Chapter 5
PI3Ks—Drug Targets in Inflammation and Cancer

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Abstract Phosphoinositide 3-kinases (PI3Ks) control cell growth, proliferation, cell survival, metabolic activity, vesicular trafficking, degranulation, and migration. Through these processes, PI3Ks modulate vital physiology. When over-activated in disease, PI3K promotes tumor growth, angiogenesis, metastasis or excessive immune cell activation in inflammation, allergy and autoimmunity. This chapter will introduce molecular activation and signaling of PI3Ks, and connections to target of rapamycin (TOR) and PI3K-related protein kinases (PIKKs). The focus will be on class I PI3Ks, and extend into current developments to exploit mechanistic knowledge for therapy.

Keywords Cancer inflammation allergy metabolism phosphatidylinositol phosphoinositide phosphoinositide 3-kinase · PI3K target of rapamycin · TOR · mTOR protein kinase B · Akt · PKB 3-phosphoinositide phosphatase and tensin homolog deleted in chromosome ten · Also PTEN wortmannin LY294002 rapamycin pharmacology signal transduction

5.1 PI3Ks—Molecular Mechanisms

5.1.1 Introduction to PI3Ks

The deregulation of phosphoinositide 3-kinase (PI3K) pathways interferes with cellular hemostasis and contributes to the over-activation of many cell types. In this respect, PI3Ks have been shown to play a central role in the control of cellular metabolism, growth, proliferation, survival and migration, intracellular membrane transport, secretion and more.

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T. Balla et al. (eds.), *Phosphoinositides I: Enzymes of Synthesis and Degradation*,
Subcellular Biochemistry 58, DOI 10.1007/978-94-007-3012-0_5,

Although cancer and inflammatory disease include a wide variety of disorders with a broad degree of severity and clinical outcome, both emerge from preexisting, but derailed physiologic repair and defense mechanisms. To enter a tissue repair or host defense mode, cells have to switch from a quiescent to an activated state. Cell surface receptor-controlled PI3Ks produce PtdIns(3,4,5)P_3 at the inner leaflet of the plasma membrane, where the lipid provides a docking site for signaling molecules with lipid receptor domains (Balla and Varnai 2002; Lemmon 2008). The prototype for these is the Ser/Thr protein kinase B (PKB, also called Akt)(Bellacosa et al. 1991), which indirectly relays the activation of PI3K to the target of rapamycin (TOR or mammalian TOR, mTOR), which in turn regulates protein synthesis and growth. Via the activation of guanine nucleotide exchange factors (GEFs), PtdIns(3,4,5)P_3 levels modulate Rho GTPase activities and thus cytoskeletal rearrangements, cell polarity and migration. The importance of PtdIns(3,4,5)P_3 levels in these processes has been validated by the loss of one of the counter-players of PI3Ks: when the expression of the lipid 3′-phosphatase PTEN (Phosphatase and Tensin homolog deleted on chromosome Ten) is lowered, PtdIns(3,4,5)P_3 rises, and develops its oncogenic potential (Stambolic et al. 1998; Di Cristofano et al. 1998; Leslie et al. 2008; Zhang and Yu 2010). In fact, most cases of Cowden Syndrome are due mutations in PTEN (Eng 1998), and result in the formation of hyperplasia and adenoma formation in various tissues, which constitute early forms of cancer (Liaw et al. 1997; Sansal and Sellers 2004; Hobert and Eng 2009; Farooq et al. 2010). But also the genetic attenuation of lipid 3′-phosphatases degrading PtdIns(3,5)P_2 or PtdIns-3-*P* gives rise to progressive disease, as mutations in the gene coding for myotubularin 1 (MTM1) cause X-linked myotubular myopathy (XLMTM), and loss of phosphatase activity in myotubularin-related protein 2 (Mtmr2) correlates with Charcot-Marie-Tooth disease type 4B1 (CMT4B1, Berger et al. 2002; Cao et al. 2008). This demonstrates that a delicate balance between lipid kinase and lipid phosphatase activities controls the flux through the phosphoinositide pathway, and that phosphoinositide levels play an important part in cellular homeostasis (Fig. 5.1).

Initially, PI3Ks were discovered as lipid kinases associated with viral oncogens (Whitman et al. 1985; Sugimoto et al. 1984; Macara et al. 1984), and for the last two decades the link between cancer and elevated PtdIns(3,4,5)P_3 levels has been corroborated (Vivanco and Sawyers 2002; Wymann and Marone 2005; Cully et al. 2006; Engelman et al. 2006; refer to Vol. 1, Chap. 4). While there is a clear-cut connection between elevated PtdIns(3,4,5)P_3 levels and progression of cancer, chronic inflammation, allergy, metabolic disease, diabetes and cardiovascular problems, it has been challenging to associate specific PI3K isoforms with defined disease states. The combination of genetic and pharmacological approaches has somewhat elucidated the integration of distinct PI3K isoforms in disease-associated signaling cascades during the past decade, which has helped to validate PI3Ks as drug targets in cancer and chronic inflammation.

