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REVIEW

The PTEN–PI3K pathway: of feedbacks and cross-talks

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The tumor suppressor PTEN was originally identified as a negative regulator of the phosphoinositide 3-kinase (PI3K) signaling, a main regulator of cell growth, metabolism and survival. Yet this function of PTEN is extremely relevant for its tumor-suppressive ability, albeit the recent characterization of many PI3K-independent tumor-suppressive activities. PI3K-mediated PIP_3 production leads to the activation of the canonical AKT-mTORC1 pathway. The implications of this signaling cascade in health and disease have been underscored by the high number of regulators within the pathway whose alterations give rise to different malignancies, including familiar syndromes, metabolic dysfunctions and cancer. Moreover, PI3K is tightly buffered at multiple levels by downstream components, which have turned this signaling pathway literally upside down. PI3K and its downstream components in turn cross-talk with a number of other pathways, thereby leading to a complex network of signals that may have dramatic consequences when perturbed. Here, we review the current status of the PTEN–PI3K signaling pathway with special emphasis on the most recent data on targets and regulation of the PTEN–PI3K axis. This provides novel provocative therapeutic implications based on the targeted modulation of PI3K-cross-talking signals.
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Components of the PI3K pathway

Phosphoinositide 3-kinases (PI3Ks) are a family of proteins involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis and vesicle trafficking (Engelman *et al*., 2006). Most of the members of this family are bound to regulatory subunits, which determine the signals modulating its activity. There are three members in the family (Cantley, 2002): class I PI3K, which is divided into IA and IB, is activated by receptor tyrosine kinases (RTKs, PI3K1A) and G-protein-coupled receptors (GPCRs, PI3K1B). Class IA and IB PI3Ks have different regulatory subunits, p85α/p85β/p55 for IA and p101/p84/p87PIKAP for IB. This class is characterized for generating primarily PI-3, 4.5-P_3 (PIP_3) (Katso *et al*., 2001).

PI3K class II utilizes *in vitro* PI-3-P to generate PI-3, 4-P_2 and can also produce PI-3-P from PI. This class does not require a regulatory subunit to function, and comprises three different isoforms (α, β and γ) that diverge in the N terminus and present different domains within the C terminus. Class II PI3K is involved in membrane trafficking and receptor internalization and can be activated in response to RTKs, integrins and cytokine receptors (Engelman *et al*., 2006).

Class III PI3K (Vps34), which was first identified in the budding yeast (Herman and Emr, 1990), is involved in vesicle trafficking (Backer, 2008) and cross-talks with class I PI3K through the regulation of mTORC1 signaling, as we will discuss later.

Class I PI3K is the most studied among the three members of the family. For the purpose of this review, we will focus mostly on this class, referring to it as PI3K unless otherwise specified.

PTEN

The Phosphatase and Tensin homolog deleted on chromosome TEN was originally discovered as a candidate tumor suppressor mutated and lost in various cancers (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). Several lines of evidence soon highlighted PTEN as a lipid phosphatase-hydrolysing phosphates in position 3′ from phosphoinositides (Maehama and Dixon, 1998; Stambolic *et al*., 1998). The major function of PTEN is the buffering of PI3K signaling; yet recent studies point to additional novel, lipid phosphatase-independent functions that may contribute to its tumor-suppressive activity (Carracedo *et al*., 2008; Salmena *et al*., 2008). The loss and mutation of *PTEN* in various cancers lead to hyperactive PI3K signaling. For example, *PTEN* is commonly mutated in its phosphatase domain (Eng, 2003); and in glioblastoma, mutations that impair its proper membrane localization might result in deficient tumor-suppressive activity (Walker *et al*., 2004). It is therefore clear that PTEN is a main player in the regulation of PI3K signaling and, as we will discuss, perturbations in its levels or function can dramatically impact on this pathway.

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PIP_3-dependent pathways

Upon 3′ phosphorylation of PI-4,5-P_2 by PI3K, proteins containing pleckstrin homology (PH) and PH-like domains are recruited to the plasma membrane, thereby transmitting the signal elicited by PI3K activation (Engelman *et al*., 2006). One of the best-characterized members of this group of proteins is the pro-survival AKT kinase. AKT contains a PH domain; upon PIP_3 production it anchorages to the membrane, where another phosphoinositide-binding protein, PDK1 (Alessi *et al*., 1997), and a recently discovered protein complex, mTORC2 (Sarbassov *et al*., 2004, 2005), phosphorylate and activate the kinase. Activated AKT mediates several of the well-described PI3K responses, mainly growth, metabolism, survival and glucose homeostasis (Manning and Cantley, 2007). Therefore, the PI3K-AKT axis is considered the canonical PI3K signaling. But, as we will discuss later, PI3K leads to the modulation of other pathways that are of great importance for the described function of this kinase.

AKT phosphorylates up to 100 substrates thereby modulating a variety of cellular functions. First, AKT signaling exerts a strong antiapoptotic effect through the phosphorylation and inhibition of key pro-apoptotic proteins, such as BAD, MDM2 and members of the Forkhead family (del Peso *et al*., 1997; Biggs *et al*., 1999; Brunet *et al*., 1999; Guo *et al*., 1999; Kops *et al*., 1999; Nakae *et al*., 1999; Rena *et al*., 1999; Takaishi *et al*., 1999; Tang *et al*., 1999; Mayo and Donner, 2001; Ashcroft *et al*., 2002). Second, AKT activates cell proliferation by inactivating p27 (Fujita *et al*., 2002) and inhibiting glycogen synthase kinase 3 (GSK3)-mediated Myc and cyclin D1 inhibition (Vivanco and Sawyers, 2002). Third, this kinase regulates a subset of proteins involved in growth, metabolism and angiogenesis. AKT phosphorylates and inactivates GSK3β (Cross *et al*., 1995), increases glucose transporter Glut4 translocation to plasma membrane by blocking AS160 (Sano *et al*., 2003) and, through FOXO inactivation, inhibits phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Burgering, 2008). All these actions converge in an increased glucose catabolism rate. Additionally, AKT is one of the main regulators of a complex involved in protein translation and ribosome biogenesis; this is mTORC1, which is composed of the protein kinase mTOR and a series of interactors (Guertin and Sabatini, 2007). AKT regulates this complex through the phosphorylation of two different substrates. On the one hand, this kinase phosphorylates and inactivates the GTPase-activating protein (GAP) TSC2 (Dan *et al*., 2002; Inoki *et al*., 2002; Potter *et al*., 2002), which forms a complex with TSC1 to inhibit a small GTPase, Rheb (Ras-homolog enriched in brain) (Garami *et al*., 2003; Inoki *et al*., 2003; Tee *et al*., 2003; Zhang *et al*., 2003b). Rheb activates mTORC1 through the inhibition of the FKBP38, a novel negative regulator of mTORC1 (Bai *et al*., 2007). On the other hand, AKT phosphorylates and inhibits PRAS40 (proline-rich AKT substrate of 40 KDa), which negatively regulates the activity of mTORC1 through the competition with the GTPase Rheb (Kovacina *et al*., 2003; Sancak *et al*., 2007; Vander Haar *et al*., 2007). Through this dual regulation, AKT promotes the activation of mTORC1 pathway, which turns on the translational machinery to produce ribosomes and increases the protein synthesis rate.

