from mice in which both p110δ alleles are replaced with p110δ constitutive activated mutants ($\delta^{S1039A/S1039A}$) (Okkenhaug et al, 2002). Pharmacological intervention with p110δ in cancer cells and WT BMMs was achieved by the use of IC87114, a small molecule inhibitor with selectivity for p110δ (Sadhu et al, 2003). To interfere with the activity of PI3K isoforms p110α and p110β, the small molecule inhibitors PW12, TGX155 were used respectively (Papakonstanti et al., 2008).

Inactivation of FAK: Cells were pretreated with PF-228 (1μM) (Sigma) for 1 hour before CSF1 stimulation and lysis.

Inactivation of ROCK: Cells were pretreated with Y27632 (25mM) (Calbiochem-Novabiochem) for 15min before CSF1 stimulation and lysis.

Cell Lysis and Immunoprecipitation (IP)

Whole cell Lysis and P-Tyr or FAK Immunoprecipitation: Cells were lysed in lysis buffer containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 50 mM NaF, and 1% Triton X-100 supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate, followed by clearing of the lysate by centrifugation in a cooled microcentrifuge. Supernatants were directly used for analysis by SDS-PAGE, or immunoprecipitated at 4°C overnight using mouse monoclonal anti-P-Tyr (Santa cruz) or rabbit polyclonal anti-FAK (Santa cruz) antibodies. Immune complexes were collected with 40μl of 50% slurry of protein A- or protein G-Sepharose after incubation for 2 h and washed with lysis buffer.

Cell Lysis and PTEN Immunoprecipitation: For detection of Tyr-phosphorylated PTEN cells pre-incubated with 30 μM pervanadate for 10 min and after stimulation lysed in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 25 mM β-glycerol phosphate, 1 mM DTT. IP was for 90 min at 40C using goat polyclonal antibody to PTEN (N19) (Santa Cruz) followed by 2 h incubation with protein G-Sepharose.

Western Blot

Whole proteins or IP samples were resolved on 8-10% SDS-PAGE and transferred onto PVDF membranes. The blots were probed with the indicated antibodies, followed by detection using enhanced chemiluminescence. For analysis of total cell lysates, 80–100 μg cell extract/lane was loaded on an SDS-PAGE gel. Polyclonal rabbit antibodies to phosphoS473-Akt, total Akt and total PTEN, were obtained from Cell Signaling, and Santa cruz was the source of mouse monoclonal anti P-Tyr and rabbit monoclonal anti FAK.

Cell viability assay

Cells were seeded at 10^4cells/well in 96-well plates and starved for 24 h, followed by stimulation as indicated. After 48h, a mixture of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt]-tetrazoleum/PMS (phenazine methosulfate) was added and further processed according to the manufacturer's protocol (CellTiter 96; Promega, Madison, WI, USA). The absorbance of the formazan produced was measured at 490 nm directly from 96-well assay plates. Each experiment was done in triplicate and repeated at least twice.

In vitro kinase assay

Human recombinant PTEN (Cayman) solution (1μg/ml in PBS+DTT) and control substrate solution (1μg/ml Poly (Glu4-Tyr) Peptide) were added to 96 well ELISA high binding plates (Costar) and incubated O.N. at 4^0 C. The other day, the PTEN solution was been removed from experimental wells and the Tyrosine kinase assay was performed in 50 μl kinase reaction buffer (100mM Hepes pH 7.5, 250 mM NaCl, 96 mM MgCl2 , 2 mM DTT, 100μM ATP) containing 0,1 μg/μl FAK active enzyme (SignalChem) and 50mM sodium Orhovanadate, at RT for 15min. Where indicated, 50μM of FAK Inhibitor was added on kinase reaction buffer. The phosphorylation

reaction was stopped by removing the solution from experimental wells and by several washes with cold washing buffer (PBS 0,05 Tween). The determination of PTEN phosphorylation on Tyr was achieved by the incubation of wells with a anti-phospho-tyrosine (PY20) antibody, followed by horseradish peroxidase-conjugated goat anti-mouse IgG antibody. PTEN Tyrosine phosphorylation was measured using TMB solution (Sigma T0440) according to the manufacturer's instructions.

[3H]Thymidine incorporation assay:

Cells were seeded at 2.105 cells per well in 96-well plate, starved for 16 h, followed by stimulation as indicated in medium containing [3H]Thymidine. 24 or 48 h later the cells were harvested and the [3H]Thymidine incorporated in DNA measured by scintillation counting.

RESULTS

1. FAK phosphorylates PTEN on Tyrosine Residues

To investigate whether PTEN is a substrate for FAK, we first carried out an *in vitro* kinase assay and we tested if FAK could induce PTEN tyrosine phosphorylation (Figure 1). We found that addition of an active recombinant FAK enzyme on the reaction buffer increase the phosphorylation of PTEN whereas, the additional adding of the FAK inhibitor, PF-228, could reverse the phosphorylation levels at basal levels (Figure 1). Phosphorylation of control substrate solution was kept under the basal PTEN phosphorylation levels, confirming that FAK react with PTEN to induce its phosphorylation (Figure 1).

- These data indicate that FAK is able of phosphorylating PTEN *in vitro.*

2. Inactive p110δ induces whereas constitutively active p110δ suppresses FAK phosphorylation

We next tested whether FAK phosphorylation/activation is affected by the class IA PI3K isoforms. To pharmacologically interfere with the activity of PI3K isoforms in wild type (WT) bone marrow derived macrophages (BMMs), we used the small molecule inhibitors PW12 (0.5μM), TGX155 (0.5 μM) and IC87114 (5μM), which have selectivity for p110α, p110β and P110δ, respectively (Papakontsanti et al., 2008). We also derived BMMs from mice homozygous for inactive germline alleles of p110δ (δD910A/D910A) and from mice expressing constitutively active p110δ ($\delta^{S1039A/S1039A}$), using cells derived from WT mice as controls.

We first tested whether Tyrosine phosphorylation of FAK is affected by treatment of WT macrophages with extracellular CSF1 and different isoform inhibitors. Pharmacological inactivation of p110δ by IC87114 led to increased Tyr phosphorylation of FAK both under basal and CSF1-stimulated conditions on WT BMMs (Figure 2), whereas pharmacological inactivation of p110β or p110α did not affect significantly FAK phosphorylation (Figure 2).

Next, we investigated the effect of genetic inactivation or constitutively activation of p110δ on the phosphorylation levels of FAK. Similar to pharmacological inactivation, genetic inactivation of p110δ led to increased Tyrosine phosphorylation of FAK, both under basal and CSF-1-stimulated conditions in $\delta^{D910A/D910A}$ macrophages, compared to WT cells (Figure 3A). In contrast, both basal as well as CSF-1-stimulated levels of phosphorylated FAK were decreased in $\delta^{S1039A/S1039A}$ macrophages derived from mice expressing constitutively active p110δ, compared to WT cells (Figure 3B).

A similar increase in FAK phosphorylation levels was also observed in EGF-treated MDA-MB-231 breast cancer cell line which express high levels of p110δ in response to IC87114 treatment (Figure 3C and 3E). In contrast, pretreatment of HeLa cervical cancer cells that express very low p110δ levels with IC87114 did not affect the phosphorylation of FAK (Figure 3D and 3E).

- These data collectively suggest a reverse correlation between the presence and magnitude of p110δ activity and the phosphorylation of FAK in p110δ-expressing cells.

3. Inactive p110δ suppresses whereas constitutively active p110δ induces Akt phosphorylation and cell proliferation.

Then we examined if the Akt phosphorylation is affected similar to FAK phosphorylation by p110δ deficiency or p110δ overexpression. As was expected from previous data (Papakonstanti et al., 2008) CSF1 stimulated Akt phosphorylation is kept lower in $\delta^{D910A/D910A}$ compared to WT cells (Figure 4A). This effect is reversed in $\delta^{S1039A/S1039A}$ cells, where the phosphorylation of Akt is higher compared to WT BMMs at all stimulation time points (Figure 4B). CSF1 stimulated cell proliferation was also decreased in $\delta^{D910A/D910A}$ (Figure 4C) and increased in $\delta^{S1039A/S1039A}$ BMMs (Figure 4D) when compared to WT cells, as was indicated by the thymidine incorporation at the same stimulation time points.

- These data show that Akt phosphorylation and cell proliferation, reversely to FAK phosphorylation, are positively regulated by p110δ activity.

4. FAK is downstream of RhoA and upstream of PTEN in the p110δ signaling pathway

Previous studies from our lab have documented that p110δ, but not p110α or p110β, negatively controls PTEN activity through a RhoA-based feedback mechanism in primary and transformed macrophages (Papakonstanti et al., 2007; Papakonstanti et al., 2008). Recently, we demonstrated that PTEN activity is induced by inactivation of p110δ PI3K in breast and prostate cancer cells that express p110δ levels in the range of 50–80% of those found in leukocytes (Figure 3E) (Tzenaki et al., 2012). Modulation of PTEN activity by p110δ was not observed in ovarian or cervical cancer cell lines, which expressed around 5–15% of the p110δ levels found in leukocytes (Figure 3E) (Tzenaki et al., 2012). These data suggested that PTEN activity is under the negative regulation of p110δ in high p110δ expressing cells such as macrophages and breast cancer cells.

RhoA was previously shown to be negatively controlled by p110δ PI3K and to mediate the activation of PTEN (Papakonstanti et al., 2007). The similar mode of RhoA (Papakonstanti et al., 2007), PTEN (Papakonstanti et al., 2007; Tzenaki et al., 2012) and FAK (Figure 3A and 3C) activation upon p110δ inhibition and the reverse correlation of FAK and Akt activity with p110δ activity (Figure 4) led us to investigate if there is a correlation between RhoA/PTEN activation and FAK phosphorylation in the p110δ signaling cascade. For this purpose we treated cells with a small inhibitor of ROCK, the downstream kinase of RhoA and checked for FAK activation.

Treatment of BMMs and MDA-MB231 breast cancer cells with the ROCK inhibitor Y27632 blocked the increase of Tyrosine phosphorylation of FAK in $\delta^{D910A/D910A}$ cells and the induced increase of FAK phosphorylation by IC87114 in MDA-MB321 cells respectively (Figure 5A and 5B). In contrast, pretreatment of HeLa cancer cells with Y27632, similar with IC87114, did not affect the phosphorylation of FAK (Figure 5C).

- These data imply that FAK becomes Tyrosine phosphorylated downstream the activation of RhoA/ROCK upon inactivation of the p110δ signaling.

Furthermore, according to previous data, the increased activity of PTEN upon genetic inactivation of p110δ was characterized by increased PTEN-Tyrosine phosphorylation, both under basal and CSF-1-stimulated conditions (Papakonstanti, 2007). Considering that FAK is a tyrosine kinase interacting with PTEN (Figure 1) and that it is under the positive regulation of RhoA (Figure 4), we examined if FAK mediates the Tyrosine phosphorylation of PTEN observed upon p110δ Inhibition. Indeed, we found that FAK inhibition affect PTEN Tyrosine phosphorylation. Specifically, the pretreatment of BMM cells with PF-228, a small molecule inhibitor of FAK, inhibited the increased Tyrosine-phosphorylation of PTEN in $\delta^{D910A/D910A}$ cells (Figure 6), whereas genetic inactivation of p110δ led to constitutively increased PTEN-Tyr phosphorylation (Figure 6). Importantly, treatment of cells with PF-228 reduced PTEN-Tyrosine phosphorylation and thus activity in $\delta^{D910A/D910A}$ cells to the levels seen in WT cells (Figure 6).

- The above results collectively are in line with a model in which FAK is Tyrosine phosphorylated and activated downstream of RhoA/ROCK in the p110δ signaling cascade regulating then the phosphorylation and activity of its downstream effector PTEN.

5. Inhibition of FAK overcomes the biological defects of p110δ inactivation

We next set out to investigate the biological importance of the FAK activity in the responses controlled by p110δ, such as the phosphorylation of Akt and cell proliferation. We found that the decreased P-Akt levels in $\delta^{D910A/D910A}$ BMMs were recovered to those of WT cells by pretreatment of cells with the ROCK inhibitor Y27632 (Figure 7A). Importantly, in line with our results, showing that FAK acts downstream of ROCK, pretreatment of cells with the FAK inhibitor PF-228 also recovered the P-Akt levels in $\delta^{D910A/D910A}$ BMMs to the same extend as that seen with Y27632 (Figure 7A). Similar results were obtained when cell proliferation was examined. The defects in proliferation of $\delta^{D910A/D910A}$ cells were overcome by pretreatment of cells by either the FAK inhibitor PF-228, or the ROCK inhibitor Y27632 or the PTEN inhibitor VO-HOpic (Figure 7B).

- Collectively, these data are consistent with a signalling model in which inactivation of p110δ leads to enhanced FAK activity downstream of ROCK and upstream of PTEN activity contributing to the observed phenotypes.