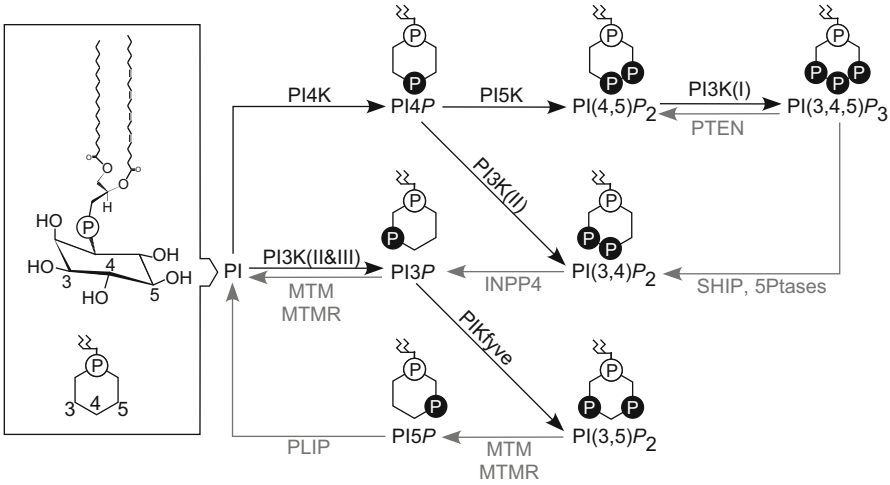

Fig. 5.1 Lipid kinases (indicated in *black*) and lipid phosphatases (*grey*) mediate the flow of phosphoinositides from phosphoinositol (PtdIns, PI) to PtdIns(3,4,5)P_3 [PI(3,4,5)P_3]. The main synthesis pathways relevant in physiology and disease are indicated: PI is phosphorylated by phosphatidylinositol 4-kinase (PI4K) to PI4P (Balla and Balla 2006; Graham and Burd 2011), which is turned over into PI(4,5)P_2 by PI5K (type I phosphatidylinositol phosphate kinase; PIPKI (Anderson et al. 1999; Ling et al. 2006)). PI(4,5)P_2 is then converted to PI(3,4,5)P_3 by class I PI3Ks [PI3K(I)]. The 3′-lipid phosphatase PTEN (Stambolic et al. 1998; Di Cristofano et al. 1998; Leslie et al. 2008; Zhang and Yu 2009) reverses the action of PI3K(I), while the 5′-lipid phosphatase SHIP (Krystal et al. 1999) produces PI(3,4)P_2. PI(3,4)P_2 could eventually also be produced by class II PI3Ks [PI3K(II)] from PI4P. PI(3,4)P_2 is degraded mostly by inositol polyphosphate 4-phosphatases, e.g. INPP4, to form PI3P. The latter is formed directly from PI by class III PI3K (PI3K(III)/Vps34) or class II PI3Ks from PI. PI3P can be converted to PI(3,5)P_2 by PIKfyve (yeast Fab1) as response to cellular stress (Dove and Johnson 2007; Dove et al. 2009). Myotubularins (MTM) and myotubularin-related (MTMR) constitute a family of lipid phosphatases that dephosphorylate PI3P and PI(3,5)P_2 (Laporte et al. 2001; Mruk and Cheng 2010). Simplified, PI(4,5)P_2 and PI(3,4,5)P_3 are localized in the plasma membrane, golgi and ER are rich in PI4P (Graham and Burd 2011); PI3P is a marker for early endosomes; and late endosomes and multi-vesicular bodies contain PI(3,5)P_2. Excellent reviews elucidation phosphoinositide localization and conversions further are (Di Paolo and De Camilli 2006; Sasaki et al. 2009; Bunney and Katan 2010). Other useful source: http://www.genome.jp > KEGG Pathway > map04070

5.1.2 The PI3K Family and PI3K-related Protein Kinases

The core PI3K family consists of three PI3K classes (Wymann and Pirola 1998; Vanhaesebroeck et al. 2001; Wymann et al. 2003b; Vanhaesebroeck et al. 2010), which have been defined according to structural characteristics and their *in vitro* substrate specificity (Fig. 5.2). The complete set of all PI3K family members has first been identified in the fruit fly *Drosophila melanogaster* (MacDougall et al. 1995; Leevers et al. 1996), and work in model organisms like *Caenorhabditiselegans* (Morris et al. 1996; Roggo et al. 2002), *Dictyosteliumdiscoideum* (refs see (Chen et al. 2007)) and yeast (Schu et al. 1993), has much contributed to the elucidation of the function of members of the PI3K family (Engelman et al. 2006).