Of note, the production of PIP_3 modulates a different branch of signals, which will not be discussed in depth in this review.

mTORC1 pathway

As aforementioned, a main part of PI3K signaling converges in mTORC1 activation. mTORC1 phosphorylates and activates S6 kinases 1 and 2 (Guertin and Sabatini, 2007), which in turn activates protein translation and ribosome biogenesis through phosphorylation of different substrates in addition to ribosomal protein S6, including PDCD4, eEF2 kinase and eIF4B (Wang *et al*., 2001; Raught *et al*., 2004; Dorrello *et al*., 2006). On the other hand, mTORC1 phosphorylates and inactivates 4E-binding protein 1 (4EBP1), thereby releasing the inhibition of the eukaryotic initiation factor 4E (eIF4E) (Guertin and Sabatini, 2007). Taken together, mTORC1 modulates a variety of substrates, which ultimately upregulate protein synthesis. This pathway has turned out to be enormously interesting for the treatment of several diseases due to the discovery of a specific inhibitor, rapamycin, a macrolide extracted from a fungi *Streptomyces hygroscopicus* (Vezina *et al*., 1975). Rapamycin binds an intracellular protein, FKBP12, which blocks mTORC1 activity through mechanisms not yet well understood (Sabatini *et al*., 1994). The ability of rapamycin to inhibit mTORC1, together with the observation that this complex is hyperactive in many cancers, has promoted a number of clinical trials for the treatment of a variety of cancers. Meanwhile, the benefit of mTORC1 inhibition for the treatment of many cancers remains to be established (Guertin and Sabatini, 2007).

The importance of mTOR in the response to PI3K activation goes beyond mTORC1 activation. Recently, mTOR was found to interact with a different subset of proteins in a complex called as mTORC2 (Sarbassov *et al*., 2004, 2005). This complex is now accepted as the factor responsible for AKT phosphorylation in serine 473 (Ser473), thus locating mTOR both at the top and at the bottom of this pathway.

PI3K pathway in disease

Since its discovery, the role of PI3K pathway in disease has been highlighted by the number of members of the pathway whose aberrant function associates with the development of malignancies (Engelman *et al*., 2006). As this pathway is a central integrator of metabolism and survival/growth signals, it is not surprising that dysfunctional PI3K cascade would lead to metabolic diseases and cancer.

With respect to metabolism, many studies in mouse models and the further support of clinical data have made it evident that reduced PI3K activity can

play a role in insulin sensitivity and non-insulin-dependent diabetes mellitus (type II diabetes). Data coming from mice lacking p110α regulatory subunits have shed light on the dual role of p85 in PI3K regulation in response to insulin (Katso *et al*., 2001). Heterozygous deletion of either *p85α* or *β* in mice leads, surprisingly, to increased sensitivity to insulin, whereas compound heterozygous mutants fail to activate PI3K in response to insulin. The explanation for this dual effect is based on the fact that p85 can buffer the binding of p110α/p85 complex when expressed in excess within the cell. Therefore, partial p85 reduction would favor PI3K activation by reducing insulin receptor substrate-1 (IRS-1)-bound p110α-free p85, whereas a further decrease would attenuate p110α activation through disruption of p110α/p85 complexes (Luo *et al*., 2005a). In support of a role for PI3K in diabetes, *p110α* and *β* double heterozygous mutant mice show glucose intolerance. On the other hand, Pten loss of function exhibits dramatic consequences for metabolism homeostasis. As an example, *Pten*-deleted muscle, liver or fat tissue exhibits increased glucose sensitivity (Stiles *et al*., 2004; Kurlawalla-Martinez *et al*., 2005; Wijesekara *et al*., 2005).

The recent discovery of a role for class 3 PI3K (VPS34) in amino-acid sensing might similarly provide a new angle for the role of this PI3K in metabolism. VPS34 was originally involved in vesicle trafficking (Herman and Emr, 1990). Further studies proposed a role for this kinase in the formation of autophagosomes, double-membrane organelles involved in the digestion of intracellular components in response to starvation and stress (Backer, 2008). The role of VPS34 in autophagy already implicates this kinase in aging and response to nutrient deprivation. But, recently, a novel function for VPS34 in amino-acid sensing was discovered by the group of Drs Thomas and Kozma. In an elegant study, they found that the ability of mTORC1 to sense amino acid was not dependent on the class 1 PI3K, but on the class 3 (Nobukuni *et al*., 2005). A more recent study by this group reveals the mechanism of how VPS34 senses amino acids, a phenomenon that depends on the release on calcium from the endoplasmic reticulum to the cytoplasm (Gulati *et al*., 2008). Therefore, this finding suggest that aberrant Class 3 PI3K signaling could have a deep impact in proper amino-acid sensing by mTOR, with possible implications in metabolic processes.

The evidence of a role for this pathway in diabetes is underscored in humans by the presence of genetic alterations of PI3K regulators in patients with this disease. p85 levels are found increased in some patients with insulin resistance (Bandyopadhyay *et al*., 2005) Moreover, polymorphism of *PI3KR1* (which encodes for p85) together with those found in IRS-1 and IRS-2 has been associated with diabetes (Almind *et al*., 1996; Kawanishi *et al*., 1997; D'Alfonso *et al*., 2003; Esposito *et al*., 2003). On the other hand, *AKT2* has been found to be mutated in familiar insulin resistance (George *et al*., 2004), thereby enforcing the concept that PI3K, through modulation of targets involved in metabolic regulation, fine-tunes glucose metabolism in response to extracellular stimuli.

Alterations in PI3K pathway have been largely associated with cancer as well as different cancer susceptibility syndromes. From the top of the pathway, PI3K class IA is mutated and amplified in a variety of cancers (Samuels *et al*., 2004). The most frequently activating mutations (*Hot Spots*) in PI3K are located within the kinase domain, H1074, and in the helical domain, E542-5. Although these two mutations may have different impacts on PI3K targets, both drive transformation *in vitro* (reviewed in Zhao and Vogt, 2008). Interestingly, there appears to be a tissue-specific preference for mutation or amplification of PI3K. Although amplifications but not mutations are frequent in ovarian and gastric tumors, mutations in the absence of amplifications of this gene often occur in breast and colorectal cancer (Bader *et al*., 2005). Of interest, somatic mutations in *PIK3R1* have been found in ovarian and colon cancer, and in fact partial reduction of p85 levels enforces the tumorigenesis by heterozygous *Pten* loss in mice (Luo *et al*., 2005b), providing support to the hypothesis that alteration of PI3K signaling at multiple levels can result in cancer (Bader *et al*., 2005). Of note, although the implications of PI3K1A mutation and amplification are widely characterized, the role of other PI3K isoforms is still unclear. Indeed, according to recent studies different PI3K isoforms would require distinct signaling pathways to drive transformation (Denley *et al*., 2008), which in turn further highlights the relevance of cross-talks involving PI3K and other pathways for tumor progression.