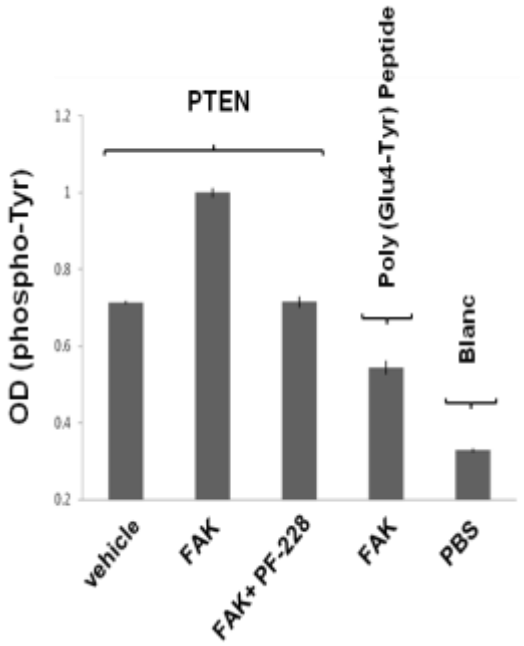


Figure 1: PTEN is a FAK substrate*: In vitro* phosphorylation of PTEN by FAK. Human recombinant PTEN and control substrate solution [(Poly (Glut4-Tyr) peptide] were bound in ELISA binding plates and their Tyrosine-phosphorylation level was determined using an anti phospho-tyrosine antibody. Experimental wells were incubated for 15 min with kinase reaction buffer containing active recombinant FAK enzyme (0,1 µg/µl) and/or FAK inhibitor PF-228 or buffer control. Substrate control wells were incubated similarly with kinase reaction buffer supplemented with FAK. Tyrosine phosphorylation intensity was measured by the absorbance of TMB solution. Average OD of one representative experiment out of two independent experiments carried out in triplicate is shown.

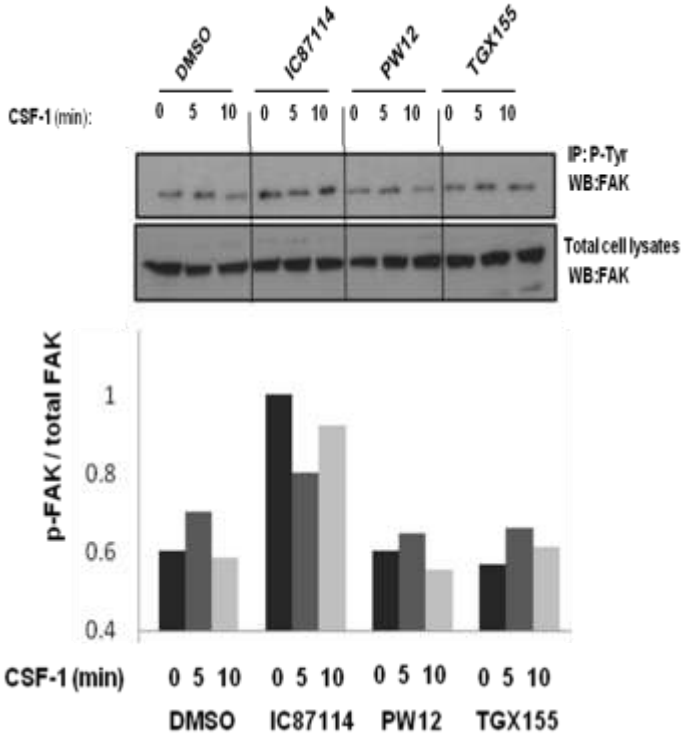


Figure 2: p110δ is the only Class IA PI3K isoform that control FAK phosphorylation. Effect of pharmacological inactivation of class IA PI3K isoforms on CSF1-induced phosphorylation of FAK on BMMs derived from WT mice. Cells were pretreated for 1 h with PW12 (0.5 µM), TGX155 (0.5 µM), or IC87114 (5 µM), followed by incubation with CSF1 for the indicated time points. Protein lysates were immunoprecipitated (IP) for Phospho-tyrosine proteins and western blotted (WB) for FAK. Total cell lysates of the same samples were immunoblotted for total FAK. The graph illustrate the average ratio of Tyr-phosphorylated FAK (P-FAK) to total FAK from three separate experiments with similar results; one representative blot is provided.

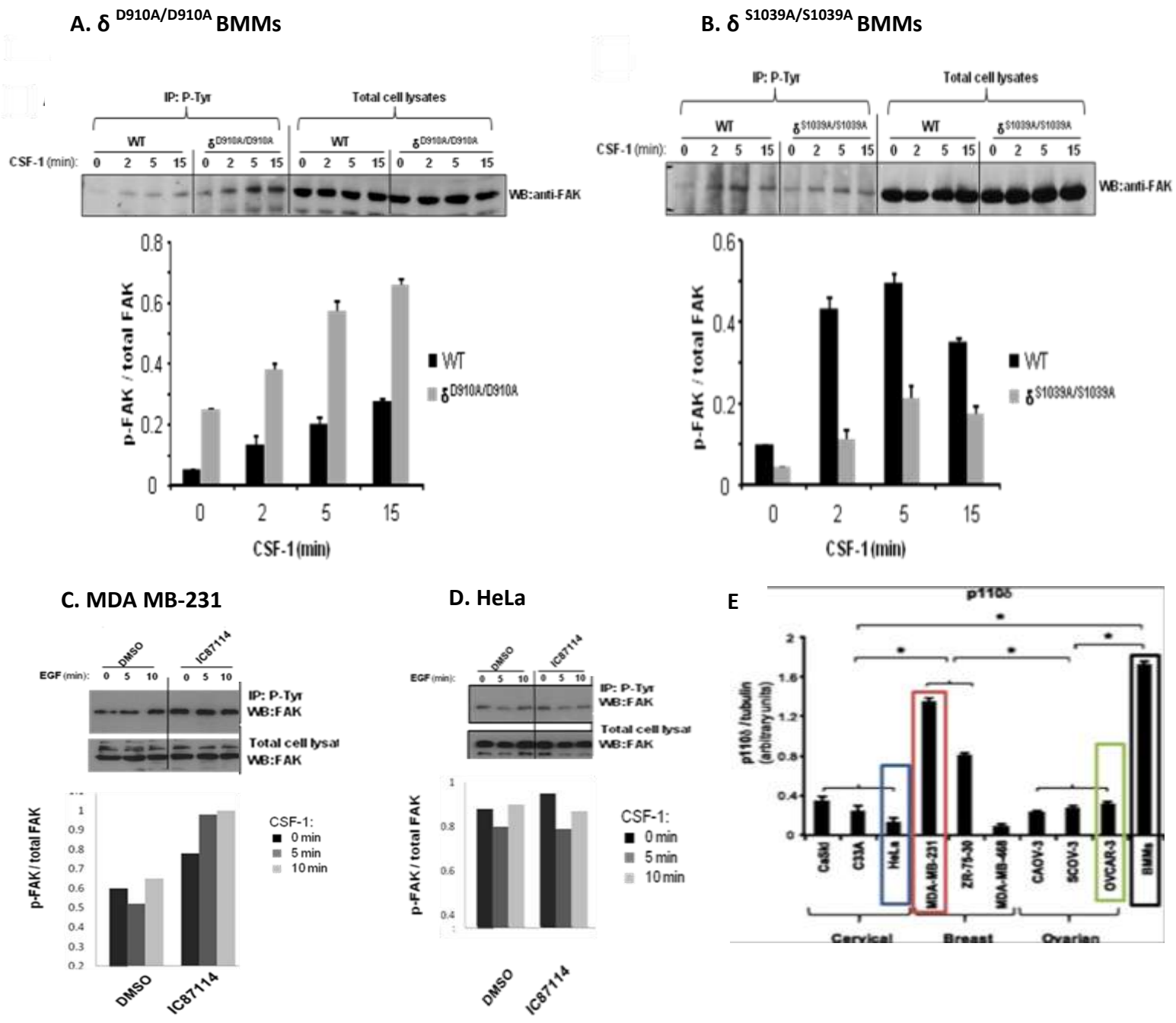


Figure 3: p110δ activity controls FAK phosphorylation. (A) BMMs derived from $\delta^{D910A/D910A}$ mice and (B) $\delta^{S1039A/S1039A}$ mice and their WT littermates were starved of CSF-1 for 16–20 and stimulated with 30 ng/ml of CSF-1 for the indicated time points, followed by IP of P-Tyr proteins and WB for FAK. (C) MDA-MB 231 and (D) HeLa cancer cells were pretreated for 1 h with p110δ inhibitor IC87114 (5μM) and stimulated with EGF (50 ng/ml) for the indicated time points followed by IP of P-Tyr proteins and WB for FAK. Total lysates of the same samples were immunobloted for the total FAK. The ratio of WB signal of Tyr-phosphorylated FAK over that of total FAK is illustrated. Three independent experiments were performed for each case with similar results. One representative blot is provided. (E) The graph illustrates the p110δ expression levels of the different cell types used for this study (Tzenaki et al., 2012).

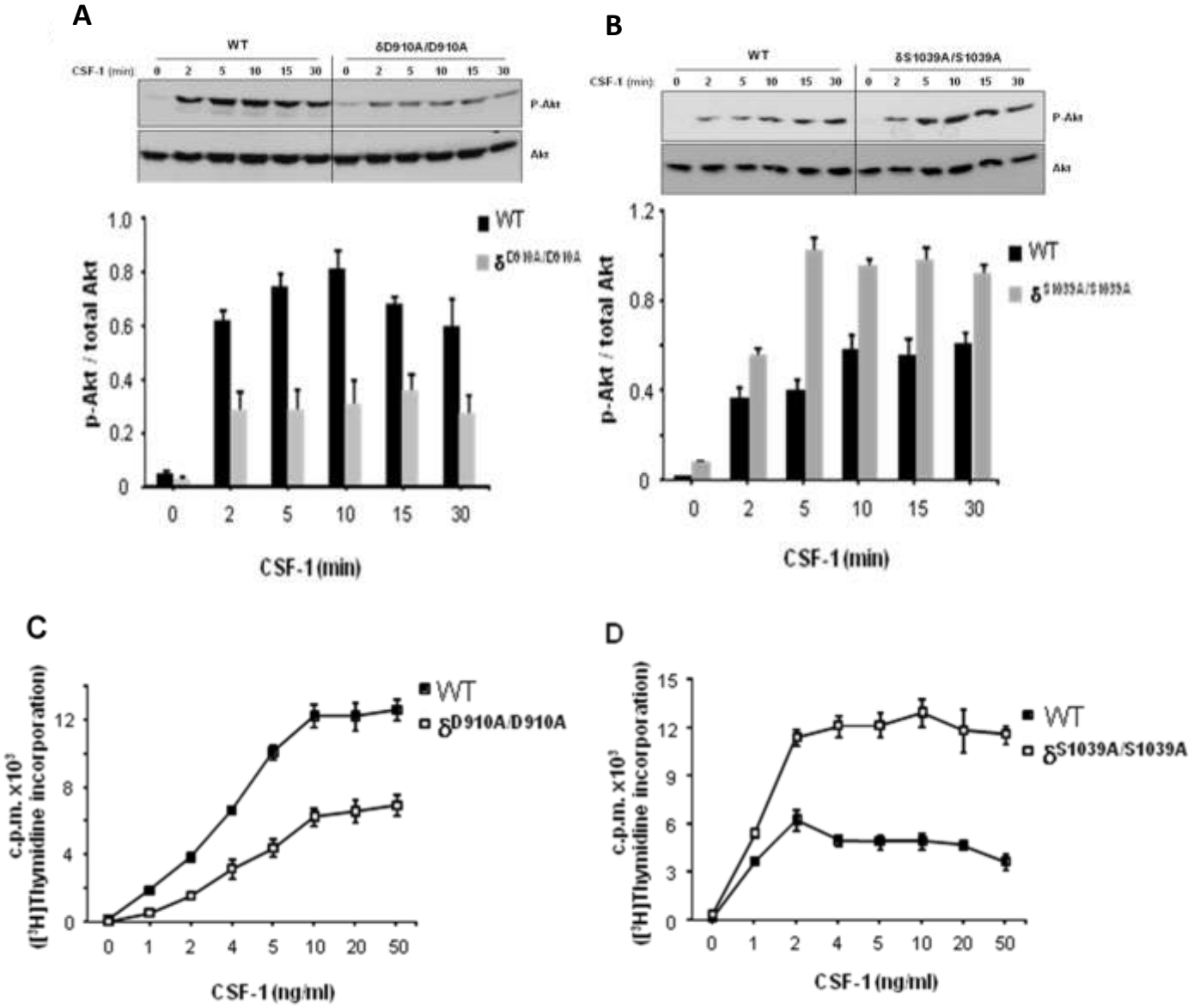
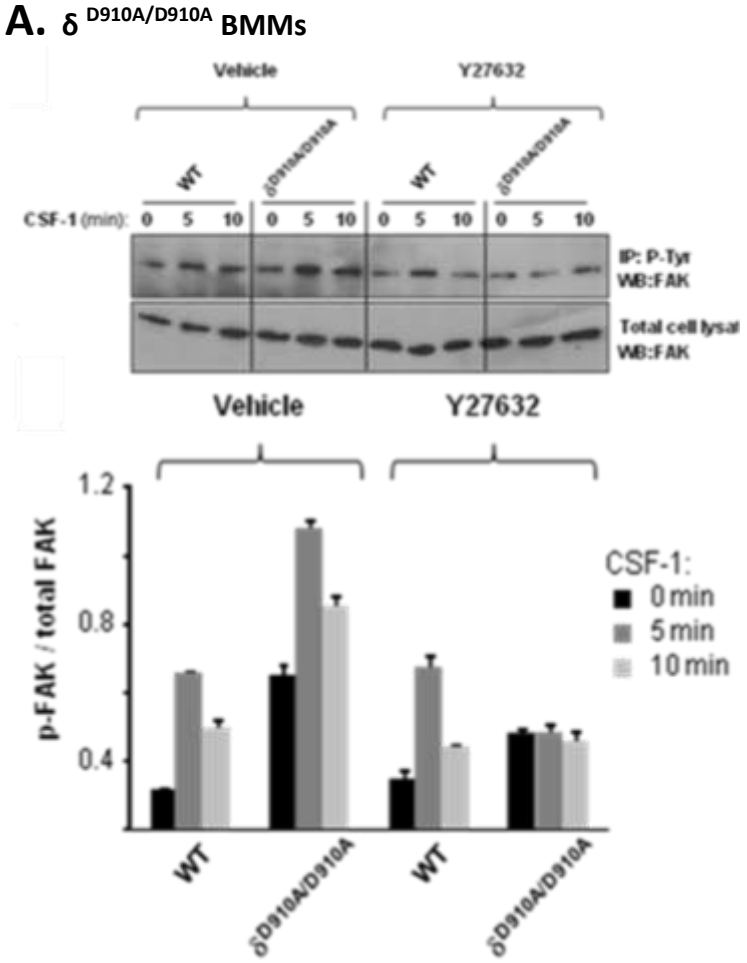
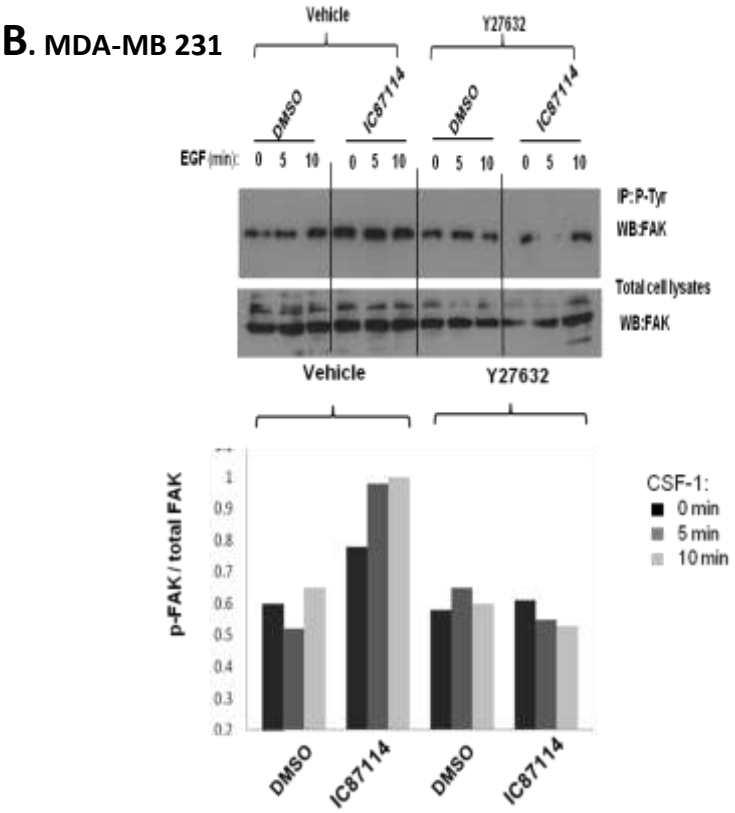