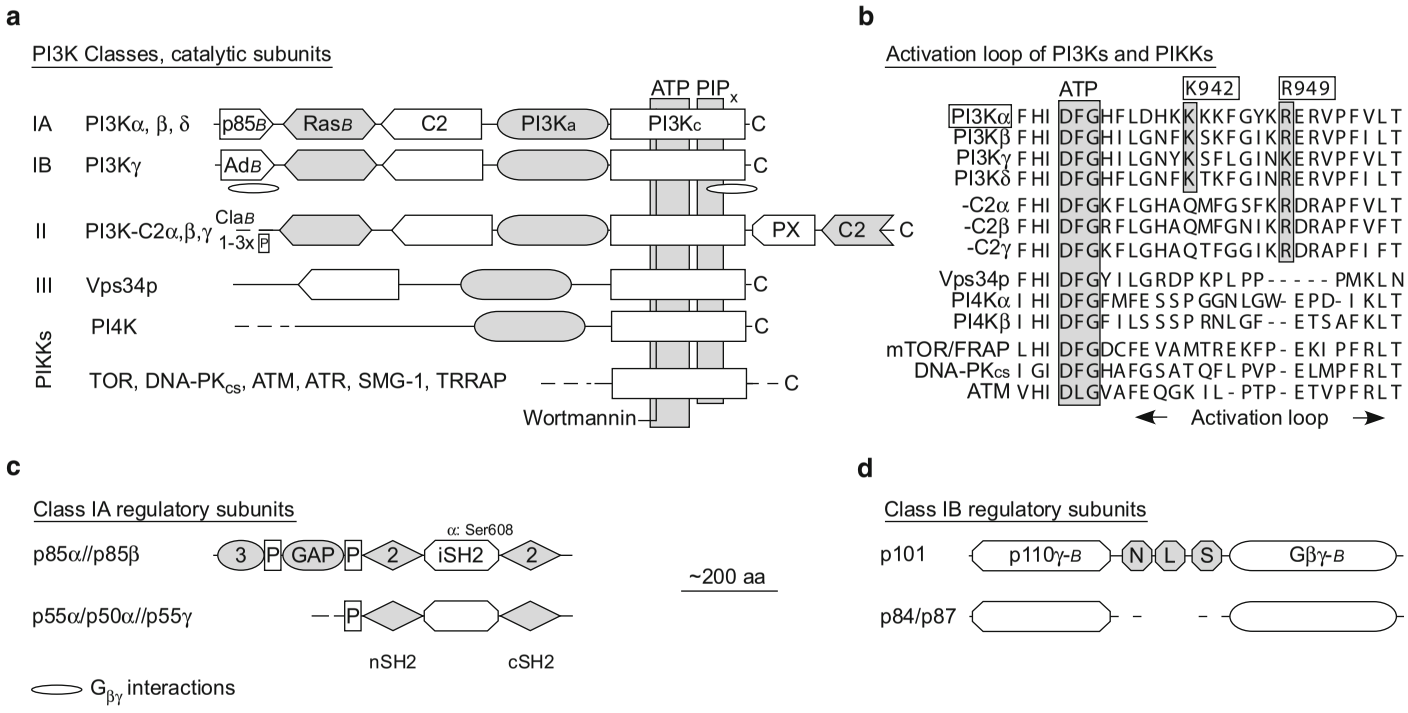

Fig. 5.2 Schematic representation of the domain structure of the phosphoinositide 3-kinase (PI3K) family, and PI3K-related protein kinases (PIKKs). **a** the catalytic subunits of class IA enzymes (PI3Kα, β, δ) are composed of a *N*-terminal p85-binding (p85*B*) region, followed by a Ras-binding (Ras*B*) domain, a C2 domain, a PI3K accessory (or helical, PI3K*a*) domain and a catalytic core (PI3K*c*) domain. The latter constitutes the ATP-binding site, and also accommodates the head group of the class I substrate PtdIns(4,5)P_2 (labeledPIP$_x$-binding site here). Binding of the PI3K inhibitor wortmannin occurs within the ATP-binding

Fig. 5.2 (Continued) site, and wortmannin forms a covalent link with a conserved lysine in the PI3K and PIKK enzymes' catalytic core pocket (see text). All PI3Ks and PIKKs share a core catalytic domain (PI3K*c*), which displays lipid or protein kinase activity, except for the TRRAP protein, where two mutations in conserved residues abrogate the catalytic activity completely. Class IB enzyme PI3Kγ (p110γ) does not bind p85-like regulatory subunits, but interacts instead with the p101 or p84(also called $p87^{PIKUP}$) adapter proteins and Gβγ subunits of trimeric G proteins. PI3Kγ was proposed to bind adaptor proteins mainly, but not exclusively, via its N-terminus (Ad*B*). Class II (PI3K-C2α, β, γ) enzymes lack an adapter-binding site, but were reported to interact with clathrin (Cla*B*) or SH3 domains via N-terminal proline-rich sequences (P, only present in PI3K-C2β). Class II enzymes are characterized by a C-terminal extension containing a phox (PX) and a C2 domain (for a concise review on class II enzymes see (Falasca and Maffucci 2007)). Class III PI3Ks and PI4Ks display PI3K*a* and PI3K*c* domains, and both are only capable to accept PtdIns as a substrate. The PI4K members shown here belong to the type III PI4K enzymes. The type II PI4K enzymes are structurally and biochemically distinct from the PI3K family, are insensitive to wortmannin, and show even little resemblance to other phosphotransferase enzymes (Minogue et al. 2001; Barylko et al. 2001; Balla and Balla 2006; refer to chapter on PI4K, Vol. 1, Chap. 1). Target of rapamycin (TOR or FRAP), the catalytic subunit of DNA-dependent protein kinase ($DNA\text{-}PK_{cs}$), the gene mutated in the human genetic disorder ataxia telangiectasia (ATM), and ATM and Rad3-related (ATR) contain a PI3Kc domain, and possess activities as serine/threonine protein kinases. Numerous protein/protein interaction domains present in the N-terminal part of PIKKs are not depicted here (for a reviews on PIKKs see (Zoncu et al. 2011; Shiloh 2003; Cimprich and Cortez 2008)). **b** The activation loop in PI3Ks and PIKKs is located C-terminally to the prominent, ATP-interacting DFG motif (*boxed*). Two positive charges (K942 and R949 in p110α) have to be positioned correctly to allow a productive accommodation of the PtdIns(4,5)P_2 head group (Bondeva et al. 1998; Pirola et al. 2001). Class II PI3K provide no charge compensation at the corresponding position to K942, which explains why they make only PtdIns(3,4)P_2*in vitro*. Class III PI3K and PI4K seem to have a more rigid activation loop by the presence of multiple prolines, and accept only PtdIns as a substrate. Class III PI3K interact with a regulatory kinase, yeast Vps15p or mammalian p150 not depicted here (Vanhaesebroeck et al. 2010; Funderburk et al. 2010; Stack et al. 1995). **c** Class IA PI3K catalytic subunits are tightly associated with the inter-SH2 (iSH2) domain of p85-like regulatory subunits. The long forms of these regulatory subunits present N-terminally an SH3 and a RhoGAP (GAP) domain flanked by proline-rich motifs (P). Although interactions of these domains with Shc, Cbl, dynamin, Grb2 and Src-like protein kinases have been demonstrated (for references see (Wymann and Pirola 1998)), their importance in physiologic processes is ill defined. **d** Class IB PI3K, PI3Kγ, binds adapter subunits p101 and p84 (also named $p87^{PIKUP}$), which C-terminally interact with Gβγ subunits. Regulatory functions of these adapters are localized N-terminally, but binding to the PI3Kγ catalytic subunits was reported to involve various regions in the adapter subunits (Bohnacker et al. 2009; Krugmann et al. 1999; Voigt et al. 2005). Abbreviations (alphabetical): 2/3 src-homology 2/3 domains, ATM ataxia telangiectasia mutated, ATR ataxia telangiectasia related, C2 protein kinase C homology domain 2, Cla*B* clathrin-binding sites, $DNA\text{-}PK_{cs}$ catalytic subunit of DNA-dependent protein kinase, Gβγ-*B* Gβγ-binding domains, iSH2 inter-SH2 domain, NLS nuclear localization signals, P proline-rich region, p85*B* p85-binding domain, PIK3Ka PI3K accessory domain, also called helical domain, PI3Kc PI3K catalytic domain, PX phox domain or phagocyte oxidase domain, GAP: RhoGAP domain, also referred to as (BH) BCR homology domain, SMG1 suppressor of morphogenesis in genitalia-1, TOR/mTOR mammalian target of rapamycin, TRRAP transformation/transcription domain-associated protein

Ligand-activated receptors located in the plasma membrane relay their signals to class I PI3Ks, which are the only PI3K members able to convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Although class I PI3K are capable to phosphorylate PtdIns to PtdIns-3-*P*, and PtdIns-4-*P* to PtdIns(3,4)P_2 *in vitro*, they have a preference for PtdIns(4,5)P_2 as a substrate *in vivo* (Stephens et al. 1993; Cantley 2002). All members of the class I PI3Ks are heterodimers, contain a catalytic subunit of 100–120 kD (referred to as p110 proteins and genes named *PIK3c*).