The relevance of PTEN in cancer arises from its discovery, when three independent groups characterized a gene located in chromosome 10 as highly mutated and lost in several cancers (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). PTEN is a lipid phosphatase with a lipid-binding domain that allows anchorage to the plasma membrane. Although described as a highly stable protein, different groups have recently reported PTEN is tightly regulated at the transcriptional level, as well as by post-translational modifications (Salmena *et al*., 2008). Mutations either in the catalytic domain, C2 domain (Eng, 2003) or in ubiquitinylation sites (Trotman *et al*., 2007) have been shown to correlate with the development and progression of cancer. Moreover, germline heterozygous alterations in *PTEN* locus are associated with the development of a group of syndromes generally referred as PTEN hamartoma tumor syndromes, which includes Cowden, Lhermitte–Duclos, Bannayan–Riley–Rubalcaba, Proteus and Proteus-like syndromes (Eng, 2003). This disease is characterized by the presence of developmental and neurological defects, hamartomas and cancer susceptibility. Mouse models of *Pten* loss have shown that deletion of one allele of *Pten* results in lethal autoimmune disorders, and various forms of epithelial cancers (Di Cristofano *et al*., 1998, 1999). These data have led to the notion that, at least in the mouse, Pten is haploinsufficient for its tumor-suppressive activity.

Because of the lethal nature of complete *Pten* loss, several groups have addressed the effect of the acute loss of this tumor suppressor through tissue-specific *Pten*-conditional deletion. Surprisingly, complete *Pten* deletion does not have the pro-tumorigenic action predicted. Indeed, acute *Pten* loss in the hematopoietic stem cell compartment leads to exhaust of the hematopoietic stem cell (Yilmaz *et al*., 2006; Zhang *et al*., 2006b) and acute *Pten* loss in the prostate leads to a strong p53-dependent senescence response that opposes cancer progression (Chen *et al*., 2005). On the other hand, *Pten* deletion in neural cells increases the size of the brain and perturbs its function without leading to cancer. At the cellular level, *Pten* loss induces glial cell proliferation but does not have any effect on neurons (Fraser *et al*., 2004, 2008; Endersby and Baker, 2008). These studies suggest that tumors may not select for a complete loss of function of PTEN in the initial steps of tumorigenesis (as happens in prostate cancer, where approximately 70% of the tumors have heterozygous alterations in PTEN at presentation and lose the other allele at later stages), which may have relevance in the understanding of the 'just-right' dose (Albuquerque *et al*., 2002) of PI3K that would be of advantage for cancer to progress.

The relevance of AKT in the transduction of PI3K signals suggests that the activity of this kinase could be altered through mutations in *AKT* genes. Indeed, myristoylated constitutively active Akt1 promotes aberrant prostate growth (prostate intraepithelial neoplasia) in transgenic mice where the gene is set under prostate-specific promoter regulation (Majumder *et al*., 2003). Interestingly, Akt overexpression leads to an mTORC1-dependent (rapamycin-sensitive) glucose metabolism signature, which underscores the major role of mTORC1 in AKT-driven tumorigenesis (Majumder *et al*., 2004).

The role of AKT in human cancers was for a long time thought to be restricted to AKT (AKT1 and 2) gene amplification, as found in gastric, ovarian, pancreas and stomach cancers (Engelman *et al*., 2006). But recently, the group of Thomas reported a novel point mutation in the lipid-binding pocket of AKT1 in breast, colorectal and ovarian cancer patients that leads to AKT1 targeting to plasma membrane and activation of downstream effectors (Carpten *et al*., 2007). Mutant Akt1 is able induce leukemia in mice and recent studies have reported the same mutation in a subset of squamous cell lung carcinoma and breast cancer (Kim *et al*., 2008; Malanga *et al*., 2008).

Lastly, alterations in *TSC* genes (TSC1 and TSC2) result in the development of an autosomal dominant disease, tuberous sclerosis, characterized hamartomas in brain, heart, lung and kidney, neurological disorders, angiomyolipomas and rhabdomyomas (Jozwiak *et al*., 2008). Moreover, most of the patients who develop these malignancies (with the exception of the cerebral tubers) exhibit loss of the intact allele, a compelling example of a tumor suppressor that conforms to the 'two-hit hypothesis' for tumor suppression proposed by Knudson (Knudson, 1996).

Upstream regulators of PI3K signaling

As mentioned above, PI3K is activated mainly upon nutrient and growth factor stimulation. The regulatory subunit of PI3K1α, p85 binds to tyrosine-phosphorylated proteins, typically receptor tyrosin kinases and adaptor proteins, to activate PI3K (Cantley, 2002). In a prototypical example, the engagement of the IRS protein by the tyrosine kinase receptors leads to the binding of the p85–PI3K1A heterodimer, which mediates the transmission of the extracellular signal (that is, insulin) to the interior of the cell. RTK can also activate PI3K through the engagement of Ras and consequential binding of the GTPase to the Ras-binding domain (RBD) of PI3K (Engelman *et al*., 2006). There are other mechanisms of PI3K activation by extracellular signals. Another family of plasma membrane receptors, the GPCRs, can activate some PI3K isoforms directly (Schwindinger and Robishaw, 2001), which is thought to occur through the βγ subunits of activated heterotrimeric G proteins and indirectly (Schafer *et al*., 2004) through transactivation of RTK.

The intensity of PI3K activation is negatively regulated by a number of proteins. The main negative regulator is the phosphatase PTEN. As aforementioned, PTEN dephosphorylates phosphoinositides in position 3′, being therefore in charge of the termination of the signal transmitted by PI3K. The levels of PTEN are controlled at the transcriptional level by PI3K. Through the regulation of the transcription factor NF-κB (Vasudevan *et al*., 2004), PPARβ/δ agonists (Han *et al*., 2008) and TNFα (Kim *et al*., 2004) repress the expression of PTEN. This negative regulation is of importance in lung cancer cells, where downregulation of PTEN by PPARs promotes cancer cell proliferation. On the other hand, ubiquitinylation-dependent proteasomal degradation of PTEN reduces its levels (Torres and Pulido, 2001; Wu *et al*., 2003; Wang *et al*., 2007). To date, NEDD4-1 is the first and only identified E3 ligase for PTEN (Wang *et al*., 2007). Interestingly, NEDD4-1 transcription is positively regulated by PI3K pathway (Ahn *et al*., 2008), therefore representing a positive feedback for PTEN degradation and PI3K activation. Although the biological conditions where NEDD4-1 would ubiquitinylate PTEN are unclear, the existence of this or other E3 ligases targeting PTEN would represent potential targets for cancer therapy in tumors where PTEN is downregulated.