Figure 4: Inactive p110δ suppresses whereas active p110δ induces Akt phosphorylation and cell proliferation. (A) Effect of genetic inactivation of p110δ on Akt phosphorylation. WT and δD910A/D910A BMMs were stimulated with 30ng/ml CSF1 for different time points followed by WB for P-Akt (S473) and total Akt. The ratio of phosphorylated Akt over that of total was calculated from 3 independent experiments with similar results; one representative blot is illustrated. (B) Effect of p110δ overexpression on Akt phosphorylation. WT and δS1039A/S1039A BMMs were stimulated with 30ng/ml CSF1 for different time points followed by WB for P-Akt (S473) and total Akt. The ratio of phosphorylated Akt over that of total was calculated from 3 independent experiments with similar results; one representative blot is illustrated. (C, D) Effect of genetic inactivation and overexpression of p110δ on CSF1 induced cell proliferation of BMMs as measured by thymidine incorporation.





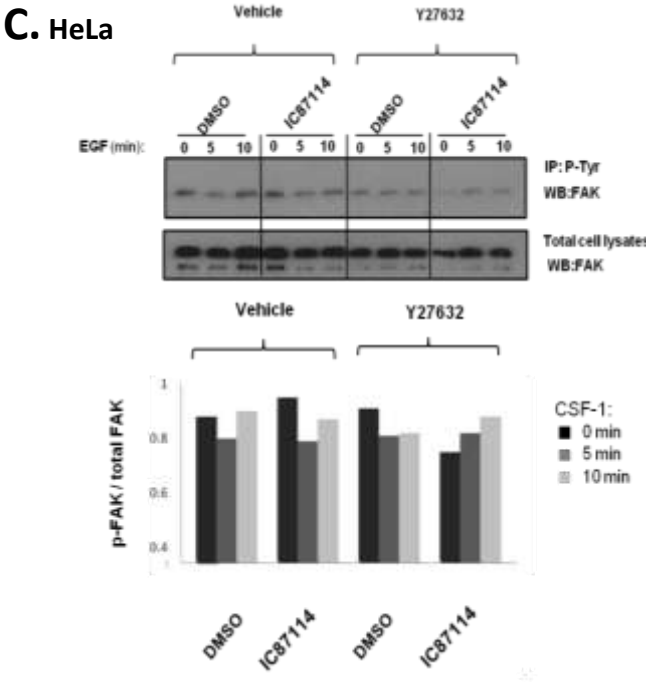


Figure 5. FAK is downstream of RhoA in the p110δ signaling pathway. (A) WT and δD910A/D910A BMMs were pretreated with 25 mM of Y27632 or its vehicle for 15 min, stimulated with CSF-1 (30 ng/ml) for the indicated time points followed by IP of P-Tyr proteins and WB for FAK. (B) MDA-MB 231 and (C) HeLa cancer cells were pretreated for 1 h with p110δ inhibitor IC87114 (5μM) and/or for 15min with ROCK inhibitor Y27632 (25mM) or its vehicle, stimulated with EGF (50 ng/ml) for the indicated time points followed by IP of P-Tyr proteins and WB for FAK. Total lysates of the same samples were immunoblotted for total FAK. Ratio of the WB signal of P-FAK over that of total FAK was calculated from 3 independent experiments with similar results; one representative blot is illustrated.

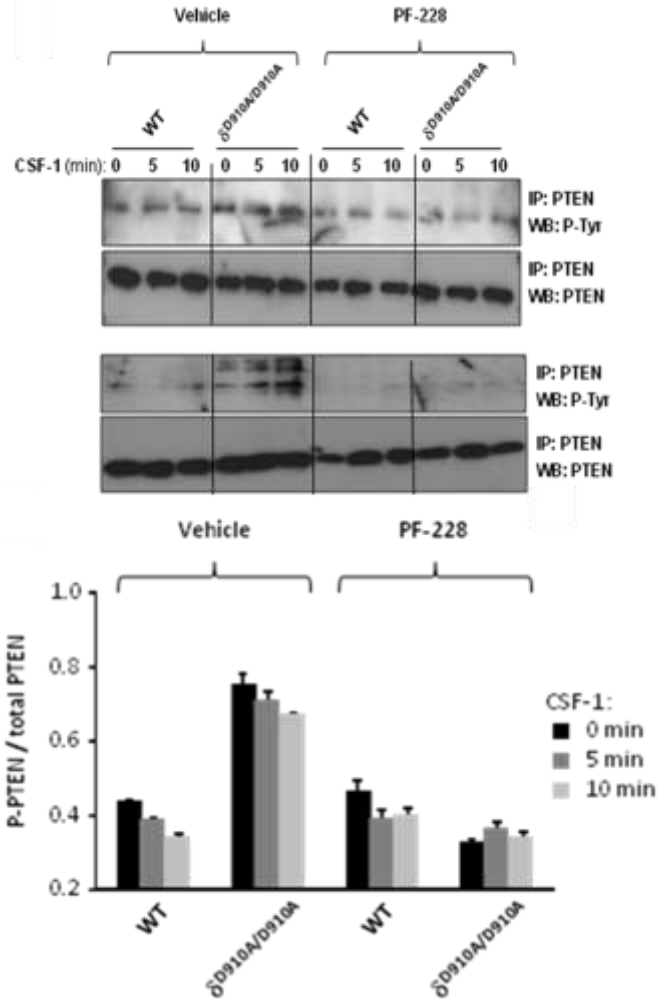


Figure 6. FAK is upstream of PTEN in the p110δ signaling pathway. WT and δD910A/D910A BMMs were pretreated with PF228 (1μM) or its vehicle for 1h, stimulated with CSF-1 (30 ng/ml) for the indicated time points followed by IP for PTEN and WB for P-Tyr or Total PTEN. The ratio of phosphorylated PTEN over that of total was calculated from 4 independent experiments; two representative blots are illustrated.

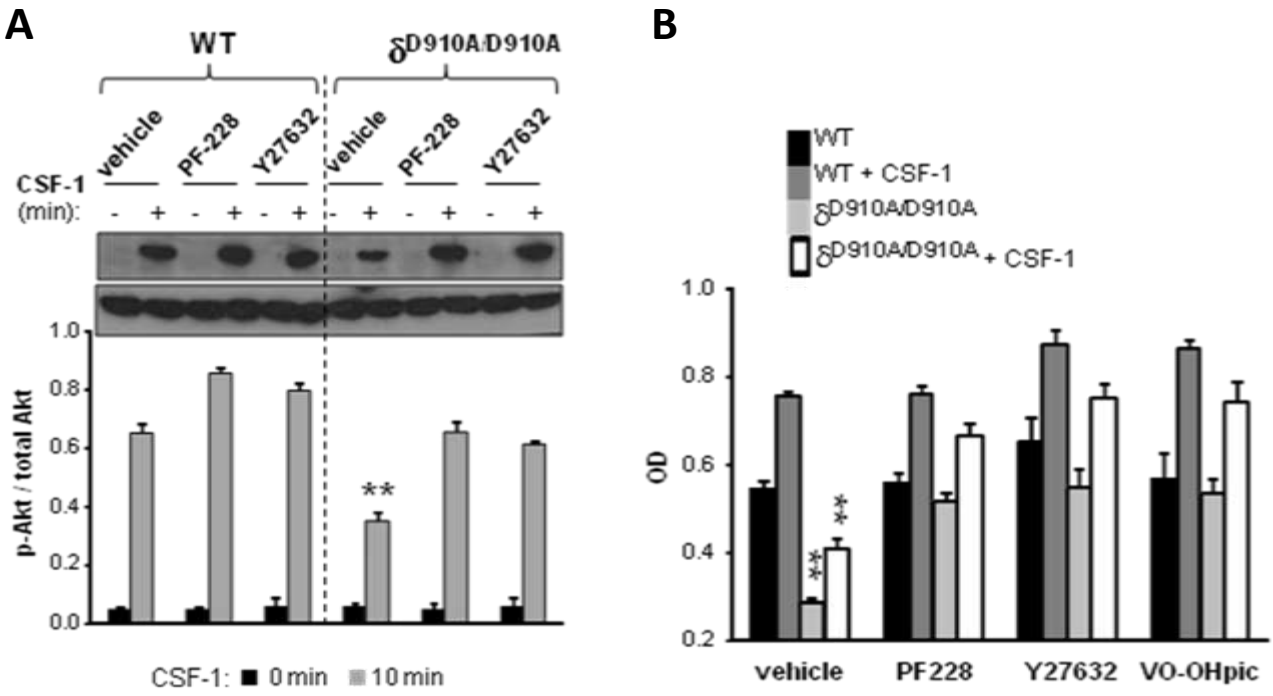


Figure 7: Inhibition of FAK overcomes the biological defects of p110δ inactivation. (A) Effect of FAK and ROCK inhibition on Akt phosphorylation. WT and δD910A/D910A BMMs were pretreated with ROCK inhibitor Y27632 (25mM) for 15min or with FAK inhibitor PF228 for 1 h, stimulated with 30ng/ml CSF1 for the indicated time points different time points followed by WB for P-Akt (S473) and total Akt. The ratio of phosphorylated Akt over that of total was calculated from 3 independent experiments with similar results; one representative blot is illustrated. (B) Effect of FAK, ROCK and PTEN inhibition on CSF1 induced cell proliferation. WT and δD910A/D910A BMMs were treated with ROCK inhibitor Y27632 (25mM) or with FAK inhibitor PF228 (1μM) or with PTEN inhibitor VO-OHpic (500 nM) in the presence or absence of CSF1 (30 ng/ml). Cell survival is measured by the formazan production.

DISCUSSION

PTEN is a phosphatase that can dephosphorylate both phosphoinositide substrates and proteins. The tumor suppressor activity of PTEN has been mostly attributed to its lipid phosphatase activity because of the antagonizing effect of PTEN on PI3K signaling and the consequent control of cell survival (Leslie and Downes, 2002; Sulis and Parsons, 2003). However, PTEN has been also shown to dephosphorylate *in vitro* protein substrates such as FAK and itself (Raftopoulou et al., 2004; Tamura et al., 1999a). The class IA subset of PI3Ks are acutely activated by membrane-bound tyrosine kinase pathways and consists of heterodimers made up of a 110-kDa catalytic subunit (p110α, p110β, or p110δ) in a complex with one of 5 regulatory subunits (collectively called the p85s) (Vanhaesebroeck et al., 2010). The inhibitory effect of PTEN on PI3K signaling does not discriminate among the different PI3K isoforms. However, different PI3K isoforms seem to have distinct effects on PTEN function. Indeed, PTEN activity was found to become enhanced by binding of PTEN to the p85/p110β complex (Chagpar et al., 2010; Rabinovsky et al., 2009). In contrast, the p110δ isoform was found to inactivate PTEN activity through a pathway involving RhoA with the latter to induce paradoxically the tyrosine phosphorylation of PTEN (Papakonstanti et al., 2007). The mechanism though by which this occurs has remained so far elusive.