Although class II PI3Ks were reported to be direct downstream targets of growth factor receptors like the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) and stem cell factor receptor (SCFR, c-kit; Arcaro et al. 2000, 2002), their main connection to signaling at the plasma membrane is likely to be mediated via clathrin-mediated endocytosis (Domin et al. 2000; Gaidarov et al. 2001; Falasca and Maffucci 2007). Class II PIKs were proposed to produce PtdIns-3-*P* and PtdIns(3,4)P_2. Class III PI3Ks are represented by Vps34p (vacuolar protein sorting mutant 34; Schu et al. 1993), which generates PtdIns-3-*P* only. Vps34p is involved in vesicular transport to yeast vacuolar and mammalian early endosomal compartments, protein sorting (Simonsen et al. 2001), autophagy (Backer 2008; Simonsen and Tooze 2009; Funderburk et al. 2010) and has recently been proposed to promote cytokinesis (Sagona et al. 2010; Nezis et al. 2010).

The members of the PI3K family have close relatives, which are the type III phosphoinositide 4-kinases (PI4Ks; Minogue et al. 2001; Barylko et al. 2001; Balla and Balla 2006) refer to Vol. 1, Chap. 1 and protein kinases referred to as class IV PI3Ks or phosphoinositide 3-kinase-related kinases (PIKKs). These include the target of rapamycin (TOR; also dubbed FRAP or mTOR), DNA-dependent protein kinase (DNA-PK_{cs}), ATM (ataxia telangiectasia mutated), ataxia telangiectasia-related (ATR), suppressor of morphogenesis in genitalia-1 (SMG-1) and transformation/transcription domain-associated protein (TRRAP). DNA-PK_{cs}, ATM, ATR and SMG-1 take part in DNA-damage repair responses. DNA-PK_{cs} and ATM respond mainly to double strand break (DSB), while ATR and SMG-1 are activated due to ultraviolet-induced stress, DNA-damage, and DSB (Durocher and Jackson 2001; Hiom 2005). SMG-1 is involved also in mRNA surveillance mechanisms, genotoxic stress responses and non-sense-mediated mRNA decay (Yamashita et al. 2005; Oliveira et al. 2008). TOR, on the other hand, is regulated by growth factors receptors and the availability of nutrients. Two distinct TOR complexes coordinate protein and lipid synthesis, cell growth and proliferation (Wullschleger et al. 2006; Laplante and Sabatini 2009; Sancak et al. 2010; Kapahi et al. 2010).

5.1.3 The PI3K Catalytic Core, Enzymatic Activities

PI3Ks and PIKKs share a similar catalytic core (PI3Kc), where the ATP- and lipid and protein substrate-binding sites are localized (Fig. 5.2). Interestingly, early PI3K inhibitors like wortmannin (Arcaro and Wymann 1993; Yano et al. 1993; Wymann and Arcaro 1994) and LY294002 (Vlahos et al. 1994) inhibit multiple enzymes of

the PI3K and PIKK family at elevated concentrations (Wymann and Arcaro 1994; Marone et al. 2008). For class I PI3Ks wortmannin's IC_{50} is 1–5 nM, for class II PI3K the IC_{50} was reported to be isoform-dependent [PI3K-C2α: $IC_{50} \cong 500$ nM; PI3K-C2β: $IC_{50} \cong 2$–5 nM; for human Vps34p class III PI3K the IC_{50} is ca. 3 nM, while yeast Vps34p was reported to be rather resistant to wortmannin (Panaretou et al. 1997; Woscholski et al. 1994); and for type III PI4Ks $IC_{50} \cong 100$–300 nM (Falasca and Maffucci 2007; Balla and Balla 2006). PIKKs were inhibited at somewhat higher concentrations: IC_{50}s for wortmannin have been reported for SMG-1 around 60 nM, for DNA-PKcsfrom 20–120 nM, for mTOR and ATM from 100–200 nM, while ATR requires ca. 1.8 μM (IC_{50}) of wortmannin (Yamashita et al. 2005; Brunn et al. 1996; Izzard et al. 1999; Chan et al. 2000; Sarkaria et al. 1998).

Wortmannin binds covalently to a conserved lysine residue in the catalytic pocket of PI3Ks (Lys 802 in p110α (Wymann et al. 1996); Lys833 in p110γ (Stoyanova et al. 1997; Walker et al. 2000)) and PIKKs (e.g. Lys2187 in TOR (Brunn et al. 1996)). This active site Lys residue is conserved in protein and lipid kinases, and is critical for the transfer of the γ-phosphate group of ATP to kinase substrates. The ε-amino group of this Lys residue is especially nucleophilic and is capable to form a Schiff-base with the carbon-20 of the furan ring of wortmannin. This adduct is stable at a physiologic pH and can be detected by immunoblotting with anti-wortmannin antibodies (Balla and Balla 2006; Marone et al. 2008; Wymann et al. 1996). Data obtained using wortmannin as a PI3K inhibitor above 100 nM must therefore be interpreted with great caution, last but not least because it was reported that also polo-like protein kinases involved in the control of mitosis are inhibited by wortmannin in the nM range ($IC_{50} \cong 30$–50 nM; Liu et al. 2005). Although wortmannin and LY294002 remain useful tools to explore the importance of PI3K signaling in a cellular context, panels of more selective inhibitors are now available, and should be used complementary (see Table 5.1 and sections below).