The activity of PTEN is also controlled by the PI3K pathway. The loss of the regulatory subunit of PI3K1A, p85, results in the decrease of Pten activity in p85 liver conditional knockout mice (Barber *et al*., 2006). Similarly, δ-isoform of PI3K has been shown to regulate PTEN activity through a RhoA-ROCK-dependent signaling, therefore impacting on chemotaxis and proliferation of macrophages (Papakonstanti *et al*., 2007). Lastly, PTEN nuclear–cytoplasmic shuttling is of great importance for the proper tumor-suppressive activity of the phosphatase (Denning *et al*., 2007; Trotman *et al*., 2007). NEDD4-1-mediated PTEN monoubiquitinylation has been defined as the key for

PTEN to shuttle to the nucleus (Trotman *et al*., 2007). Similarly, activated PI3K promotes PTEN nuclear exclusion through a chromosome region maintenance 1/S6K-dependent mechanism (Liu *et al*., 2007). The effect of aberrant PTEN localization in PI3K signaling is not yet completely understood. Although forced nuclear PTEN expression can reduce AKT phosphorylation (Trotman *et al*., 2007), it has also been proposed that PTEN is less efficient in hydrolysing nuclear PIP3 (Lindsay *et al*., 2006), and could have other functions that do not depend on dephosphorylating phosphoinositides (Salmena *et al*., 2008).

Downstream PI3K, the activation of AKT is also negatively regulated by different proteins. On the one hand, protein phosphatase (PP) 2A dephosphorylates primarily Thr308, whereas the phosphatase PHLPP dephosphorylates Ser473 (Bayascas and Alessi, 2005; Gao *et al*., 2005). On the other hand, a pseudokinase, namely Tribbles homolog 3, binds to and inhibits AKT, which leads to insulin resistance (Du *et al*., 2003).

Recently, a well-characterized tumor suppressor, the promyelocytic leukemia (PML) protein was uncovered as a key regulator of the PI3K at multiple levels. In one study, it was found that Pml regulates PP2A activity toward nuclear AKT residing in the nuclear bodies, which may account for the acceleration observed in tumors *Pten* heterozygous/*Pml*-defective mice (Trotman *et al*., 2006). In an independent study, Pml loss was also shown to promote neo-angiogenesis in femoral arterial ligation experiments. The mechanism of this phenomenon depends on the sequestration of mTOR to the nuclear bodies in hypoxic conditions, which accounts for the reduction in hypoxia-inducible factor 1α translation and ribosomal protein S6 phosphorylation (Bernardi *et al*., 2006).

Feedback regulators of PI3K signaling

In the past years, several reports have described that PI3K is tightly controlled by downstream components of the pathway, thus providing feedback regulation in response to extracellular signals (Figure 1).

The most prominent negative feedback regulating PI3K pathway is the one triggered downstream of mTORC1. The first observation of the ability of PI3K downstream components to regulate PI3K activation arose from a series of studies showing that chronic insulin stimulation led to the phosphorylation and proteasomal degradation of the adaptor protein IRS-1 (Haruta *et al*., 2000; Tremblay and Marette, 2001; Berg *et al*., 2002; Hartley and Cooper, 2002; Zhande *et al*., 2002; Greene *et al*., 2003; Pirola *et al*., 2003). Since then, a number of groups have shown that insulin-mediated PI3K activation can lead to the downregulation of IRS-1 activation through transcriptional regulation and post-translational modifications. Further studies showed that loss of TSC complex resulted in impaired activation of AKT upon insulin (Harrington *et al*., 2004; Shah *et al*., 2004). The analysis of the gene expression profile in *Tsc2*-expressing versus -defective cells unraveled that *Tsc1/2* loss results in a strong repression of IRS-1 transcription (Harrington *et al*., 2004). The mechanism of this transcriptional repression depends on the activation of mTORC1 signaling, as rapamycin treatment and silencing of S6K genes restores IRS-1 levels. Moreover, as mentioned above, *TSC1* or *TSC2* loss in human patients results mostly in benign tumors. Our group, along with the Cantley lab, recently demonstrated that *Tsc2* heterozygous mice develop malignancies in which this feedback inhibition is overactive (Ma *et al*., 2005b; Manning *et al*., 2005). Indeed, PI3K activation induced by heterozygous *Pten* deletion is able to accelerate the progression of *Tsc2* loss-derived malignancies (Manning *et al*., 2005). This suggests that one of the mechanisms by which *Tsc* loss would not result in more aggressive tumors is based on the strong brake provided by the negative feedback signaling at the very top of PI3K pathway, to the extent that forced PI3K activation induced by heterozygous *Pten* deletion is able to accelerate the progression of *Tsc2* loss-derived malignancies (Manning *et al*., 2005). Similarly, in the fly, Radimerski *et al*. (2002) showed that dS6K reduction could restore PI3K activation status and rescue larval lethality induced by upon *Tsc1/2* loss.

Loss of function of the TSC complex has also been shown to impact RTK levels. Studies from the group of Dr Kwiatkowski demonstrated that mTORC1 hyperactivation by either *Tsc2* or *Pten* loss, as well as by PI3K activation, leads to the repression of PDGFRA and B transcription, which impacts not only in PDGF signaling to AKT but also on the proper transmission of the signal from other growth factor receptors (Zhang *et al*., 2003a, 2007). Overall, these data provide support for the concept that this negative regulation of PI3K by downstream components may be of great importance in cell physiology, as well as in pathological conditions (Manning, 2004).

Although IRS-1 transcriptional repression might be a critical component of the negative feedback upon chronic PI3K activation, Harrington *et al*. (2004) showed in the same study that a downstream target of mTORC1, S6K1 was able to phosphorylate and inhibit IRS-1 in Ser302. This phosphorylation of IRS-1 is thought to inhibit its activity, preventing its recruitment and binding to RTKs. Another study has recently suggested that in conditions of obesity, as in db/db mice (in which a mutation in the cytoplasmic domain of the long form of the leptin receptor, Ob-Rb, results in loss of expression of this isoform and therefore alters STAT3 signaling in satiety response; Chen *et al*., 1996; Ghilardi *et al*., 1996), S6K can phosphorylate IRS-1 on a different residue, Ser1101, thus reducing its function and leading to insulin resistance *in vivo* in skeletal muscle (Tremblay *et al*., 2007). mTOR kinase itself might be able to phosphorylate this adaptor protein on Ser636/639 in certain circumstances (Ozes *et al*., 2001; Tzatsos and Kandror, 2006), and other key regulators of this pathway can similarly modulate this adaptor protein (Jakobsen *et al*., 2001). Therefore, both transcriptional repression and inhibitory phosphoryla-