PTEN is often mutationally inactivated in cancer cells, contributing to the constitutive activation of the PI3K pathway (Cully et al., 2006; Leslie and Downes, 2004; Parsons, 2004; Sansal and Sellers, 2004). Reduced or lost activity of PTEN also occurs through deletions, transcriptional silencing, protein interactions and posttranslational modifications such as phosphorylation (Chalhoub and Baker, 2009; Fine et al., 2009; Koul et al., 2002; Li et al., 2005; Miller et al., 2002; Sanchez et al., 2005; Torres and Pulido, 2001; Vazquez et al., 2000). The phosphorylation of PTEN on serine and threonine residues has been extensively studied (Al-Khouri et al., 2005; Miller et al., 2002; Torres and Pulido, 2001, Okahara et al., 2006; Yim et al., 2007, Li et al., 2005). Inductive phosphorylation of PTEN on Tyr residues has been reported on several studies but the potentially phosphorylated residues have not yet been found or confirmed by high-quality mass spectrometry (Koul et al., 2002; Papakonstanti et al., 2007; Sanchez et al., 2005; Yim et al., 2009).

Here, our data show that PTEN is actually tyrosine phosphorylated by the protein FAK *in vitro* and that, in cells the activation of FAK is under the negative control of p110δ PI3K and the positive control of RhoA. Our results were revealed by multiple methods including cell-free assays and assays on cells from mice expressing kinase-dead or constitutively active p110δ and cancer cell lines with differential expression levels of p110δ. Importantly, we document for the first time here that PTEN is a substrate of FAK and we provide the first molecular link between RhoA and PTEN on the previously documented p110δ/RhoA/PTEN pathway (Papakonstanti et al., 2007).

PTEN has previously been shown to interact and dephosphorylate FAK leading to the regulation of various processes such as cell spreading and the formation of focal adhesions (Tamura et al., 1999a; Tamura et al., 1998; Tamura et al., 1999b). We now show that the relationship of PTEN with FAK is bidirectional whereby FAK phosphorylates PTEN (Figure 1). We also show that the FAK-mediated regulation of PTEN has a significant role in the regulation of the biological functions of PTEN (Figure 7).

An unexpected finding reported here are the increased levels of phosphorylated FAK in macrophages derived from mice expressing kinase-dead p110δ ($\delta^{D910A/D910A}$) and the decreased phosphorylation of FAK in cells from mice expressing constitutively active p110δ ($\delta^{S1039A/S1039A}$) (Figure 3A and 3B). Increased levels of phosphorylated FAK upon pharmacological inhibition of p110δ were also found in high-p110δ expressing cells such as breast cancer cells (Figure 3C) whereas the phosphorylation of FAK remained unaffected in cervical cancer cells not expressing high p110δ (Figure 3D). In all these cells, the overall pattern of changes in phosphorylated FAK levels following Tyr kinase receptor (CSF-1R or EGFR) stimulation was not affected by p110δ inactivation but the levels of tyrosine phosphorylated FAK remained consistently above of those in cells with active p110δ. That PI3K can be negatively involved in CSF-1-induced FAK phosphorylation has also been previously suggested by the observation that treatment of macrophages with a pan-PI3K inhibitor resulted in FAK hyperphosphorylation (Rovida et al., 2005). No explanation for this observation has been put forward so far but this is in line with our findings, showing that p110δ negatively controls FAK, especially given that we did not find any changes in FAK phosphorylation upon p110α or p110β inhibition (Figure 2). In contrast to FAK phosphorylation, Akt activation and cell proliferation were decreased in $\delta^{D910A/D910A}$ and increased in $\delta^{S1039A/S1039A}$ BMMs compared to WT cells (Figure 4), showing a reverse correlation between FAK activation and the observed phenotypes under the regulation of p110δ.

Moreover, inhibition of RhoA or ROCK restored the elevated levels of FAK phosphorylation in $\delta^{D910A/D910A}$ BMMs and MDA-MB 231 cells treated with a p110δ inhibitor to those observed in WT cells (Figure 5A) and in untreated cancer cells respectively (Figure 5B), suggesting that FAK is a downstream effector of RhoA/ROCK in p110δ PI3K signaling cascade. The fact that pharmacological inhibition of FAK recovered the biological defects (Akt phosphorylation and cell proliferation) in $\delta^{D910A/D910A}$ BMMs (Figure 7) while in parallel prevented the hyperphosphorylation and hyperactivation of PTEN induced by p110δ inactivation (Figure 6) is consistent with a model in which p110δ negatively controls FAK upstream of PTEN and suggests that the negative regulation of FAK and PTEN are needed for p110δ to exert its biological functions properly (Figure 8). At present, it is not clear how RhoA and its downstream kinase ROCK control the Tyr-phosphorylation of FAK. However, it is of interest to note that inhibition of RhoA or ROCK resulted in the inhibition of Tyr-phosphorylation of FAK in a variety of other cell systems (Chaturvedi et al., 2011; Goundiam et al., 2012; Sinnett-Smith et al., 2001; Torsoni et al., 2005; Yagi et al., 2006). A molecular link that could explain the RhoA-dependent increase in levels of phosphorylated FAK is the RhoAGEF Net1A. Indeed, a recent study has shown that Net1A is located at focal adhesions, interacts with FAK and is necessary for FAK activation (Carr et al., 2013).

FAK is a cytoplasmic tyrosine kinase that has been shown to play critical roles in development and in pathogenesis of human diseases, including cancer (Schaller, 2010). Although a lot of studies have linked the inhibition of FAK with reduced proliferative responses (Zhao and Guan, 2009), other published data have correlated reduced levels of FAK with elevated survival signals, growth and angiogenesis (Nakamura et al., 2008; Sweeney et al., 2003; Wang and Basson, 2011). A more recently published study has shown that the B16F0 melanoma and CMT19T lung carcinoma tumor growth as well as angiogenesis are enhanced in FAK-heterozygous mice (expressing the half of the normal FAK protein level) compared to wild-type mice indicating a negative role of stromal-FAK to tumor growth and angiogenesis (Kostourou et al., 2013). The mechanism by which stromal cells in FAK-heterozygous mice induced tumor growth is not clear (Kostourou et al., 2013), but the notion that a large amount of tumor-associated stroma consists of macrophages that promote tumor growth and angiogenesis (Condeelis and

Pollard, 2006; Owen and Mohamadzadeh, 2013) makes it likely that the reduced PTEN activity and the consequent induced survival of macrophages contributes to the effect of FAK-heterozygosity on tumor growth in these mice.

Moreover, it has been suggested that the levels of FAK expression, phosphorylation and activity can differentially affect the cancer phenotype. Indeed, low doses of the FAK-inhibitor PF-228, that do not totally abolished FAK activity, mimicked the FAK-heterozygosity and induced tumor growth and angiogenesis (Kostourou et al., 2013). Treatment also of cancer cell lines with PF-228 at concentrations that significantly inhibited the phosphorylation of FAK failed to block cell growth and instead, at least in some cancer cell lines, increased the cells growth (Slack-Davis et al., 2007). On the other hand, endothelial-specific loss of FAK in mice inhibited angiogenesis (Tavora et al., 2010) while reduced levels of FAK in endothelial cells derived from FAK-heterozygous mice led to increased proliferation and Akt activity levels (Kostourou et al., 2013). Notably, the angiogenesis and tumor growth were enhanced in FAK-heterozygous mice (Kostourou et al., 2013). Low concentrations of RGD-mimetic integrin inhibitors, that inhibit FAK signalling, have also been shown to stimulate tumour growth and angiogenesis (Reynolds et al., 2009). These data are in line with our findings which show that the activation of p110δ does not abolish FAK phosphorylation but keeps it constantly at low levels resulting in the inhibition of PTEN activity and in induction of Akt phosphorylation and cell survival. Our data also showed that FAK is downstream of RhoA/ROCK and that inhibition of the latter leads to inhibition of FAK. Additionally, we observed that the expression levels of p110δ is correlated with the negative effect of p110δ activation on FAK and consequently on PTEN activity.

In summary, our work shows for the first time that tyrosine residues on PTEN are directly phosphorylated by FAK. Our data also indicate that this role of FAK is operational in cells since FAK was found to be negatively regulated by the p110δ isoform of PI3K and to act downstream of RhoA (Figure 8). The important role of PTEN activity in cell growth together with the fact that FAK inhibitors have been developed as anti-proliferative agents raise the question if the efficacy of FAK inhibition might be counterbalanced by the PTEN inhibition. Our data show a novel mechanism of PTEN regulation and provide new information for the biological roles of FAK that have to be taken into consideration when the intervention of cancer cells growth is designed.

p110δ (→) ┤ RhoA → ROCK (→) → FAK → PTEN

Figure 8: FAK regulates PTEN under the control of p110δ PI3 kinase. FAK directly phosphorylates PTEN, comprising the molecular link between RhoA and PTEN on the previously documented p110δ/RhoA/PTEN pathway.

REFERENCES

Abu-Ghazaleh, R., Kabir, J., Jia, H., Lobo, M. Zachary, I. (2001). Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. *Biochem J 360*, 255–264.

Aksoy, E., Taboubi, S., Torres, D., Delbauve, S., Hachani, A., Whitehead, M.A., Pearce, W.P., Berenjeno-Martin, I., Nock, G., Filloux, A., Beyaert, R., Flamand, V., Vanhaesebroeck, B. (2012). The p110[delta] isoform of the kinase PI(3)K controls the subcellular compartmentalization of TLR4 signaling and protects from endotoxic shock. *Nat Immunol advance*, online publication.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J 15*, 6541-6551.

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., Macdougall, C.N., Harbison, D., Asworth, A., Bownes, M. (1997a). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 7, 776-789.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., Cohen, P. (1997b). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol 7*, 261-269.

Ali, I.U., Schriml, L.M., and Dean, M. (1999). Mutational Spectra of PTEN/MMAC1 Gene: a Tumor Suppressor With Lipid Phosphatase Activity. *J Natl Cancer Inst 91*, 1922-1932.

Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gilfillan, A.M., Tkaczyk, C., Kuehn, N., Gray, A., Giddings, J., Peskett, E., Fox, R., Bruce, I., Walker, C., Sawyer, C., Okkenhaug, K., Finan, P., and Vanhaesebroeck, B. (2004). Essential role for the p110[delta] phosphoinositide 3-kinase in the allergic response. *Nature 431*, 1007-1011.

Ali, K., Camps, M., Pearce, W.P., Ji, H., Ruckle, T., Kuehn, N., Pasquali, C., Chabert, C., Rommel, C., and Vanhaesebroeck, B. (2008). Isoform-Specific Functions of Phosphoinositide 3-Kinases: p110{delta} but Not p110{gamma} Promotes Optimal Allergic Responses In Vivo. *J Immunol 180*, 2538-2544.

Alvarez, B., Martinez-a, C., Burgering, B.M.T., Carrera, A.C. (2001). Forkhead transcription factors contribute to execution of the mitotic programme in mammals. *Nature 413*, 744-747.

Al-Khouri, A. M., Ma, Y., Togo, S. H., Williams, S., Mustelin, T. (2005). 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.

Barber, M. A., Welch, H. C. (2006). PI3K and RAC signalling in leukocyte and cancer cell migration. *Bull Cancer 93*, E44-52.

Bernards, A., Settleman, J. (2005). GAPs in growth factor signalling. *Growth Factors 23*, 143-149.

Besson, A., Gurian-West, M., Schmidt, A., Hall, A., Roberts, J.M. (2004). p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev. 18*, 862-876.

Bi, L., Okabe, I., Bernard, D.J., Nussbaum, R.L. (2002). Early embryonic lethality in mice deficient in the p110β catalytic subunit of PI 3-kinase. *Mamm. Genome 13*, 169-172.

Bi, L., Okabe, I., Bernard, D.J., Wynshaw-Boris, A., Nussbaum, R.L. (1999). Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110α subunit of phosphoinositide 3-kinase. *J. Biol. Chem. 274*, 10963-10968.

Blanco-Aparicio, C., Renner, O., Leal, J. F., Carnero, A. (2007). PTEN, more than the AKT pathway. *Carcinogenesis 28*, 1379–1386.

Boller, D., Schramm, A., Doepfner, K.T., Shalaby, T., Von Bueren, A.O., Eggert, A., Grotzer, M.A., Arcaro, A. (2008). Targeting the Phosphoinositide 3-Kinase Isoform p110delta Impairs Growth and Survival in Neuroblastoma Cells. *Clin Cancer Res 14*, 1172-1181.

Brachmann, S.M., Hofmann, I., Schnell, C., Fritsch, C., Wee, S., Lane, H., Wang, S., Garcia-Echeverria, C., Maira, S.-M. (2009). Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. *Proc Natl Acad Sci USA 106*, 22299-22304.

Braren, R., Hu, H., Kim, Y. H., Beggs, H. E., Reichardt, L.F., Wang, R., .Braren, R. (2006). Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. *J Cell Biol 172*, 151–162.

Brunet, A,. Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell 96*, 857–868.

Brunton, V. G., Frame, M. C. (2008). Src and focal adhesion kinase as therapeutic targets in cancer. *Curr Opin Pharmacol 8*, 427–432.

Burgering, B.M.T., Medema, R.H. (2003). Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol 73*, 689-701.

Cai, X., Lietha, D., Ceccarelli, D. F., Karginov, A. V., Rajfur, Z., Jacobson, K., Hahn, K. M., Eck, M. J., Schaller, M. D. (2008) Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Mol Cell Biol 28*, 201–214.

Chae, H. D., Broxmeyer, H. E. (2011). SIRT1 deficiency downregulates PTEN/JNK/FOXO1 pathway to block reactive oxygen species-induced apoptosis in mouse embryonic stem cells. *Stem Cells Dev 20*, 1277–1285.