A productive transfer of the γ-phosphate group of ATP to the D3-OH position of the phosphoinositide substrate depends also on a tight interaction of the phosphoinositide head group with the PI3K catalytic pocket. For the higher phosphorylated phosphoinositides this poses the problem that the negatively charged phosphate groups must be accommodated. In class I PI3Ks, the so-called activation loop contains two essential positively charged amino acid side chains (for p110α these are Lys942 and Arg949), which interact with the 4- and the 5-phosphate groups of PtdIns(4,5)P_2. If these charges are removed, or if the class I PI3K activation loop is replaced with a sequence derived from other PI3K family members, the resulting mutant enzyme looses its ability to turn over PtdIns(4,5)P_2, but can still phosphorylate phosphatidylinositol (Fig. 5.2b, Bondeva et al. 1998; Pirola et al. 2001). As the experimental transfer of an activation loop from class II PI3Ks (containing one correctly positioned positive charged amino acid side chain corresponding to the Arg949 in p110α) to class I enzymes retains the ability to phosphorylate PtdIns-4-*P* and to generate PtdIns(3,4)P_2, while the insertion of a class III activation loop only conserves the reactivity towards phosphatidylinositol, it is possible to classify PI3Ks solely on the basis of their activation loop sequences. Based on the analysis of the activation loop, class I PI3K are the only PI3Ks to produce PtdIns(3,4,5)P_3, and class III PI3K can only generate PtdIns-3-*P*. The *in vitro* products of class II PI3K were reported to be PtdIns-3-*P*

Table 5.1 Inhibitors and drugs targeting the PI3K/PKB/TOR pathway—stages of developments to therapy

Drug name	References	Target(s)	Stage, phase	Company	Application
PI3K isoform-specific inhibitors, targeted inhibitors					
AS605240 and AS252424	(Fougerat et al. 2008; Camps et al. 2005; Barber et al. 2005; Pomel et al. 2006; Edling et al. 2010)	p110γ	Preclinical		Chronic inflammatory, allergic, cardio-vascular disease, atherosclerosis, pancreatic cancer?
BYL719		p110α	Phase I	Novartis	Advanced solid tumors with PIK3CA mutations
CAL-101 (clinical follow-up of IC87114)	(Lannutti et al. 2011; Ikeda et al. 2010; Herman et al. 2010)	p110δ	Phase I	Calistoga	B cell malignancies, CLL, allergies
CAL-120	(Lannutti et al. 2009)	Dual p110β/p110δ	Preclinical	Calistoga	Solid tumors
CAL-263		p110δ	Phase I	Calistoga	Allergic rhinitis
SF1126	(Garlich et al. 2008; Ozbay et al. 2010)	Targeted pan-PI3K inhibitor	Phase I	Semafore Pharmaceuticals	Solid tumors
TG100-115	(Doukas et al. 2006, 2007)	Dual p110γ/p110δ	Phase I, completed	TargeGen/(Sanofi-Aventis?)	Myocardial ischemia/ reperfusion injury
TGX-221	(Jackson et al. 2005)	P110β inhibitor	Preclinical		Platelet aggregation
pan-PI3K inhibitors					
BKM-120	(Aziz et al. 2010; Buonamici et al. 2010)	pan-PI3K	Phase I/II	Novartis	Breast, colon, ovarian, endometrium cancer, solid tumors
GDC-0941	(Raynaud et al. 2009; Folkes et al. 2008)	pan-PI3K (p110α/p110δ)	Phase I/II	Roche/Genentech	Advanced and metastatic solid tumors, NSCLC, Non-Hodgkin's lymphoma
GSK1059615		pan-PI3K	I/II, terminated	GlaxoSmithKline	Advanced solid tumors, lymphoma

Table 5.1 (continued)

Drug name	References	Target(s)	Stage, phase	Company	Application
XL-147	(Shapiro et al. 2009)	pan-PI3K	I/II	Exelixis/Sanofi-Aventis	Solid tumors, advanced breast, endometrial cancer, glioblastoma
ZSTK474	(Marone et al. 2009; Kong et al. 2010; Kong and Yamori 2007, 2009; Yaguchi et al. 2006)	pan-PI3K	Phase I	Zenyaku	Neoplasms
Dual PI3K/mTOR kinase inhibitors					
BEZ235	(Maira et al. 2008; Marone et al. 2009; Engelman et al. 2008; Serra et al. 2008; Brachmann et al. 2009)	PI3K/mTOR	Phase I/II, completed	Novartis	Solid tumors
BGT226		PI3K/mTOR	Phase I/II completed	Novartis	Solid tumors, Cowden disease
GDC-0980	(Wagner et al. 2009)	PI3K/mTOR	Phase I	Roche/Genentech	Refractory solid tumors, non-Hodgkin's lymphoma
GSK2126458	(Knight et al. 2009)	PI3K/mTOR	Phase I	GlaxoSmithKline	Solid malignancies
PF-04691502/	(Cheng et al. 2010)	PI3K/mTOR	Phase I	Pfizer	Advanced solid tumors
PKI-402	(Mallon et al. 2010; Dehnhardt et al. 2010)	PI3K/mTOR	Preclinical	Pfizer/Wyeth	
PKI-587, PF-05212384	(Venkatesan et al. 2010)	PI3K/mTOR	Phase I	Pfizer/Wyeth	Advanced solid tumors
PX-866	(Ihle et al. 2004)	PI3K/mTOR	Phase I/II	Oncothyreon	Solid tumors, glioblastoma
XL-765	(Laird et al. 2008)	PI3K/mTOR	Phase II	Exelixis/Sanofi-Aventis	Solid tumors, glioblastoma
mTORC1/mTORC2 kinase inhibitors					
AR-mTOR-1	(Wallace et al.)	mTOR kinase	Preclinical	Array Biopharma	
AZD2014		mTOR kinase	Phase I	AstraZeneca	Advanced solid tumors

Table 5.1 (continued)