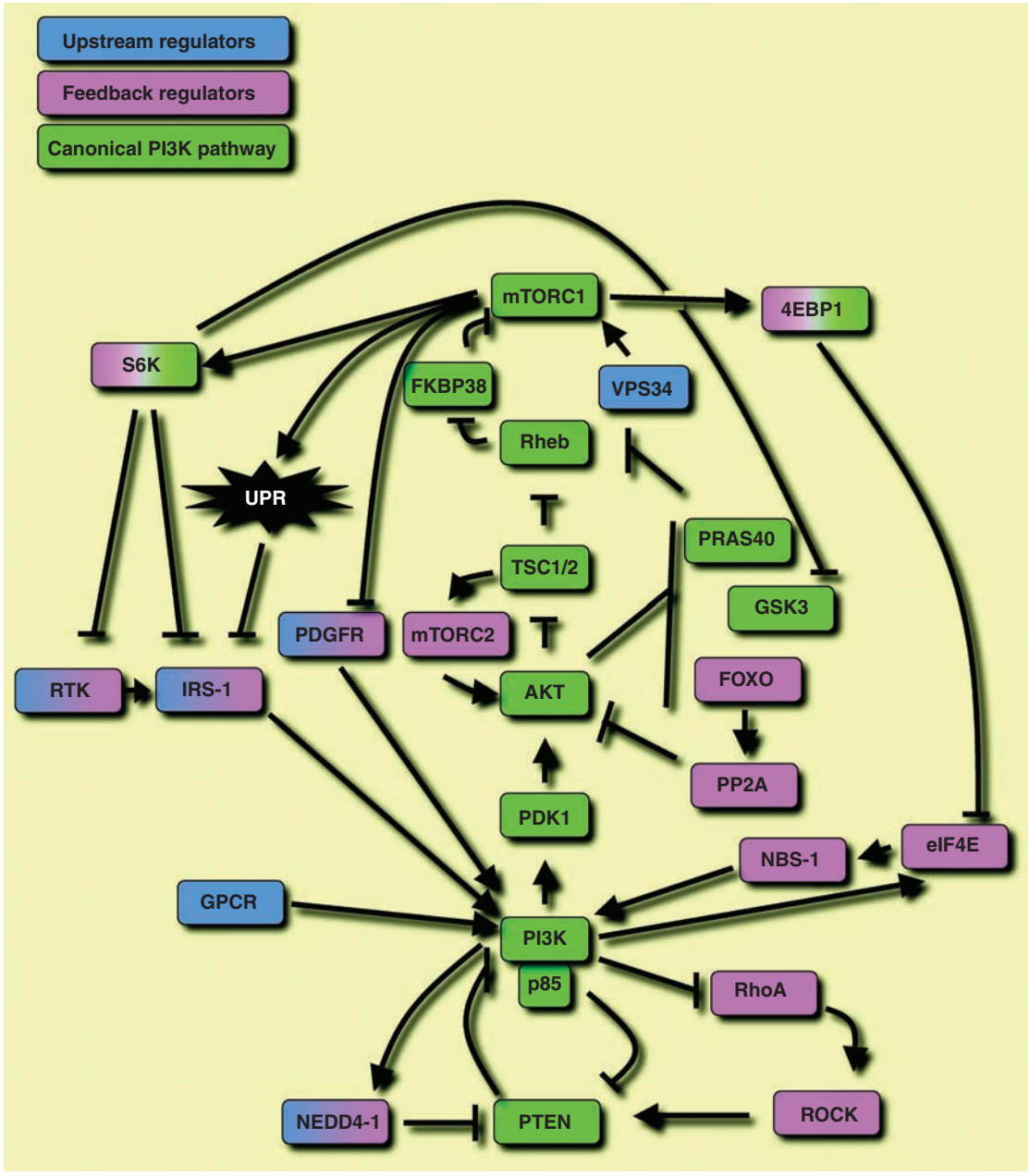

Figure 1 Feedback regulation of the PTEN–PI3K pathway. The canonical PTEN–PI3K pathway (green) is regulated at multiple levels. First, membrane receptor (RTK and GPCR) and its adaptors activate PI3K isoforms leading to the transmission of the signal to the interior of the cell (blue). PI3K signal intensity is controlled by a number of proteins, including, but not limited to PTEN and TSC1/2. Moreover, PI3K activation activates several feedback loops (rose), some of them promote further pathway activation (TSC1/2–mTORC2, NEDD4-1–PTEN, PI3K–RhoA–ROCK–PTEN) and some of them exert a negative effect on it (multiple signals from S6K, eIF4E—NBS-1–PI3K, FOXO–PP2A–AKT). GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; NBS-1, Nijmegen breakage syndrome 1; UPR, unfolded protein response.

tion of IRS-1 by downstream components account for the feedback inhibition of PI3K.

The concept that mTORC1 hyperactivation would result in the inhibition of PI3K also raised the question of whether mTORC1 inhibitors would have the undesirable effect of activating PI3K. Although previous studies had shown that the consequence of functional loss of the TSC complex on PI3K was prevented by rapamycin (Harrington *et al*., 2004), it was not until 2006 that the group of Dr Rosen demonstrated that rapamycin treatment induced AKT activation in cancer patients, a strong drawback for the use of this family of inhibitors as single agent in the clinic (O'Reilly *et al*., 2006). In line with this idea, many groups have subsequently proved in different types of human cancers that mTORC1 inhibition can lead to PI3K activation

and therefore proposed patient stratification for the utilization of mTORC1 inhibitors and dose adjustment to improve the clinical outcome (Baselga *et al*., 2008; Cloughesy *et al*., 2008).

Although the feedback signaling to AKT activation is widely accepted and provides an explanation to the unexpected poor results with rapamycin in the treatment of certain cancers, the effect of mTORC1 inhibitors on other pathways has not been yet achieved. To answer this question, our group recently investigated the effect of mTORC1 inhibition on a pro-survival pathway other than PI3K in patients subjected to RAD001 treatment. We surprisingly found that RAD001 treatment induces a profound activation of the ERK–MAPK pathway. Furthermore, we find that this activation relies on a negative feedback involving S6K—IRS-1–PI3K–RAS and that the combination of MEK and mTORC1 inhibitors already being tested in clinical trials provides a more efficient antitumoral effect *in vitro* as well as *in vivo* (Carracedo *et al*., 2008). This study therefore provides the basis for further patient stratification and the rationale for the combination of this class of inhibitors in the treatment of cancer.

Although the S6K-IRS-1 negative feedback might be the most prominent mechanism leading to PI3K activation, other negative feedbacks have also been proposed. mTORC1 inhibition in rhabdomyosarcoma cells leads to AKT activation through a S6K–IRS-1-independent signal that relies on IGF1R activation (Wan *et al*., 2007). Along the same line, acute myeloid leukemia cells upregulate IGF1 and IRS-2, but not IRS-1, upon RAD001 treatment (Tamburini *et al*., 2008). Another study proposes that eIF4E activation leads to AKT activation through a Nijmegen breakage syndrome 1-PI3K-dependent mechanism (Culjkovic *et al*., 2008) and conversely, PI3K has been proposed to signal directly to eIF4E upon rapamycin treatment (Sun *et al*., 2005). Downstream PI3K, members of the FOXO family can inhibit PP2A phosphatase, thereby increasing AKT phosphorylation (Ni *et al*., 2007), and activated S6K can overcome the effect of AKT inactivation through direct phosphorylation of GSK3 (Zhang *et al*., 2006a). In the context of *Tsc1/2* loss, Ozcan *et al*. (2008) recently showed that mTORC1 hyperactivation leads to aberrant protein synthesis rate and activation of the unfolded protein response. Importantly, the activation of this pathway impacts in IRS-1 phosphorylation status, therefore reducing the ability of extracellular signals to activate PI3K. Finally, a recent study by the Manning group showed that the TSC complex, binds to and regulates mTORC2 activity, therefore promoting an mTORC1 ON/mTORC2 OFF versus an mTORC1 OFF/mTORC2 ON status (Huang *et al*., 2008).

Overall, the mechanisms developed by the cell to sense the intensity of PI3K activation by extracellular signals are complex and involve different pathways, and may vary from tissue to tissue. Whereas in physiological conditions the proper functioning of this feedback might be of importance for the metabolic processes, re-activation of this brake in conditions of inhibition of mTORC1 could represent a very attractive therapeutic target to oppose tumor progression (Lopiccolo *et al*., 2007).