Calalb, M. B., Zhang, X., Polte, T. R., Hanks, S. K. (1996). Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun 228*, 662–668.

Calvanese, V., Fernández, A.F., Urdinguio, R.G., Suárez-Alvarez, B., Mangas, C., Pérez-García, V., Bueno, C., Montes, R., Ramos-Mejía, V., Martínez-Camblor, P., Ferrero, C., Assenov, Y., Bock, C., Menendez, P., Carrera, A.C., Lopez-Larrea, C., Fraga, M.F. (2012). A promoter DNA 599 demethylation landscape of human hematopoietic differentiation. *Nucl Acids Res 40*, 116-131.

Campbell, I.G., Russell, S.E., Choong, D.Y.H., Montgomery, K.G., Ciavarella, M.L., Hooi, C.S.F., Cristiano, B.E., Pearson, R.B., and Phillips, W.A. (2004). Mutation of the PIK3CA Gene in Ovarian and Breast Cancer. *Cancer Res 64*, 7678-7681.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science 296*, 1655–1657.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T., Stanbridge, E., Frisch, S., Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science 282*, 1318–1321.

Carelli, S., Zadra, G., Vaira, V., Falleni, M., Bottiglieri, L., Nosotti, M., Di Giulio, A. M., Gorio, A., Bosari, S. (2006). Up-regulation of focal adhesion kinase in non-small cell lung cancer. *Lung Cancer 53*, 263–271.

Carr, H. S., Zuo, Y., Oh, W., Frost, J. A. (2013). Regulation of focal adhesion kinase activation, breast cancer cell motility, and amoeboid invasion by the RhoA guanine nucleotide exchange factor Net1. *Mol Cell Biol 33*, 2773-86.

Castillo, J.J., Furman, M., and Winer, E.S. (2012). CAL-101: a phosphatidylinositol-3-kinase p110-delta inhibitor for the treatment of lymphoid malignancies. *Expert Opin Investig Drugs 21*, 15-22.

Chagpar, R. B., Links, P. H., Pastor, M. C., Furber, L. A., Hawrysh, A. D., Chamberlain, M. D., Anderson D. H. (2010). Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3‐kinase. *Proc Natl Acad Sci USA 107*, 5471–5476.

Chalhoub, N., Baker, S.J. (2009). PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol 4*, 127-150.

Chan, K. T., Cortesio, C.L., Huttenlocher, A. (2009). FAK alters invadopodia and focal adhesion composition and dynamics to regulate breast cancer invasion, *J Cell Biol 185*, 357–370.

Chantry, D., Vojtek, A., Kashishian, A., Holtzman, D.A., Wood, C., Gray, P.W., Cooper, J.A., Hoekstra, M.F. (1997). p110delta , a Novel Phosphatidylinositol 3-Kinase Catalytic Subunit That Associates with p85 and Is Expressed Predominantly in Leukocytes. *J Biol Chem 272*, 19236-19241.

Chaturvedi, L. S., Marsh, H. M., Basson, M. D. (2011). Role of RhoA and its effectors ROCK and mDia1 in the modulation of deformation-induced FAK, ERK, p38, and MLC motogenic signals in human Caco-2 intestinal epithelial cells. *Am J Physiol Cell Physiol 301*, C1224-1238.

Chen, H. C., Guan, G. L. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA 91*, 10148–10152.

Chen, H. C., Appeddu, P. A., Isoda, H., Guan, J. L. (1996). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem 271*, 26329–26334.

Chung, C. Y., Funamoto, S., Firtel, R. A. (2001). Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem Sci 26*, 557-66.

Ciraolo, E., Iezzi, M., Marone, R., Marengo, S., Curcio, C., Costa, C., Azzolino, O., Gonella, C., Rubinetto, C., Wu, H., Dastrù, W., Martin, E.L., Silengo, L., Altruda, F., Turco, E., Lanzetti, L., Musiani, P., Rückle, T., Rommel, C., Backer, J.M., Forni, G., Wymann, M.P., Hirsch, E. (2008). Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development. *Sci Signal 1*, ra3.

Clayton, E., Bardi, G., Bell, S.E., Chantry, D., Downes, C.P., Gray, A., Humphries, L.A., Rawlings, D., Reynolds, H., Vigorito, E., Turner, M. (2002). A Crucial Role for the p110{delta} Subunit of Phosphatidylinositol 3-Kinase in B Cell Development and Activation. *J. Exp. Med. 196*, 753-763.

Condeelis, J. and Pollard, J. W. (2006). Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis. *Cell 124*, 263-266.

Cotter, L., Ozçelik, M., Jacob, C., Pereira, J. A., Locher, V., Baumann, R., Relvas, J. B., Suter, U., Tricaud, N. (2010). Dlg1–PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. *Science 328*, 1415–1418

Cully, M., You, H., Levine, A.J., Mak, T.W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer 6*, 184-192.

Currie, R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R., Lucocq, J. (1999). Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem J 337*, 575-583.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E. (1997). Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery. *Cell 91*, 231-241.

Denu, J. M., Stuckey, J. A., Saper, M. A., Dixon, J. E. (1996). Form and function in protein dephosphorylation. *Cell 87*, 361–364.

Di Nicolantonio, F., Arena, S., Tabernero, J., Grosso, S., Molinari, F., Macarulla, T., Russo, M., Cancelliere, C., Zecchin, D., Mazzucchelli, L., Sasazuki, T., Shirasawa, S., Geuna, M., Frattini, M., Baselga, J., Gallicchio, M., Biffo, S., Bardelli, A. (2010). Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. *J Clin Invest 120*, 2858-2866.

Edwards, H., Xie, C., Lafiura, K.M., Dombkowski, A.A., Buck, S.A., Boerner, J.L., Taub, J.W., Matherly, L.H., and Ge, Y. (2009). RUNX1 regulates phosphoinositide 3-kinase/AKT pathway: role in chemotherapy sensitivity in acute megakaryocytic leukemia. *Blood* 114, 2744-2752.

Eickholt, B.J., Ahmed, A.I., Davies, M., Papakonstanti, E.A., Pearce, W., Starkey, M.L., Bilancio, A., Need, A.C., Smith, A.J.H., Hall, S.M., Hamers, F.P., Giese, K.P., Bradbury, E.J., Vanhaesebroeck, B. (2007). Control of Axonal Growth and Regeneration of Sensory Neurons by the p110δ PI 3-Kinase. 677 *PLoS ONE 2*, e869.

Eng, C. (2003). PTEN: one gene, many syndromes. *Hum Mutat 22*, 183–198.

Erickson, C. A. (1990). Cell migration in the embryo and adult organism. *Curr Opin Cell Biol 2*, 67–74.

Escrivà, M., Peiró, S., Herranz, N., Villagrasa, P., Dave, N., Montserrat-Sentís, B., Murray, S. A., Francí, C., Gridley, T., Virtanen, I., García de Herreros, A. (2008). Repression of PTEN phosphatase by Snail1 transcriptional factor during γ-radiation induced apoptosis. Mol Cell Biol 28, 1528–1540.

Fenteany, G., Glogauer, M. (2004). Cytoskeletal remodeling in leukocyte function. *Curr Opin Hematol 11*, 15-24.

Fine, B., Hodakoski, C., Koujak, S., Su, T., Saal, L. H., Maurer, M., Hopkins, B., Keniry, M., Sulis, M. L., Mense, S., Hibshoosh, H., Parsons, R. (2009). Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. *Science 325*, 1261–1265.

Foukas, L.C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A.J.H., Withers, D.J., Vanhaesebroeck, B. (2006). Critical role for the p110[alpha] phosphoinositide-3-kinase in growth and metabolic regulation. *Nature 441*, 366-370.

Frisch, S. M., Vuori, K., Ruoslahti, E., Chan-Hui, P. Y. (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol 134*, 793–799.

Fruman, D.A., and Rommel, C. (2011). PI3Kδ inhibitors in cancer: rationale and serendipity merge in the clinic. *Cancer Discov 1*, 562-572.

Fujita, N., Sato, S., Katayama, K., Tsuruo, T. (2002). Akt-dependent Phosphorylation of p27Kip1 Promotes Binding to 14-3-3 and Cytoplasmic Localization. *J. Biol. Chem. 277*, 28706-28713.

Furuyama, K., Doi, R., Mori, T., Toyoda, E., Ito, D., Kami, K., Koizumi, M., Kida, A., Kawaguchi, Y., Fujimoto, K. (2006). Clinical significance of focal adhesion kinase in resectable pancreatic cancer. *World J Surg 30*, 219–226.

Garami, A., Zwartkruis, F.J.T., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L., Thomas, G. (2003). Insulin Activation of Rheb, a Mediator of mTOR/S6K/4E-BP 699 Signaling, Is Inhibited by TSC1 and 2. *Mol Cell 11*, 1457-1466.

Garces, C. A., Kurenova, E. V, Golubovskaya, V. M., Cance W. G. (2006). Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. *Cancer Res 66*, 1446–1454.

Geering, B., Cutillas, P.R., Nock, G., Gharbi, S.I., Vanhaesebroeck, B. (2007). Class IA phosphoinositide 3-kinases are obligate p85-p110 heterodimers. *Proc Natl Acad Sci USA 104*, 7809-7814.

Girault, J. A., Labesse, G., Mornon, J. P., Callebaut, I. (1999). The N-termini of FAK and JAKs contain divergent band domains. *Trends Biochem Sci 24*, 54–57.

Golubovskaya, V., Kaur, A., Cance, W. (2004). Cloning and characterization of the promoter region of human focal adhesion kinase gene: nuclear factor kappa B and p53 binding sites. *Biochim Biophys Acta 1678*, 111–125.

Golubovskaya, V. M., Cance, W. G. (2007). Focal adhesion kinase and p53 signaling in cancer cells. *Inter Rev Cytol 263*, 103–153.

Golubovskaya, V. M., Kweh F. A., Cance W. G. (2009). Focal adhesion kinase and cancer. *Histol Histopath 24*, 503–510.

Goundiam, O., Nagel, M. D., Vayssade, M. (2012). Akt and RhoA inhibition promotes anoikis of aggregated B16F10 melanoma cells. *Cell Biol Int 36*, 311-319.

Graupera, M., Guillermet-Guibert, J., Foukas, L.C., Phng, L.-K., Cain, R.J., Salpekar, A., Pearce, W., Meek, S., Millan, J., Cutillas, P.R., Smith, A.J.H., Ridley, A.J., Ruhrberg, C., Gerhardt, H., Vanhaesebroeck, B. (2008). Angiogenesis selectively requires the p110[alpha] isoform of PI3K to control endothelial cell migration. *Nature 453*, 662-666.

Gu, T., Zhang, Z., Wang, J., Guo, J., Shen, W. H., Yin, Y. (2011). CREB is a novel nuclear target of PTEN phosphatase. *Cancer Res 71*, 2821–2825.

Guillermet-Guibert, J., Bjorklof, K., Salpekar, A., Gonella, C., Ramadani, F., Bilancio, A., Meek, S., Smith, A.J.H., Okkenhaug, K., Vanhaesebroeck, B. (2008). The p110b isoform of phosphoinositide 3- kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110g. *Proc Natl Acad Sci U S A 105*, 8292-8297.

Hawkins, P.T., Anderson, K.E., Davidson, K., Stephens, L.R. (2006). Signalling through Class I PI3Ks in mammalian cells. *Biochem Soc Trans 34*, 647-662.

He, L., Ingram, A., Rybak, A. P., Tang, D. (2010). Shank interacting protein-like 1 promotes tumorigenesis via PTEN inhibition in human tumor cells. *J Clin Invest 120*, 2094–2108.

He, L., Fan, C., Kapoor, A., Ingram, A. J., Rybak, A. P., Austin, R. C., Dickhout, J., Cutz, J. C., Scholey, J., Tang, D. (2011). α-mannosidase 2C1 attenuates PTEN function in prostate cancer cells. *Nature Commun 2*, 307.

Hill, K., Welti, S., Yu, J., Murray, J.T., Yip, S.-C., Condeelis, J.S., Segall, J.E., Backer, J.M. (2000). Specific Requirement for the p85-p110alpha Phosphatidylinositol 3-Kinase during Epidermal Growth Factor-stimulated Actin Nucleation in Breast Cancer Cells. *J Biol Chem 275*, 3741-3744.

Hollander, M. C., Blumenthal, G. M., Dennis, P. A. (2011). PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nature Rev Cancer 11*, 289–301.

Hooshmand-Rad, R., Hajkova, L., Klint, P., Karlsson, R., Vanhaesebroeck, B., Claesson-Welsh, L., Heldin, C.H. (2000). The PI 3-kinase isoforms p110α and p110β have differential roles in PDGF- and insulin-mediated signaling. *J Cell Sci 113*, 207-214.

Hungerford, G. H., Compton, M. T., Matter, M. L., Hoffstrom, B. G., Otey, C. A. (1996). Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J Cell Biol 135*, 1383–1390.

Ihle, N.T., Lemos, R., Wipf, P., Yacoub, A., Mitchell, C., Siwak, D., Mills, G.B., Dent, P., Kirkpatrick, D.L., Powis, G. (2009). Mutations in the Phosphatidylinositol-3-Kinase Pathway Predict for Antitumor Activity of the Inhibitor PX-866 whereas Oncogenic Ras Is a Dominant Predictor for Resistance. *Cancer Res 64*, 143-150.

Inoki, K., Li, Y., Xu, T., Guan, K.-L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev 17*, 1829-1834.