Drug name	References	Target(s)	Stage, phase	Company	Application
AZD8055	(Chresta et al. 2010)	Selective mTOR kinase	Phase I/II	AstraZeneca	Liver cancer; advanced tumors
CC-223		mTOR kinase	Phase I/II	Celgene	Advanced Solid Tumors, Non-Hodgkin Lymphoma, Multiple Myeloma
INK128	(Jessen et al. 2009)	mTOR kinase	Phase I	Intellikine	Advanced tumors, relapsed/refractory multiple myeloma, Waldenström's macroglobulinemia
OSI-027	(Carayol et al. 2010; Vakana et al. 2010)	mTOR kinase	Phase I	OSI Pharmaceuticals	Advanced solid tumors, lymphoma
Palomid 529, P529	(Diaz et al. 2009; Xue et al. 2008)	Affects mTORC1 and mTOR2 activity. Mechanism?	Phase I	Paloma Pharmaceuticals	Advanced Neovascular Age-Related Macular Degeneration (AMD)
PP242/PP30	(Feldman et al. 2009; Hoang et al. 2010)	Selective mTOR kinase inhibitor	Preclinical		
XL388	(Miller 2009)	mTOR kinase inhibitor	Preclinical	Exelixis	
mTORC1 allosteric inhibitors (rapamycin derivatives or rapalogs)					
Everolimus, Afinitor, Certican, RAD001	(Motzer et al. 2008; Ryan et al. 2011; Yao et al. 2010; Ellard et al. 2009; Baselga et al. 2009; Amato et al. 2009; Gridelli et al. 2007)	mTORC1	Phase II/III	Novartis	Pancreatic neuroendocrine tumors/2nd line elderly NSCLC; Approved for organ rejection; was approved for renal cell carcinoma 3/2009 (Motzer et al. 2008)

Table 5.1 (continued)

Drug name	References	Target(s)	Stage, phase	Company	Application
Temsirolimus, Torisel, CCI-779	(Hess et al. 2009; Hudes et al. 2007; Galanis et al. 2005; Witzig et al. 2011, 2005; Atkins et al. 2004; Sarkaria et al. 2010; Johnston et al. 2010a; Ghobrial et al. 2010)	mTORC1	Phase II/III	Wyeth	Approved for renal carcinoma, relapsed mantle cell lymphoma /newly diagnosed GBM/ non-Hodgkin's lymphoma (NHL), Waldenström's macroglobulinemia
Ridaforolimus, Deforolimus, AP23573, MK-8669	(Rizzieri et al. 2008; Sessa et al. 2010)	mTORC1	Phase III, Ib	Merck/ARIAD Pharmaceuticals	Sarcoma, relapsed/refractory hematologic malignancies
PKB/Akt inhibitors					
A-443654	(de Frias et al. 2009; Han et al. 2007; Liu et al. 2008; Zhuang et al. 2010)	pan-PKB/Akt	Preclinical	Abbott Labs	
AT-13148	(Lyons et al. 2007)	PKB/Akt	Phase I	AstraZeneca/Astex Therapeutics	
CCT129254, AT11854	(Davies et al. 2009)	PKB/Akt	Preclinical	AstraZeneca	
GSK690693	(Altomare et al. 2010; Carol et al. 2010; Heerding et al. 2008; Levy et al. 2009; Rhodes et al. 2008)	pan-PKB/Akt	Phase I (withdrawn)	GlaxoSmithKline	
GSK2141795 and GSK21110183 (follow-ups of GSK690693)		pan-PKB/Akt	Phase I	GlaxoSmithKline	Hematologic cancer

Table 5.1 (continued)

Drug name	References	Target(s)	Stage, phase	Company	Application
Perifosine, D21226	(Argiris et al. 2006; Bailey et al. 2006; Elrod et al. 2007; Ernst et al. 2005; Knowling et al. 2006; Posadas et al. 2005; Van Ummersen et al. 2004; Vink et al. 2006; Chee et al. 2007)	PKB/Akt	Phase I/II	Keryx	Advanced solid tumors, ovarian cancer, metastatic melanoma, multiple myeloma
MK-2206	(Tolcher et al. 2009)	PKB/Akt & mTOR	Phase I	Merck	Advanced solid tumors
XL-418		PKB/Akt	I, suspended	Exelixis	Solid tumors

For updates of study progress and status consult http://www.clinicaltrials.gov; http://clinicaltrialsfeeds.org; http://www.cancer.gov; compounds in clinical trials can be retrieved by entering keywords [e.g. Akt, PKB, PI3K, mTOR] at http://nci.nih.gov/drugdictionary. Recent, excellent reviews covering PI3K pathway drug development include (Liu et al. 2009; Albert et al. 2010). Compound structures were reviewed in depth in (Marone et al. 2008).

and PtdIns(3,4)P_2, but it was shown that PI3K-C2α bound to clathrin was able to generate PtdIns(3,4,5)P_3 *in vitro* (Gaidarov et al. 2001). Activation loop sequences would predict that this does not happen in a PI3K-C2α monomer, and suggests that clathrin takes part in the presentation of PtdIns(4,5)P_2 as a substrate for this lipid kinase. Presently, the physiologic role, lipid product(s) and selective downstream targets of class II PI3Ks remain largely undefined.