Cross-talk of PI3K and other signaling pathways

Regulation of other signaling cascades by PI3K pathway
The PI3K pathway is not limited to the regulation of the canonical AKT-mTORC1 cascade. Indeed, PI3K can modulate the activation status of other pathways (Figure 2). PI3K pathway impacts on Ras–MAPK at multiple levels. Ras activation by PI3K has been well characterized in (lysophosphatidic acid) LPA-induced MAPK activation, where PI3K pharmacological inhibitors blunt the activation of ERK kinase. This activation partially relies on Gβγ subunits and Shc-Grb2-Sos activation as well as in PIP3-mediated PKCζ activation (Yart *et al*., 2002). Nevertheless, the specific requirement of PI3K for Ras activation might be tissue-specific and involve additional signaling molecules (Yart *et al*., 2002). Similarly, somatostatin receptor (Lahlou *et al*., 2003), as well as insulin and low doses of EGF (Wennstrom and Downward, 1999) can drive Ras activation through PI3K, which may be due to the ability of PIP_3 to recruit GAP/Shp2 in some circumstances (Yart *et al*., 2001; Sampaio *et al*., 2008). On the other hand, some PI3K inhibitors such as the cytokine β-galactosidase-binding protein (βGBP) inhibit MAPK (Wells *et al*., 2007). The cellular context in which PI3K signals to Ras is not yet elucidated, and further research on the role of PI3K-Ras binding, PI species production and involvement of the different PI3K isoforms is required for the proper delineation of this signaling cross-talk. Similarly, the biological consequences of Ras activation by PI3K need to be investigated as they may selectively impact in some cellular responses but not others.

PI3K pathway can directly modulate the Ras target Raf therefore bypassing the GTPase. AKT phosphorylates c-Raf in Ser259 (Zimmermann and Moelling, 1999) and B-Raf (Guan *et al*., 2000). This inhibitory phosphorylation on Raf reduces the activity of MAPK pathway (Zimmermann and Moelling, 1999). This action of AKT on MAPK pathway is crucial for proper regulation of differentiation in some tissues and for cell survival (Rommel *et al*., 1999; Reusch *et al*., 2001; Mabuchi *et al*., 2002; Lee *et al*., 2008) and may as well impact on the response to growth factors (Guan *et al*., 2000). Another protein kinase with similar catalytic domain to AKT, SGK has also been suggested to phosphorylate Raf in an AKT site (Zhang *et al*., 2001). In which circumstances AKT inhibits Raf is still unclear; and the strength of PI3K activation may regulate this cross-talk, as AKT phosphorylation of Raf is more prominent at higher PI3K activity (Moelling *et al*., 2002).

The small GTPase Rheb also regulates Raf (Yee and Worley, 1997; Im *et al*., 2002; Karbowniczek *et al*., 2004, 2006), and so do PDK1 and GSK3 on MEK and ERK kinases, respectively (Sato *et al*., 2004; Wang *et al*., 2006). Finally, PTEN inhibits MAPK through the negative regulation of integrin signaling (Gu *et al*., 1998).

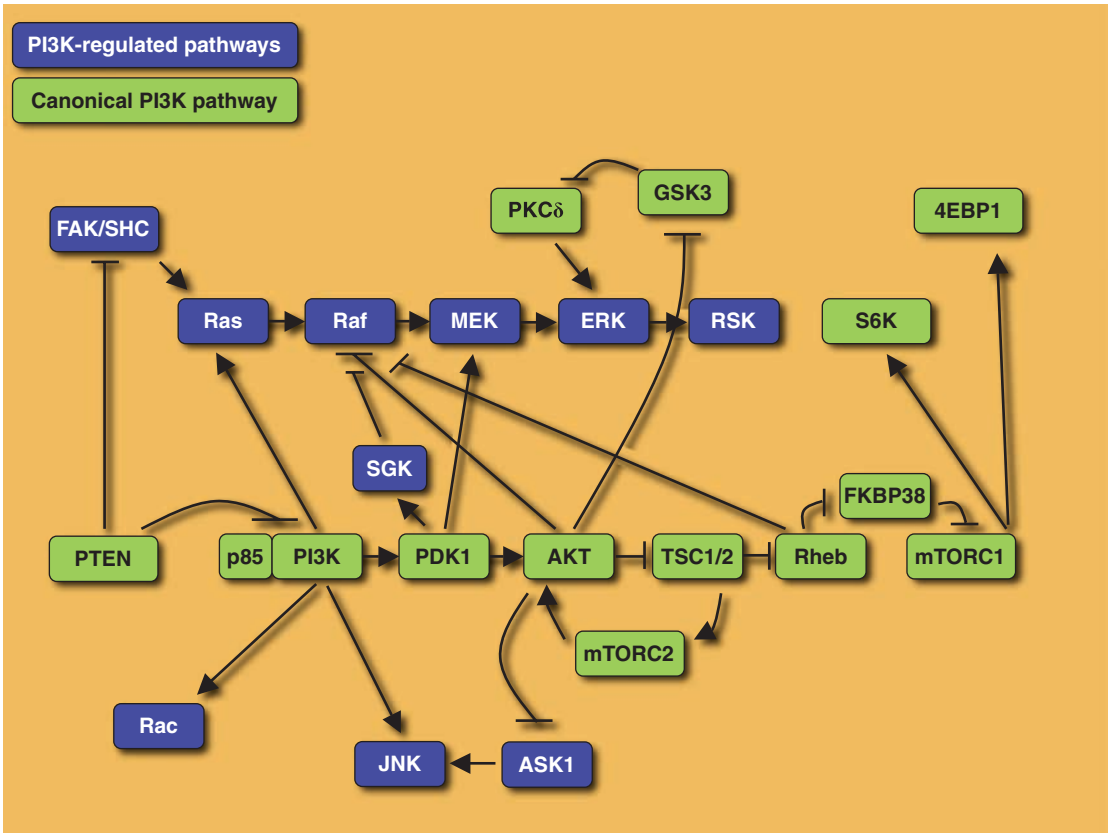

Figure 2 Pathways regulated by PI3K. The cascades modulated upon PI3K activation go beyond the canonical PI3K pathway (green). Several members of this pathway control the activation status of the Ras-MAPK pathway (PTEN, PI3K, PDK1, AKT, Rheb). On the other hand, PI3K and AKT also regulate other pathways (ASK1–JNK, Rac). The pathways under the control of PI3K pathway are shown in blue. PI3K, phosphoinositide 3-kinase; Rheb, Ras-homolog enriched in brain.

Importantly, PI3K regulates the activity of Rac G monomeric proteins through the modulation of Rac-GEFs by PIP3 (Welch *et al*., 2003). This cross-talk is of importance for the regulation of actin cytoskeleton and other functions such as cell cycle and transcription. Conversely, several studies point out to the regulation of p85 by Rac *in vitro*, which might be of importance for neutrophil chemotaxis (Welch *et al*., 2003).