Jackson, S.P., Schoenwaelder, S.M., Goncalves, I., Nesbitt, W.S., Yap, C.L., Wright, C.E., Kenche, V., Anderson, K.E., Dopheide, S.M., Yuan, Y., Sturgeon, S.A., Prabaharan, H., Thompson, P.E., Smith, G.D., Shepherd, P.R., Daniele, N., Kulkarni, S., Abbott, B., Saylik, D., Jones, C., Lu, L., Giuliano, S., Hughan, S.C., Angus, J.A., Robertson, A.D., Salem, H.H. (2005). PI 3-kinase p110β: a new target for antithrombotic therapy. *Nat Med 11*, 507.

Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S.H., Zhang, J., Signoretti, S., Loda, M., Roberts, T.M., and Zhao, J.J. (2008). Essential roles of PI(3)K-p110β in cell growth, metabolism and tumorigenesis. *Nature 454*, 776-779.

Jiang, X., Chen, S., Asara, J.M., Balk, S.P. (2010). Phosphoinositide 3-Kinase Pathway Activation in Phosphate and Tensin Homolog (PTEN)-deficient Prostate Cancer Cells Is Independent of Receptor Tyrosine Kinases and Mediated by the p110β and p110δ Catalytic Subunits. *J Biol Chem 295*, 14980-14989.

Jope, R.S., Johnson, G.V.W. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci 29*, 95-102.

Jou, S. T., Carpino, N., Takahashi, Y., Piekorz, R., Chao, J.-R., Carpino, N., Wang, D., Ihle, J.N. (2002). Essential, nonredundant role for the phosphoinositide 3-kinase p110δ in signaling by the B-Cell receptor complex. *Mol Cell Biol 22*, 8580-8591.

Juhasz, G., Hill, J., Yan, Y., Sass, M., Baehrecke, E. H., Backer, J., Neufeld, T. P. (2007). The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in *Drosophila*. *J Cell Biol 181*, 655–666.

Kang, S., Denley, A., Vanhaesebroeck, B., Vogt, P.K. (2006). Oncogenic transformation induced by the p110beta, -{gamma}, and -{delta} isoforms of class I phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A 103*, 1289-1294.

Kharas, M. G., Okabe, R., Ganis, J. J., Gozo, M., Khandan, T., Paktinat, M., Gilliland, D. G., Gritsman, K. (2010). Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. Blood 115, 1406–1415

Karreth, F. A., Tay, Y., Perna, D., Ala, U., Tan, S. M., Rust, A. G., DeNicola, G., Webster, K. A., Weiss, D., Perez-Mancera, P. A., Krauthammer, M., Halaban, R., Provero, P., Adams, D. J., Tuveson, D. A., Pandolfi, P. P. (2011). In vivo identification of tumorsuppressive PTEN ceRNAs in an oncogenic BRAF induced mouse model of melanoma. Cell 147, 382–395.

Kim, R. H., Peters, M., Jang, Y., Shi, W., Pintilie, M., Fletcher, G. C., DeLuca, C., Liepa, J., Zhou, L., Snow, B., Binari, R. C., Manoukian, A. S., Bray, M. R., Liu, F. F., Tsao, M. S., Mak, T. W. (2005). DJ‑1, a novel regulator of the tumor suppressor PTEN. Cancer Cell 7, 263–273.
Kim, Y. C., Kitaura, H., Taira, T., Iguchi-Ariga, S. M., Ariga, H. (2009). Oxidation of DJ‑1‑dependent cell transformation through direct binding of DJ‑1 to PTEN. Int J Oncol 35, 1331–1341.

Klarlund, J., Guilherme, A., Holik, J., Virbasius, J., Chawla, A., Czech, M. (1997). Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. Science 275, 1927-1930.

Knobbe, C.B., and Reifenberger, G. (2003). Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 13, 507-518.

Kok, K., Geering, B., Vanhaesebroeck, B. (2009a). Regulation of phosphoinositide 3-kinase expression in health and disease. Trends Biochem Sci 34, 115-127.

Kok, K., Nock, G.E., Verrall, E.A.G., Mitchell, M.P., Hommes, D.W., Peppelenbosch, M.P., Vanhaesebroeck, B. (2009b). Regulation of p110δ PI 3-Kinase Gene Expression. PLoS ONE 4, e5145.

Kostourou, V., Lechertier, T., Reynolds, L. E., Lees, D. M., Baker, M., Jones, D. T., Tavora, B., Ramjaun, A. R., Birdsey, G. M., Robinson, S. D., Parsons, M., Randi, M., Harts, l, R., Hodivala-Dilke, K. (2013). FAK-heterozygous mice display enhanced tumour angiogenesis. Nat Commun 4, 2020.

Koul, D., Jasser, S. A., Lu, Y., Davies, M. A., Shen, R., Shi, Y., Mills, G. B., Yung, W. K. (2002). Motif analysis of the tumor suppressor gene MMAC/PTEN identifies tyrosines critical for tumor suppression and lipid phosphatase activity. Oncogene 21,2357-64.

Krugmann, S., Cooper, M.A., Williams, D.H., Hawkins, P.T., and Stephens, L.R. (2002). Mechanism of the 797 regulation of type IB phosphoinositide 3OH-kinase by G-protein bg subunits. Biochem. J. 362, 725-798 731

Lahlou, H., Sanguin-Gendreau, V., Zuo, D., Cardiff, R. D., McLean, G. W., Frame, M. C., Muller, W. J. (2007). Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc Natl Acad Sci USA 104*, 20302–20307.

Lee, J. O., Yang, H., Georgescu, M. M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P., Pavletich, N. P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell 99*, 323–334.

Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., Rhee, S.G. (2002). Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem 277*, 20336–20342.

Lee, J. Y., Kang, M. B., Jang, S. H., Qian, T., Kim, H. J., Kim, C. H., Kim, Y., Kong, G. (2009). Id‑1 activates Akt-mediated Wnt signaling and p27(Kip1) phosphorylation through PTEN inhibition. *Oncogene 28*, 824–831.

Lehmann, B.D., Bauer, J.A., Chen, X., Sanders, M.E., Chakravarthy, A.B., Shyr, Y., Pietenpol, J.A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest 121*, 2750-2767.

Leslie, N.R., and Downes, C.P. (2002). PTEN: The down side of PI 3-kinase signalling. *Cell Signal 14*, 285-295.

Leslie, N. R. and Downes, C. P. (2004). PTEN function: how normal cells control it and tumour cells lose it. *Biochem J 382*, 1-11.

Leverrier, Y., Okkenhaug, K., Sawyer, C., Bilancio, A., Vanhaesebroeck, B., Ridley, A.J. (2003). Class I phosphoinositide 3-kinase p110beta is required for apoptotic cell and Fcgamma receptor-mediated phagocytosis by macrophages. *J Biol Chem 278*, 38437-38442.

Li, S., Hua, Z. (2008). FAK expression regulation and therapeutic potential. Adv *Cancer Res 101*, 45–61.

Li, Y., Corradetti, M.N., Inoki, K., Guan, K. L. (2004). TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem Sci 29*, 32-38

Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., Wu, D. (2005). Regulation of PTEN by Rho small GTPases. *Nat Cell Biol 7*, 399-404.

Liang, J., and Slingerland, J.M. (2003). Multiple Roles of the PI3K/PKB (Akt) Pathway in Cell Cycle Progression. *Cell Cycle 2*, 336-342.

Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., Franssen, E., and Slingerland, J.M. (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med 8*, 1153 - 1160.

Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H., Wu, H. (2000). Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. *Curr Biol 10*, 401–404.

Lim, S. T., Chen, X. L., Lim, Y., Hanson, D. A., Vo, T. T., Howerton, K., Larocque, N., Fisher, S. J., Schlaepfer, D. D., Ilic, D. (2008). Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol Cell 29*, 9–22.

Low, P.C., Misaki, R., Schroder, K., Stanley, A.C., Sweet, M.J., Teasdale, R.D., Vanhaesebroeck, B., Meunier, F.A., Taguchi, T., Stow, J.L. (2010). Phosphoinositide 3-kinase δ regulates membrane fission of Golgi carriers for selective cytokine secretion. *J Cell Biol 190*, 1053-1065.

Luo, J., Manning, B.D., Cantley, L.C. (2003). Targeting the PI3K-Akt pathway in human cancer: Rationale and promise. *Cancer Cell 4*, 257-262.

Luo, M., Guan, J. (2010). Focal adhesion kinase: a prominent determinant in breast cancer initiation, progression and metastasis. *Cancer Lett 289*, 127–139.

Ma, X., Kumar, M., Choudhury, S. N., Becker Buscaglia, L. E., Barker, J. R., Kanakamedala, K., Liu, M. F., Li, Y. (2011). Loss of the miR‐21 allele elevates the expression of its target genes and reduces tumorigenesis. *Proc Natl Acad Sci USA 108*, 10144–10149.

Maehama, T., Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5‐trisphosphate. *J Biol Chem 273*, 13375–13378.

Manning, B. D. and Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell 129*, 1261–1274.

Marinov, M., Fischer, B., Arcaro, A. (2007). Targeting mTOR signaling in lung cancer. *Crit Rev Oncol Hematol 63*, 172-182.

Marone, R., Cmiljanovic, V., Giese, B., Wymann, M.P. (2008). Targeting phosphoinositide 3-kinaseβ "Moving towards therapy. *Biochim Biophys Acta 1784*, 159-185.

Mavrakis, K. J., Wolfe, A. L., Oricchio, E., Palomero, T., de Keersmaecker, K., McJunkin, K., Zuber, J., James, T., Khan, A. A., Leslie, C. S., Parker, J. S., Paddison, P. J., Tam, W., Ferrando, A., Wendel, H. G. (2010). Genome-wide RNA-mediated interference screen identifies miR‐19 targets in Notch-induced T‐cell acute lymphoblastic leukaemia. *Nature Cell Biol 12*, 372–379.

Mayo, L. D., Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Pro Natl Acad Sci USA 98*, 11598–11603.

McLean, G. W., Carragher, N. O., Avizienyte, E., Evans, J., Brunton, V. G., Frame, M. C. (2005). The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nat Rev Cancer 5*, 505-515.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S. T., Patel, T. (2007). MicroRNA‐21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology 133*, 647–658.

Meric-Bernstam, F., Akcakanat, A., Chen, H., Do, K.-A., Sangai, T., Adkins, F., Gonzalez-Angulo, A.M., Rashid, A., Crosby, K., Dong, M., Phan, A.T., Wolff, R.A., Gupta, S., Mills, G.B., Yao, J. (2012). PIK3CA/PTEN Mutations and Akt Activation As Markers of Sensitivity to Allosteric mTOR Inhibitors. *Clin Cancer Res 18*, 1777-1789.

Merlot, S., Firtel, R. A. (2003). Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J Cell Sci 116*, 3471-3478.

Meuillet, E., Zuohe, S., Lemos, R., Ihle, N., Kingston, J., Watkins, R., Moses, S., Zhang, S., Du-Cuny, L., Herbst, R., Jacoby, J., Zhou, L., Ahad, A., Mash, E., Kirkpatrick, D., and Powis, G. (2010). Molecular pharmacology and antitumor activity of PHT-427, a novel Akt/phosphatidylinositide-dependent protein kinase 1 pleckstrin homology domain inhibitor. *Mol Cancer Ther 9*, 706-717.

Miller, S. J., Lou, D. Y., Seldin, D. C., Lane, W. S., Neel, B. G. (2002). Direct identification of PTEN phosphorylation sites. *FEBS Lett 528*, 145-153.

Mitra, S. K., Hanson, D. A., Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol 6*, 56–68.

Mitra, S. K, Schlaepfer, D. D. (2006). Integrin-regulated FAK–Src signaling in normal and cancer cells. *Curr Opin Cell Biol 18*, 516–523.

Mizoguchi, M., Nutt, C.L., Mohapatra, G., Louis, D.N. (2004). Genetic alterations of phosphoinositide 3-kinase subunit genes in human glioblastomas. *Brain Pathol 14*, 372-377.

Molina, J. R., Agarwal, N. K., Morales, F. C., Hayashi, Y., Aldape, K. D., Cote, G., Georgescu, M. M. (2012). NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma. *Oncogene 31*, 1264–1274.

Mounir, Z., Krishnamoorthy, J. L., Robertson, G. P., Scheuner, D., Kaufman, R. J., Georgescu, M. M., Koromilas, A. E. (2009). Tumor suppression by PTEN requires the activation of the PKR–eIF2α phosphorylation pathway. *Sci. Signal. 2*, ra85.

Mu, P., Han, Y. C., Betel, D., Yao, E., Squatrito, M., Ogrodowski, P., de Stanchina, E., D'Andrea, A., Sander, C., Ventura, A. (2009). Genetic dissection of the miR‐17~92 cluster of microRNAs in Myc-induced B‐cell lymphomas. *Genes Dev 23*, 2806–2811.

Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R., Tonks, N. K. (1997). P‐TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci USA 94*, 9052–9057.

Nakamura, J., Shigematsu, S., Yamauchi, K., Takeda, T., Yamazaki, M., Kakizawa, T., Hashizume, K. (2008). Biphasic function of focal adhesion kinase in endothelial tube formation induced by fibril-forming collagens. *Biochem Biophys Res Commun 374*, 699–703.

Niggli, V., Keller, H. (1997). The phosphatidylinositol 3-kinase inhibitor wortmannin markedly reduces chemotactic peptide-induced locomotion and increases in cytoskeletal actin in human neutrophils. *Eur J Pharmacol 335*, 43-52.

Niggli, V. A. (2000). A Membrane-permeant ester of phosphatidylinositol 3,4, 5-trisphosphate (PIP(3) is an activator of human neutrophil migration. *FEBS Lett* 473, 217-221.