5.1.4 Structural Features and Activation of Class IA PI3Ks

Class IA PI3K catalytic subunits p110α (encoded by the gene named *PIK3ca*), p110β (*PIK3cb*), and p110δ (*PIK3cd*) bind tightly to a regulatory subunit harboring two src-homology 2 (SH2) domains. The latter docks specifically to phosphorylated tyrosines in pYxxM (phospho-Tyr-x-x-Met) motifs on growth factor receptors or protein tyrosine kinase substrates. Mammalia have three genes encoding five major p85-like regulatory subunits subunits (*PIK3r1* encodes p85α and splice variants p55α, and p50α; *PIK3r2* yields p85β; and *PIK3r3* gives rise to p55γ). Each regulatory subunit contains a coiled-coil region located between the N- and C-terminal SH2 domains (dubbed interSH, or iSH domain), which tightly binds to the N-terminus (designated as p85-binding region, p85*B*) of the catalytic subunits p110α, p110β, and p110δ (Fig. 5.2). The class IA regulatory p85-like subunits exert an inhibitory action onto the catalytic subunit, which keeps the potentially oncogenic class I enzymes silent (Zhao et al. 2005; Kang et al. 2006). Inhibition is released by the translocation of p85 regulatory subunits to growth factor receptors and binding of the SH2 domains to pYxxM motifs (for reviews see (Wymann and Marone 2005; Vanhaesebroeck et al. 2001; Wymann et al. 2003b; Cantley 2002; Backer 2010)). The isolated domain structures of the regulatory p85 subunit have been determined early on (for the SH3 domain of p85α see (Batra-Safferling et al. 2010); the N-terminal SH2 domain of p85α (nSH2) see (Nolte et al. 1996); for the iSH2 of p85β see (Schauder et al. 2010); for the C-terminal SH2-domain (cSH2) of p85α see (Hoedemaeker et al. 1999)), but the mechanism of this important inhibitory action has only been elucidated recently: the structural determination of p85-fragments bound to p110α (Miled et al. 2007) and the resolution of a p85α iSH-cSH2 fragment in a complex with p110β suggest that both SH2 domains interact with perpendicularly oriented C-terminal α-helices dubbed "the regulatory square" (Zhang et al. 2011). For class I PI3K it is thus clear, that phosphorylated YxxM motifs on growth factor receptors do not only translocate PI3Ks to the plasma membrane to secure access of the catalytic subunit to PtdIns(4,5)P_2, but that they also relieve two to three safety latches from class IA PI3K complexes.

Some growth factor receptors directly recruit class IA PI3Ks, but for other input signals, adaptor molecules are key to the activation and localization of class IA PI3Ks (Fig. 5.3). As such, Grb2-associated binders (Gab1-3) and insulin receptor substrates (IRS1-4) display pYxxM sites to class IA PI3Ks. They belong to aYxxM-multisite adaptor protein family including daughter of sevenless (Dos). The insulin and IGF-1

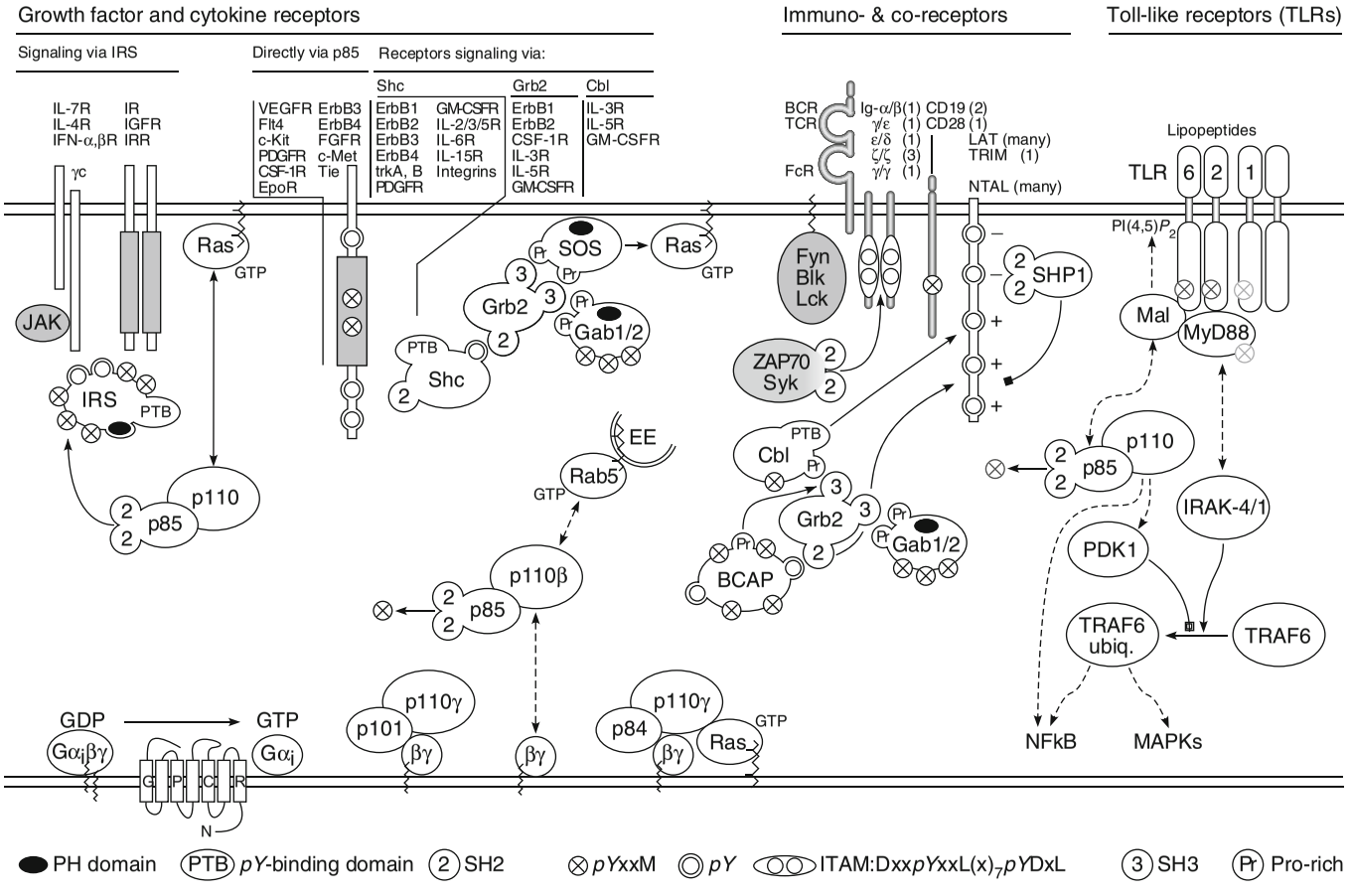
Growth factor and cytokine receptors
Signaling via IRS
Directly via p85
Receptors signaling via:
Shc
Grb2
Cbl
IL-7R
IL-4R
IFN-α,βR
IR
IGFR
IRR
γc
VEGFR
Flt4
c-Kit
PDGFR
CSF-1R
EpoR
ErbB3
ErbB4
FGFR
c-Met
Tie
ErbB1
ErbB2
ErbB3
ErbB4
trkA, B
PDGFR
GM-CSFR
IL-2/3/5R
IL-6R
IL-15R
Integrins
ErbB1
ErbB2
CSF-1R
IL-3R
IL-5R
GM-CSFR
IL-3R
IL-5R
GM-CSFR
Immuno- & co-receptors
BCR
TCR
FcR
Ig-α/β(1)
γ/ε (1)
ε/δ (1)
ζ/ζ (3)
γ/γ (1)
CD19 (2)
CD28 (1)
LAT (many)
TRIM (1)
NTAL (many)
Toll-like receptors (TLRs)
Lipopeptides
TLR
6
2
1
PI(4,5)P2
Ras
GTP
JAK
IRS
PTB
p110
p85
SOS
Grb2
Gab1/2
Shc
Fyn
Blk
Lck
ZAP70
Syk
SHP1
Mal
MyD88
EE
Rab5
p110β
Cbl
BCAP
IRAK-4/1
PDK1
TRAF6
ubiq.
TRAF6
NFkB
MAPKs
GDP
GTP
Gαiβγ
Gαi
p101
p110γ
βγ
p84
N
PH domain
pY-binding domain
SH2
pYxxM
pY
ITAM:DxxpYxxL(x)7pYDxL
SH3
Pro-rich