PI3K pathway can also regulate JNK pathway. AKT phosphorylates and inhibits apoptosis signal-regulating kinase 1 (ASK1) at Ser83, thereby promoting cell survival (Yuan *et al*., 2003; Zhang *et al*., 2005; Wu *et al*., 2006). On the other hand, the group of Sawyers recently identified JNK pathway activation as a major consequence of PTEN loss in an elegant screening, suggesting that PI3K induces a parallel activation of AKT and JNK to promote cancer progression (Vivanco *et al*., 2007).

Overall, the multiple cross-talks arising from PI3K pathway toward other signaling cascades suggest that many of the PI3K-regulated functions actually go beyond PI3K canonical signaling.

Signaling pathways regulating PI3K pathway

PI3K pathway receives regulatory signals from other pathways at the transcriptional and post-translational level (Figure 3). PTEN transcription is regulated by RAS and JNK pathway in cancer cells leading to tumor progression (Beck and Carethers, 2007; Vasudevan *et al*., 2007; Xia *et al*., 2007). Overexpression of mutant Ras promotes a MAPK-dependent repression of *PTEN* transcription (Vasudevan *et al*., 2007). A MEK-dependent pathway increase c-Jun levels and increases the binding of this transcription factor to the promoter of *PTEN* (Hettinger *et al*., 2007; Vasudevan *et al*., 2007). The downregulation of PTEN by MAPK pathway promotes the escape from apoptosis of Ras-overexpressing cells. In another study, PTEN was found downregulated in a subset of lung cancer cell lines (Xia *et al*., 2007). This downregulation of PTEN correlated with the overexpression of the JNK regulator MKK4, which increased NF-κB activity and binding to *PTEN* promoter. Therefore, PTEN downregulation might be a mechanism of other pathways to escape apoptosis or promote proliferation. Indeed, in T-cell acute lymphoblastic leukemia, aberrant Notch pathway represses *PTEN* transcription through induction of HES-1 in turn providing a novel therapeutic approach for the treatment of this malignancy with the combined inhibition of PI3K and Notch pathways (Palomero *et al*., 2007).

With regards to PI3K regulation, class I and II PI3K isoforms have a RBD by which Ras facilitates PI3K

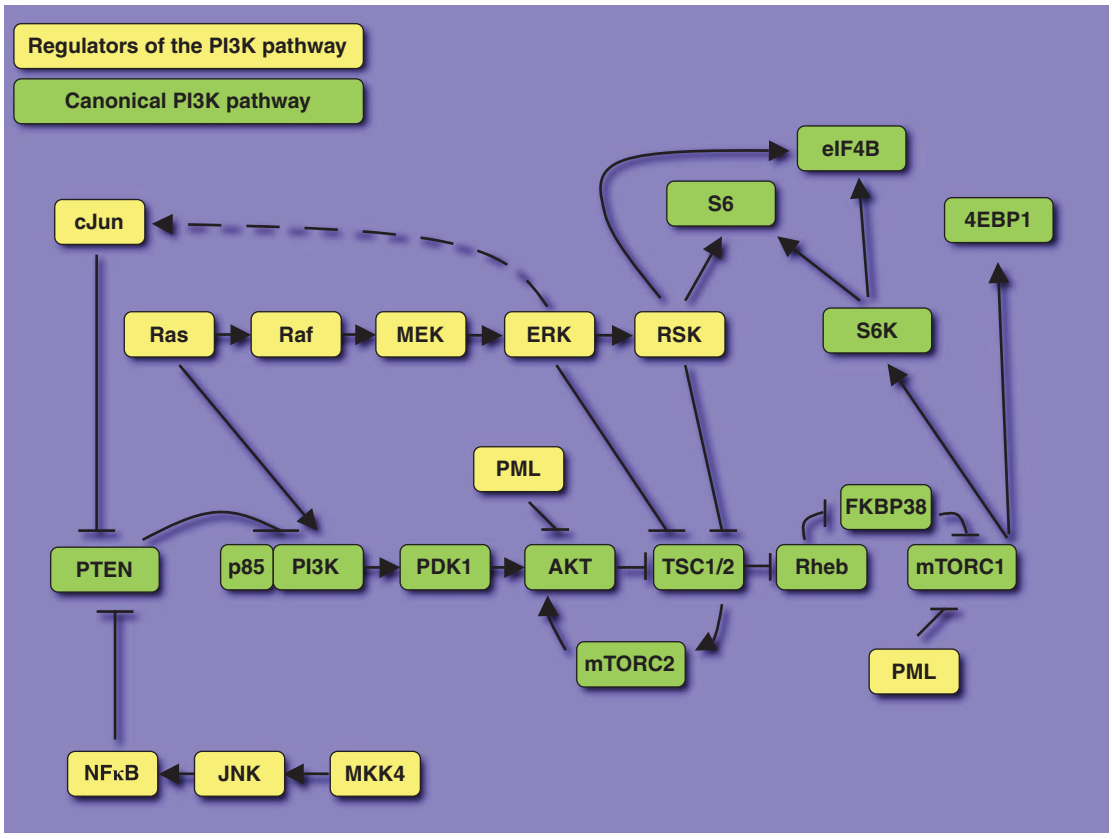

Figure 3 Pathways that regulate PI3K signaling. PI3K pathway (green) is under the control of other cascades (yellow). Ras-MAPK pathway modulates this pathway at multiple levels (MAPK–c-Jun–PTEN, Ras–PI3K, ERK–TSC2, RSK–TSC2, RSK–S6, RSK-eIF4B) and so does MKK4–JNK pathway through the activation of NF-κB. Lastly, PML inhibits PI3K signaling at the level of AKT and mTORC1. PI3K, phosphoinositide 3-kinase; PML, promyelocytic leukemia.

anchorage to the membrane and thus its full activation (Engelman *et al*., 2006). Ras binding to PI3K is required to transmit EGF and FGF2 extracellular signals, which accounts for a primary role of Ras in the regulation of PI3K signal transduction (Ramjaun and Downward, 2007). Moreover, the oncogenic ability of Ras is linked to its ability to activate this pathway (Rodriguez-Viciana *et al*., 1997; Gupta *et al*., 2007). The expression of a mutant dominant-negative form of p85 (Rodriguez-Viciana *et al*., 1997) or the mutation of PI3K1A in the RBD (Gupta *et al*., 2007) reduces Ras-mediated transformation and the aggressiveness of lung tumors in Ras mutant transgenic mice. The use of these mutant mice provided as well interesting data about the tissues that might be more sensitive to the Ras–PI3K cross-talk. As an example, the observed abnormalities of PI3K mutant mice in the development of the lymphatic system suggest that PI3K requires to bind Ras to regulate this function, at least partially through the proper activation of VEGFR3, TIE2 and FGF2 receptors (Ramjaun and Downward, 2007).

The interaction between Ras and PI3K is also critical for correct cell polarity and migration. Studies carried out in *Dictyostelium* and hippocampal neurons show that the proper cross-talk of these two pathways is essential for axon formation and cell polarity and migration (Funamoto *et al*., 2002; Sasaki *et al*., 2004, 2007; Fivaz *et al*., 2008). Moreover, in *Dictyostelium*, cytoskeleton components such as F-actin filaments play an important role in the signaling between PI3K and Ras, promoting their correct localization and interaction in the plasma membrane (Sasaki *et al*., 2007). Therefore, the PI3K, at the level of PI3K and PTEN, plays a critical role in cell polarity, chemotaxis and migration, which requires a tightly regulated cross-talk with Ras pathway.