O'brien, C., Wallin, J.J., Sampath, D., Guhathakurta, D., Savage, H., Punnoose, E.A., Guan, J., Berry, L., Prior, W.W., Amler, L.C., Belvin, M., Friedman, L.S., Lackner, M.R. (2010). Predictive Biomarkers of Sensitivity to the Phosphatidylinositol 3 Kinase Inhibitor GDC-0941 in Breast Cancer Preclinical Models. *Clin Cancer Res 16*, 3670-3683.

Okahara, F., Itoh, K., Nakagawara, A., Murakami, M., Kanaho, Y., Maehama, T. (2006). Critical Role of PICT-1, a Tumor Suppressor Candidate, in Phosphatidylinositol 3,4,5-Trisphosphate Signals and Tumorigenic Transformation. *Mol Biol Cell 17*, 4888-4895.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D., Smith, A.J.H., Vanhaesebroeck, B. (2002). Impaired B and T cell antigen receptor signaling in p110δ PI 3-kinase mutant mice. *Science 297*, 1031-1034.

Okkenhaug, K., and Fruman, D.A. (2010). PI3Ks in lymphocyte signaling and development. *Curr Top Microbiol Immunol 346*, 57-85.

Okumura, K., Zhao, M., Depinho, R. A., Furnari, F. B., Cavenee, W. K. (2005). Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor. *Proc. Natl Acad. Sci. USA 102*, 2703–2706.

Okumura, K., Mendoza, M., Bachoo, R. M., DePinho, R. A., Cavenee, W. K., Furnari, F. B. (2006). PCAF modulates PTEN activity. *J Biol Chem 281*, 26562–26568.

Olive, V., Bennett, M. J., Walker, J. C., Ma, C., Jiang, I., Cordon-Cardo, C., Li, Q.J., Lowe, S.W., Hannon, G. J., He, L. (2009). miR‑19 is a key oncogenic component of mir‑17‑92. *Genes Dev 23*, 2839–2849.

Owen, J. L. and Mohamadzadeh, M. (2013). Macrophages and chemokines as mediators of angiogenesis. *Front Physiol 4*.

Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., Cance, W.G. (1995). Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Res 55*, 2752–2755.

Ozkal, S., Paterson, J. C., Tedoldi, S., Hansmann, M. L., Kargi, A., Manek, S., Mason, D. Y., Marafioti, T. (2009). Focal adhesion kinase (FAK) expression in normal and neoplastic lymphoid tissues. *Pathol Res Pract 205*, 781–788.

Papakonstanti, E.A., Ridley, A.J., Vanhaesebroeck, B. (2007). The p110δ isoform of PI 3-kinase negatively controls RhoA and PTEN. *EMBO J 26*, 3050-3061.

Papakonstanti, E.A., Zwaenepoel, O., Bilancio, A., Burns, E., Nock, G.E., Houseman, B., Shokat, K., Ridley, A.J., Vanhaesebroeck, B. (2008). Distinct roles of class IA PI 3-kinase isoforms in primary and immortalised macrophages. *J Cell Science 121*, 4124-4133.

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L., Olivi, A., Mclendon, R., Rasheed, B.A., Keir, S., Nikolskaya, T., Nikolsky, Y., Busam, D.A., Tekleab, H., Diaz, L.A.J., Hartigan, J., Smith, D.R., Strausberg, R.L., Marie, S.K., Shinjo, S.M., Yan, H., Riggins, G.J., Bigner, D.D., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E., Kinzler, K.W. (2008). An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Science 321*, 1807-1812.

Parsons, R. (2004). Human cancer, PTEN and the PI-3 kinase pathway. *Semin Cell Dev Biol 15*, 171-176.

Peso, L.D., González-García, M., Page, C., Herrera, R., Nuñez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science 278*, 687-689.

Philipp-Staheli, J., Payne, S.R., Kemp, C.J. (2001). p27Kip1: Regulation and Function of a Haploinsufficient Tumor Suppressor and Its Misregulation in Cancer. *Exp Cell Res 264*, 148-168.

Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J., Pandolfi, P. P. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature 465*, 1033–1038.

Procko, E., McColl, S. R. (2005). Leukocytes on the move with phosphoinositide 3-kinase and its downstream effectors. *Bioessays 27*, 153-163.

Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A., and Thomas, G. (1998). Phosphorylation and activation of p70S6K by PDK1. *Science 279*, 707-710.

Qi, J. H., Claesson-Welsh, L. (2001). VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Exp Cell Res 263*, 173–182.

Rabinovsky, R., Pochanard, P., McNear, C., Brachmann, S. M., Duke-Cohan, J. S., Garraway, L. A. , Sellers W. R. (2009). p85 associates with unphosphorylated PTEN and the PTEN-associated complex. *Mol Cell Biol 29*, 5377–5388.

Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S., Hall, A. (2004). Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science 303*, 1179–1181.

Reif, K., Okkenhaug, K., Sasaki, T., Penninger, J. M., Vanhaesebroeck, B., Cyster, J. G. (2004). Cutting edge: differential roles for phosphoinositide 3-kinases, p110gamma and p110delta, in lymphocyte chemotaxis and homing. *J Immunol 173*, 2236-2240.

Reynolds, A. R., Hart, I. R., Watson, A. R, Welti J. C., Silva, R. G., Robinson, S. D., Da Violante, G., Gourlaouen, M., Salih, M., Jones, M. C., Jones, D. T., Saunders, G., Kostourou, V., Perron-Sierra, F., Norman, J. C., Tucker, G. C., Hodivala-Dilke, K. M. (2009). Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat Med 15*, 392–400.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., Horwitz, A.R. (2003). Cell Migration: Integrating Signals from Front to Back. *Science 302*, 1704-1709.

Roche, S., Downward, J., Raynal, P., Courtneidge, S.A. (1998). A function for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in fibroblasts during mitogenesis: requirement for insulin- and lysophosphatidic acid-mediated signal transduction. *Mol Cell Biol 18*, 7119-7129.

Roche, S., Koegl, M., Courtneidge, S.A. (1994). The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. *Proc Natl Acad Sci U S A 91*, 9185-9189.

Romashkova, J. A., Makarov, S. S. (1999). NF-kB is a target of AKT in anti-apoptotic PDGF signalling. *Nature 401*, 86–90.

Rommel, C. (2010). Taking PI3Kδ and PI3Kγ one step ahead: dual active PI3Kδ/γ inhibitors for the treatment of immune-mediated inflammatory diseases. *Curr Top Microbiol Immunol 346*, 279-299.

Rommel, C., Camps, M., Ji, H. (2007). PI3K[delta] and PI3K[gamma]: partners in crime in inflammation in rheumatoid arthritis and beyond. *Nat Rev Immunol 7*, 191-201.
Rovida, E., Lugli, B., Barbetti, V., Giuntoli, S., Olivotto, M. Dello Sbarba, P. (2005). Focal adhesion kinase is redistributed to focal complexes and mediates cell spreading in macrophages in response to M-CSF. *Biol Chem 386*, 919-929.

Sabbah, D.A., Brattain, M.G., Zhong, H. (2011). Dual Inhibitors of PI3K/mTOR or mTOR-Selective Inhibitors: Which Way Shall We Go? . *Curr Med Chem 18*.

Sadhu, C., Masinovsky, B., Dick, K., Sowell, C.G., Staunton, D.E. (2003). Essential Role of Phosphoinositide 3-Kinase δ in Neutrophil Directional Movement. J *Immunol 170*, 2647-2654.

Sáez, A.L., Sánchez, E., Sánchez-Beato, M., Cruz, A., Chacón, I., Muñoz E., Camacho F. I., Martínez, J., Montero, C., Mollejo, M., Garcia, G. F., a Piris, M. (1999). p27Kip1 is abnormally expressed in Diffuse Large B-Cell Lymphomas and is associated with an adverse clinical outcome. *Br J Cancer 80*, 1427-1434.

Salmena, L., Carracedo, A., Pandolfi, P. P. (2008). Tenets of PTEN tumor suppression. *Cell 133*, 403–414.

Salmena, L., Poliseno, L., Tay, Y., Kats, Lo, Pandolfi, P. P. (2011). A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell 146*, 353–358.

Samuels, Y., and Velculescu, V.E. (2004). Oncogenic Mutations of PIK3CA in Human Cancers. *Cell Cycle 3*, 1221-1224.

Sanchez, T., Thangada, S., Wu, M.-T., Kontos, C. D., Wu, D., Wu, H. and Hla, T. (2005). PTEN as an effector in the signaling of antimigratory G protein-coupled receptor. *Proc Natl Acad Sci U S A. 102*, 4312-4317.

Sansal, I., and Sellers, W.R. (2004). The biology and clinical relevance of the PTEN tumor suppressor athway. *J Clin Oncol 22*, 2954-2963.

Santiskulvong, C., Konecny, G.E., Fekete, M., Chen, K.-Y.M., Karam, A., Mulholland, D., Eng, C., Wu, H., Song, M., Dorigo, O. (2011). Dual Targeting of Phosphoinositide 3-Kinase and Mammalian Target of Rapamycin Using NVP-BEZ235 as a Novel Therapeutic Approach in Human Ovarian Carcinoma. *Clin Cancer Res 17*, 2373-2384.

Sarbassov, D., Guertin, D., Ali, S., Sabatini, D. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science 307*, 1098-1101.

Sawyer, C., Sturge, J., Bennett, D.C., O'hare, M.J., Allen, W.E., Bain, J., Jones, G.E., Vanhaesebroeck, B. (2003). Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res 63*, 1667-1675.

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., Parsons, G. T. (1992). pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci USA 89*, 5192–5196.

Schaller, M. D., Hildebrand, G. D., Shannon, J. D., Fox, J. W., Vines, R. R., Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cel Biol 14*, 1680–1688.

Schaller, M. D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci 123*, 1007-1013.

Schlaepfer D. D., Hanks, S. K., Hunter, T., van der Geer, P. (1994). Integrin mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature 372*, 786–791.

Schmitz, K. J., Grabellus, F., Callies, R., Otterbach, F., Wohlschlaeger, J., Levkau, B., Kimmig, R., Schmid, K. W., Baba, H. A. (2005). High expression of focal adhesion kinase (p125FAK) in node-negative breast cancer is related to overexpression of HER-2/neu and activated Akt kinase but does not predict outcome. *Breast Cancer Res 7*, R194–R203.

Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., Bourne, H. R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science 287*, 1037-1040.

She, Q. B., Chandarlapaty, S., Ye, Q., Lobo, J., Haskell, K.M., Leander, K.R., Defeo-Jones, D., Huber, H.E., Rosen, N. (2008). Breast tumor cells with PI3K mutation or HER2 Amplification Are Selectively Addicted to Akt Signaling. *PLoS ONE 3*, e3065.

Shen , T. L., Park, A. Y., Alcaraz, A., Peng, X., Jang, I., Koni, P., Flavell, R. A., Gu, H., Guan, J. L. (2005). Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *J Cell Biol 169*, 941–952 .

Sheppard, K., Kinross, K.M., Solomon, B., Pearson, R.B., Phillips, W.A. (2012). Targeting PI3 kinase/AKT/mTOR signaling in cancer. *Crit Rev Oncog 17*, 69-95.

Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol 2*, 249–256.

Sinnett-Smith, J., Lunn, J. A., Leopoldt, D., Rozengurt, E. (2001). Y-27632, an Inhibitor of Rho-Associated Kinases, Prevents Tyrosine Phosphorylation of Focal Adhesion Kinase and Paxillin Induced by Bombesin: Dissociation from Tyrosine Phosphorylation of p130cas. *Exp Cell Res 266*, 292-302.

Slack-Davis, J. K., Martin, K. H., Tilghman, R. W., Iwanicki, M., Ung, E. J., Autry, C., Luzzio, M. J., Cooper, B., Kath, J. C., Roberts, W. G. (2007). Cellular Characterization of a Novel Focal Adhesion Kinase Inhibitor. *J Biol Chem 282*,14845-14852.

Slack-Davis, J. K., Hershey, E. D., Theodorescu, D., Frierson, H. F., Parsons, J. T. (2009). Differential requirement for focal adhesion kinase signaling in cancer progression in the transgenic adenocarcinoma of mouse prostate model. *Mol Cancer Ther 8*, 2470–2477.

Slingerland, J.P. (2000). Regulation of the Cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol 183*, 10-17.

Song, M. S., Salmena, L., Pandolf,i P. P. (2012). The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol 13*, 283-296.

Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K., Kasahara, T. (2000). Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem 275*, 16309–16315.

Soond, D.R., Bjγérgo, E., Moltu, K., Dale, V.Q., Patton, D.T., Torgersen, K.M., Galleway, F., Twomey, B., Clark, J., Gaston, J.S.H., Tasken, K., Bunyard, P., Okkenhaug, K. (2010). PI3K p110delta regulates T-cell cytokine production during primary and secondary immune responses in mice and humans. *Blood 115*, 2203-2213.

Srinivasan, S.,Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K. (2003). Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. *J Cell Biol 160*, 375-85.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., Mak,T. W. (1998). Negative regulation of PKB/Aktdependent cell survival by the tumor suppressor PTEN. *Cell 95*, 29–39.

Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., Mak, T. W. (2001). Regulation of PTEN transcription by p53. *Mol. Cell 8*, 317–325.

Stemke-Hale, K., Gonzalez-Angulo, A.M., Lluch, A., Neve, R.M., Kuo, W.-L., Davies, M., Carey, M., Hu, Z., Guan, Y., Sahin, A., Symmans, W.F., Pusztai, L., Nolden, L.K., Horlings, H., Berns, K., Hung, M.-C., Van De Vijver, M.J., Valero, V., Gray, J.W., Bernards, R., Mills, G.B., Hennessy, B.T. (2008). An Integrative Genomic and Proteomic Analysis of PIK3CA, PTEN, and AKT Mutations in Breast Cancer. *Cancer Res 68*, 6084-6091.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reece, C.B., Mccormick, F., Tempst, P., Coadwell, J., Hawkins, P.T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science 279*, 710-714.

Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reece, C.B., Painter, G.F., Holmes, A.B., Mccormick, F., Hawkins, P.T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science 277*, 567-570.

Sulis, M.L., and Parsons, R. (2003). PTEN: from pathology to biology. *Trends Cell Biol 13*, 478-483.

Sumazin, P., Yang, X., Chiu, H. S., Chung, W. J., Iyer, A., Llobet-Navas, D., Rajbhandari, P., Bansal, M., Guarnieri, P., Silva, J., Califano, A. (2011). An extensive microRNA-mediated network of RNA–RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell 147*, 370–381.

Sweeney, S. M., DiLullo, G., Slater, S. J., Martinez, J., Iozzo, R.V., Lauer-Fields, J. L., Fields, G. B., San Antonio, J. D. (2003). Angiogenesis in collagen I requires alpha2beta1 ligation of a GFP*GER sequence and possibly p38 MAPK activation and focal adhesion disassembly. *J Biol Chem 278*, 30516–30524.

Takahashi, Y., Morales, F. C., Kreimann, E. L., Georgescu, M. M. (2006). PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. *EMBO J. 25*, 910–920.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., Yamada, K. M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science 280*, 1614–1617.

Tamura, M., Gu, J., Danen, E. H. J., Takino, T., Miyamoto, S., Yamada, K. M. (1999a). PTEN Interactions with Focal Adhesion Kinase and Suppression of the Extracellular Matrix-dependent Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway. *J Biol Chem 274*, 20693-20703.

Tamura, M., Gu, J., Takino, T., Yamada, K. M. (1999b). Tumor Suppressor PTEN Inhibition of Cell Invasion, Migration, and Growth: Differential Involvement of Focal Adhesion Kinase and p130Cas. *Cancer Res 59*, 442-449.

Tanaka, H., Yoshida, M., Tanimura, H., Fujii, T., Sakata, K., Tachibana, Y., Ohwada, J., Ebiike, H., Kuramoto, S., Morita, K., Yoshimura, Y., Yamazaki, T., Ishii, N., Kondoh, O., Aoki, Y. (2011). The Selective Class I PI3K Inhibitor CH5132799 Targets Human Cancers Harboring Oncogenic PIK3CA Mutations. *Clin Cancer Res 17*, 3272-3281.

Taniguchi, C. M., Winnay, J., Kondo, T., Bronson, R. T., Guimaraes, A. R, Alemán, J. O., Luo, J., Stephanopoulos, G., Weissleder, R., Cantley, L. C., Kahn, C. R. (2010). The phosphoinositide 3-kinase regulatory subunit p85alpha exert tumor suppressor properties through negative regulation of growth factor signaling. *Cancer Res 70*, 5305–5315.

Tavora, B., Batista, S., Reynolds, L. E., Jadeja, S., Robinson, S., Kostourou, V., Hart, I., Fruttiger, M., Parsons, M., Hodivala-Dilke, K. M. (2010). Endothelial FAK is required for tumour angiogenesis. *EMBO Mol Med 2*, 516–528.

Tay, Y., Kats, L., Salmena, L., Weiss, D., Tan, S. M., Ala, U., Karreth, F., Poliseno, L., Provero, P., Di Cunto, F., Lieberman, J., Rigoutsos, I., Pandolfi, P. P. (2011). Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. *Cell 147*, 344–357.

Theocharis, S. E., Kouraklis, G. P., Kakisis, J. D., Kanelli, H. G., Apostolakou, F. E., Karatzas, G. M., Koutselinis, A. S. (2003). Focal adhesion kinase expression is not a prognostic predictor in colon adenocarcinoma patients. *Eur J Surg Oncol 29*, 571–574.

Thomas, R.K., Baker, A.C., Debiasi, R.M., Winckler, W., Laframboise, T., Lin, W.M., Wang, M., Feng, W., Zander, T., Macconaill, L.E., Lee, J.C., Nicoletti, R., Hatton, C., Goyette, M., Girard, L., Majmudar, K., Ziaugra, L., Wong, K.-K., Gabriel, S., Beroukhim, R., Peyton, M., Barretina, J., Dutt, A., Emery, C., Greulich, H., Shah, K., Sasaki, H., Gazdar, A., Minna, J., Armstrong, S.A., et al. (2007). High-throughput oncogene mutation profiling in human cancer. *Nat Genet 39*, 347-351.

Torbett, N.E., Luna-Moran, A., Knight, Z.A., Houk, A., Moasser, M., Weiss, W., Shokat, K.M., Stokoe, D. (2008). A chemical screen in diverse breast cancer cell lines reveals genetic enhancers and 1058 suppressors of sensitivity to PI3K isoform-selective inhibition. *Biochem J 415*, 97-110.

Torres, J., and Pulido, R. (2001). The Tumor Suppressor PTEN Is Phosphorylated by the Protein Kinase CK2 at Its C Terminus. *J Biol Chem 276*, 993-998.

Torsoni, A. S., Marin, T. M., Velloso, L. A., Franchini, K. G. (2005). RhoA/ROCK signaling is critical to FAK activation by cyclic stretch in cardiac myocytes. *Am J Physiol Heart Circ Physiol 289*, H1488-H1496.

Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N. P., Carver, B. S., Cordon-Cardo, C., Erdjument-Bromage, H., Tempst, P., Chi, S. G., Kim, H. J., Misteli, T., Jiang, X., Pandolfi, P. P. (2007). Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell 128*, 141–156.

Tzenaki, N., Andreou, M., Stratigi, K., Vergetaki, A., Makrigiannakis, A., Vanhaesebroeck, B., Papakonstanti, E.A. (2012). High levels of p110δ PI3K expression in solid tumor cells suppresses 1072 PTEN activity, generating cellular sensitivity to p110δ inhibitors through PTEN activation. *FASEB J 26*, 2498-2508

Tzenaki, N. and Papakonstanti E. A. (2013). p110δ PI3 kinase pathway:emerging roles in cancer. *Front Oncol 3*, 40.

Van Diepen, M. T., Parsons, M., Downes, C. P., Leslie, N. R., Hindges, R., Eickholt, B. J. (2009). MyosinV controls PTEN function and neuronal cell size. *Nature Cell Biol 11*, 1191–1196.

Vanhaesebroeck, B., Ali, K., Bilancio, A., Geering, B., Foukas, L.C. (2005). Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem Sci 30*, 194-204.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol 11*, 329-341.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., Waterfield, M.D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem. 70*, 535-602.

Vanhaesebroeck, B., and Waterfield, M.D. (1999). Signaling by distinct classes of phosphoinisitide 3-kinases. *Exp. Cell Res.* 253, 239-254.

Vanhaesebroeck, B., Welham, M.J., Kotani, K., Stein, R., Warne, P.H., Zvelebil, M.J., Higashi, K., Volinia, S., Downward, J., Waterfield, M.D. (1997b). p110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. USA 94*, 4330-4335.

Vazquez, F., Grossman, S. R., Takahashi, Y., Rokas, M. V., Nakamura, N., Sellers, W. R. (2001). Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J *Biol Chem 276*, 48627–48630.

Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-kinase-Akt pathway in human cancer. *Nat Rev Cancer 2*, 489-501.

Vivanco, I., Palaskas, N., Tran, C., Finn, S. P., Getz, G., Kennedy, N. J., Jiao, J., Rose, J., Xie, W., Loda, M., Golub, T., Mellinghoff, I. K., Davis, R. J., Wu, H., Sawyers, C. L. (2007). Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN. *Cancer Cell 11*, 555–569.

Vogt, P.K., Gymnopoulos, M., Hart, J.R. (2009). PI 3-kinase and cancer: changing accents. *Curr Opin Genet Dev 19*, 12-17.

Wang, F., Herzmark, P.,Weiner, O. D., Srinivasan, S., Servant, G., Bourne, H. R., (2002). Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol 4*, 513-518.

Wang, X., Trotman, L. C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C., Pandolfi, P. P., Jiang, X. (2007). NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell 128*, 129–139.

Wang, S. and Basson, M. D. (2011). Akt directly regulates focal adhesion kinase through association and serine phosphorylation: implication for pressure-induced colon cancer metastasis. *Am J Physiol Cell Physiol 300*, C657-70.

Wee, S., Wiederschain, D., Maira, S.-M., Loo, A., Miller, C., Debeaumont, R., Stegmeier, F., Yao, Y.-M., Lengauer, C. (2008). PTEN-deficient cancers depend on PIK3CB. *Proc Natl Acad Sci USA 105*, 13057-13062.

Weigelt, B., and Downward, J. (2012). Genomic determinants of PI3K pathway inhibitor response in cancer. *Front Oncol 2*, 109.

Weigelt, B., Warne, P.H., Downward, J. (2011). PIK3CA mutation, but not PTEN loss of function, determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. *Oncogene 30*, 3222-3233

Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C., Bourne, H. R. (2002). A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat Cell Biol 4*, 509-513.

Welch, H.C., Coadwell, W.J., Ellson, C.D., Ferguson, G.J., Andrews, S.R., Erdjument-Bromage, H., Tempst, P., Hawkins, P.T., Stephens, L.R. (2002). P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell 108*, 809-821.

Whitehead, M.A., Bombardieri, M., Pitzalis, C., Vanhaesebroeck, B. (2012). Isoform-selective induction of human p110δ PI3K expression by TNFα: identification of a new and inducible PIK3CD promoter. *Biochem J 443*, 857-867.

Willems, L., Tamburini, J., Chapuis, N., Lacombe, C., Mayeux, P., Bouscary, D. (2012). PI3K and mTOR Signaling Pathways in Cancer: New Data on Targeted Therapies. *Curr Oncol Rep 14*, 129-138.

Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjöblom, T., Leary, R.J., Shen, D., Boca, S.M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T., Nikolsky, Y., Karchin, R., Wilson, P.A., Kaminker, J.S., Zhang, Z., Croshaw, R., Willis, J., Dawson, D., Shipitsin, M., Willson, et al. (2007). The genomic landscapes of human breast and colorectal cancers. *Science 318*, 1108-1113.

Xiao, C., Srinivasan, L., Calado, D. P., Patterson, H. C., Zhang, B., Wang, J., Henderson, J. M., Kutok, J. L., Rajewsky, K. (2008). Lymphoproliferative disease and autoimmunity in mice with increased miR‐17–92 expression in lymphocytes. *Nature Immunol 9*, 405–414.

Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D., Guan, J. L. (1994). Directed interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol Biol Cell 5*, 413-421.

Xu, L. H., Yang, X., Bradham, C. A., Brenner, D. A., Baldwin, A. S., Craven, R. J., Cance, W. G. (2000). The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. *J Biol Chem 275*, 30597–30604.

Yagi, Y., Otani, H., Ando, S., Oshiro, A., Kawai, K., Nishikawa, H., Araki, H., Fukuhara, S., Inagaki, C. (2006). Involvement of Rho signaling in PAR2-mediated regulation of neutrophil adhesion to lung epithelial cells. *Eur J Pharmacol 536*, 19-27

Yeo, M. G., Partridge, M. A., Ezratty, E. J., Shen, Q., Gundersen, G. G., Marcantonio, E.E. (2006). Src SH2 arginine 175 is required for cell motility: specific focal adhesion kinase targeting and focal adhesion assembly function. *Mol Cell Biol 26*, 4399-4409.

Yim, J. H., Kim, Y. J., Ko, J. H., Cho, Y. E., Kim, S. M., Kim, J. Y., Lee, S., Park, J. H. (2007). The putative tumor suppressor gene GLTSCR2 induces PTEN-modulated cell death. *Cell Death Differ 14*, 1872-1879.

Yim, E. K., Peng, G., Dai, H., Hu, R., Li, K., Lu, Y., Mills, G. B., Meric-Bernstam, F., Hennessy, B. T., Craven, R. J., Lin, S. Y. (2009). Rak functions as a tumor suppressor by regulating PTEN protein stability and function. *Cancer Cell 15*, 304–314.

Zhang, S., Huang, W. C., Li, P., Guo, H., Poh, S. B., Brady, S. W., Xiong, Y., Tseng, L. M., Li, S. H., Ding, Z., Sahin, A. A., Esteva, F. J., Hortobagyi, G. N., Yu, D. (2011). Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nature Med 17*, 461–469.

Zhao, J. H., Reiske, H., Guan J. L. (1998). Regulation of the cell cycle by focal adhesion kinase. *J Cell Biol 143*, 1997–2008.

Zhao, L., and Vogt, P.K. (2008a). Class I PI3K in oncogenic cellular transformation. *Oncogene 27*, 5486-1130 5496.

Zhao, J. H., Guan, J. L. (2009). Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev 28*, 35–49.

Zhou, B.P., Yong Liao, Weiya Xia, Bill Spohn, Mong-Hong Lee & Mien-Chie Hung (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol 3*, 245 - 252.

Zhu, Q., Youn, H., Tang, J., Tawfik, O., Dennis, K., Terranova, P.F., Du, J., Raynal, P., Thrasher, J.B., and Li, B. (2008). Phosphoinositide 3-OH kinase p85alpha and p110beta are essential for androgen receptor transactivation and tumor progression in prostate cancers. *Oncogene 27*, 4569-4579.

Zwaenepoel, O., Tzenaki, N., Vergetaki, K., Makrigiannakis, A., Vanhaesebroeck, B., and Papakonstanti, E.A. (2012). Functional CSF-1 receptors are located at the nuclear envelope and activated by the p110δ isoform of PI 3-kinase. *FASEB J 26*, 691-706.