TSC complex is also regulated by MAPK at two different levels. On the one hand, p90RSK1 phosphorylates and inhibits TSC2 at Ser1798, thereby promoting mTORC1 activation (Roux *et al*., 2004). On the other hand, a study from our group has recently shown that ERK can also directly phosphorylate TSC2 on Ser664, leading to the inhibition of the complex (Ma *et al*., 2005a). Thus, the regulation of mTORC1 signaling by MAPK might be an important component of tumors with aberrant Ras activation to progress. The study of this cross-talk in cancer specimens reveals that tumors with hyperactive ERK often show TSC2 phosphorylation on Ser664 and concomitant activation of mTORC1, whereas in a second group of patients, mTORC1 is found overactive even in the absence of TSC2 phosphorylation on this site (Ma *et al*., 2007).

Lastly, PI3K and MAPK signals also converge downstream of mTORC1. p90RSK phosphorylates the ribosomal protein S6 at Ser235/236 therefore promoting its activation upon Ras–MAPK upregulation (Roux *et al*., 2007). Additionally, S6K and RSK phosphorylate eIF4B at Ser422, thus enhancing its interaction with the eIF3 and promoting protein translation (Shahbazian *et al*., 2006).

Therefore, a more detailed understanding of MAPK and PI3K cross-talk in cancer, and the subsequent stratification of patients depending on the input source of mTORC1 activation will allow individualized therapy geared to singly or combinatorially target specific pathways, thus enhancing the anticancer potency of these treatment modalities, as we discuss in the following paragraph.

Clinical implications

Targeting PTEN/PI3K pathway represents a major aim for the treatment of a number of diseases. Indeed, several compounds are now being designed and tested in preclinical trials for the treatment of cancer with hyperactive PI3K (Luo *et al*., 2003), such as the PI3K SF1126 from Semaphore and XL147 from Exelixis. Although the first generation of PI3K inhibitors derived from wortmannin and LY294002 displayed high toxicity, second generation wortmannin derivatives may prove much more effective (Harvey and Lonial, 2007; Garlich *et al*., 2008; Yuan and Cantley, 2008).

Tremendous effort has also been invested at targeting molecules operating downstream of the PTEN/PI3K pathway. mTORC1 has been probably the most studied target of the PI3K pathway, perhaps due to the original identification of rapamycin (Guertin and Sabatini, 2007). But, the efficiency of this family of compounds in clinical trials has been less than satisfying. mTORC1 inhibitors failed to inhibit tumor progression in a variety of cancers, with the exception of kidney cancer, TSC-associated symptoms (Faivre *et al*., 2006) and brain tumors (Cloughesy *et al*., 2008). With the discovery of mTORC1 negative feedbacks, it became evident that the use of mTORC1 inhibitors as single agents might be limited, whereas the combinatorial use of inhibitors of other pathways would be required to target a particular feedback in stratified patients (Figure 4).

In principle, drug combinations for the inhibition of main survival pathways (mTORC1, PI3K and MAPK) might result in high toxicity due to the targeting of critical functions in non-transformed cells. However, tumor cells appear to be surprisingly more sensitive to these treatments. These data have been rationalized putting forward the hypothesis that tumor cells are addicted to oncogenic events (Weinstein and Joe, 2008). The specific genetic hit driving transformation (that is, *PTEN* loss) in a cell would promote the re-adjustment of the intracellular wiring (MAPK, translation, response to growth factors) to the aberrant activation of the pathway (PI3K), therefore resulting in a higher dependency on this cascade for cell homeostasis. The use of pharmacological inhibitors would in turn be more effective in the cancer cell as this perturbation would result in a further disequilibrium to the overall cell signaling network. This hypothesis provides support for the use of drug combinations that would inhibit a target pathway preventing feedback or cross-talks.

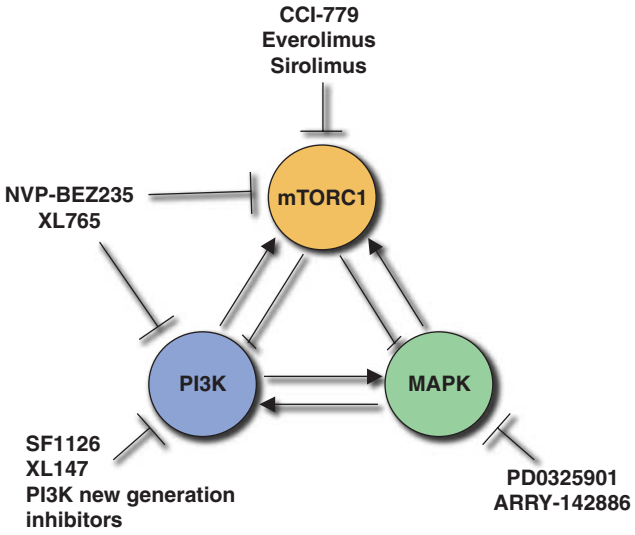

Figure 4 Therapeutic manipulation of PTEN/PI3K feedbacks and cross-talks in the treatment of human disease. PI3K, mTORC1 and MAPK conform a signaling triangle composed of feedbacks and cross-talks. Therefore, the use of selective inhibitors for a given pathway may have dramatic consequences on the other members of this triangle. This relationship highlights the requirement of patient stratification and the use of *dirty drugs* or drug combination for the increase in effectiveness in the treatment of a particular disease. PI3K, phosphoinositide 3-kinase.

The concept of signaling cross-talks and drug combinations has promoted the design of the so-called '*dirty drugs*,' which can target multiple molecules or multiple kinases at once. To this end, the design of compound mTOR kinase-PI3K inhibitors would be extremely useful in targeting in full both upstream and downstream of the PI3K pathway in the absence of undesirable feedback regulations, as modeled by recently developed compounds by Novartis (NVP-BEZ235) and Exelixis (XL765) (Garcia-Echeverria and Sellers, 2008). In this respect, it has been proposed that the activity of rapamycin on mTOR may go beyond mTORC1 at higher dose, where AKT activation through the disassembly of mTORC2 would be prevented as well (Guertin and Sabatini, 2007). Although this possibility needs to be further explored, it is tempting to speculate that mTOR kinase inhibitors could provide a double edge sword toward inhibition of the PTEN/PI3K pathway from top (mTORC2) to bottom (mTORC1).

Concluding remarks

Since the initial discovery of the PI3K pathway, multiple new components have entered this network,

thus increasing its complexity. Although represented as a linear/up-to-down cascade, in the past few years the numerous feedback regulations have put the pathway literally upside down. From PTEN to mTOR, each member of this network is either feedback-regulated or cross-talks to other signaling cascades. Therefore, the characterization of the context and genetic background where a particular signaling circuit is operational is essential both for the understanding of cell physiology and also for the appropriate design of therapeutic intervention in pathological conditions.

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Competing financial interests statement

The authors declare no competing financial interests.

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