Fig. 5.3 Class I PI3Ks—multiple ways of activation. A plethora of growth factor receptors, cytokine and immune receptors, Toll-like and G protein-coupled receptors trigger PI3K activities. The simplest mode of PI3K activation is mediated by growth factor receptors with intrinsic protein tyrosine kinase activity, of which some present phosphorylated Tyr-x-x-Met motifs (pYxxM; see explanation of symbols at the bottom of the figure) and thus directly interact with the two SH2 domains of p85-like regulatory subunits. Other receptors operate via adapter proteins like Shc, Grb2, and Cbl that are interlinked by SH3/proline-rich regions, SH2/pY and phosphotyrosine-binding/pY interaction pairs. Here, the output to PI3K is often relayed via Gab1/2 (Nishida and Hirano 2003), which also displays a PtdIns(3,4,5)P_3-selective PH domain and docking sites for p85 (pYxxM motifs). Cytokine receptors signal through Janus kinase (JAK) either via Shc/Grb2/Cbl as indicated, or via the phosphorylation of insulin receptor substrate (IRS), which is the main target of insulin receptor (IR), IGFR and IRR. Ras, e.g. activated by SOS (son of sevenless), interacts with the Ras-binding domain of class I PI3Ks. In hematopoietic cells, constitutively membrane-bound src-like protein kinases (Fyn, Blk, Lck) phosphorylate cross-linked immunoreceptors on immunoreceptor tyrosine-based activation motifs (ITAMs; Rolli et al. 2002; Hamerman et al. 2009). The immunoglobulin or antigen-binding portion of the immunoreceptors is shown schematically for the B-cell receptor (BCRs), T-cell receptors (TCRs), and immunoglobulin receptors (FcR). Accessory chains and co-receptor molecules are listed on the right of the respective immune receptors. The phosphorylated ITAM motifs of the accessory chains recruit SH2 containing tyrosine kinases ZAP70 or Syk, supporting the protein tyrosine cascade and extending phosphorylation to CD19, CD28, TRIM, LAT, and NTAL. Inhibitory ITAMs recruit the protein phosphatase SHP1 and the lipid phosphatase SHIP (not shown; Blank et al. 2009). In the BCR, complement receptor 2 (CR2/CD21, not shown) drags along CD19 with two pYxxM motifs. The B-cell adaptor for PI3K (BCAP) and Gab2 mediate translocation of PI3K (mainly p110δ) to the BCR. In T cells, tyrosine-phosphorylated CD28 and TRIM display docking sites for p85. When phosphorylated, LAT recruits phospholipase Cγ1 (PLC-γ1), Gads, Grap, 3BP2, and Shb (not shown) and Grb2. With these components SOS, Vav, SLP-76, Itk (not depicted), Gab and c-Cbl, can be recruited. For reasons of simplicity, other PH domain-containing adapters where omitted (such as SKAPs, Bam32/DAPP1 [binding PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2], and TAPP1/2 [specific for PtdIns(3,4)P_2]; more on their function can be found in (Zhang et al. 2009)). In myeloid cells, ITAMs of immunoglobulin receptors are phosphorylated by Lyn and Syk. The direct activation of PI3K in mast cells by the high affinity IgE receptor requires Gab2 (Gu et al. 2001) and LAT (Wilson et al. 2001). For further reading on PI3K in immunoreceptor complexes see (Okkenhaug and Vanhaesebroeck 2003; Wymann et al. 2003a; Werner et al. 2010). Toll-like receptors (TLRs) play an important role in the recognition of pathogens and "non-self", and prime cell of the innate immune system for activation: TLR 1,2, and 6 have been shown to contain a YxxM sequence, which can potentially recruit p85 (Li et al. 2010). Lipopeptide-dependent activation of PI3K via heterodimeric TLR2/6 has been shown to depend on a direct interaction of p85 and Mal (Santos-Sierra et al. 2009). Mal has a *N*-terminal PtdIns(4,5)P_2-binding domain that is crucial for membrane translocation. Abbreviations: CSF-1R colony stimulating factor 1 receptor, CD cluster of differentiation, ErbB epidermal growth factor receptor [family], EpoR erythropoietin receptor, FGFR fibroblast growth factor receptor, Gab Grb2-associated binder, GM-CSFR granulocyte–macrophage colony-stimulating factor, Grb2 growth factor receptor-bound protein 2, IGFR insulin-like growth factor 1 receptor, IL interleukin, IRR insulin receptor-related receptor, LAT linker for activation of T cells, Mal/TIRAP MyD88-adapter-like/TIR-domain-containing adaptor protein, MyD88 myeloid differentiation primary response gene 88, PDGFR platelet-derived growth factor receptor, PH domain pleckstrin homology domain, PTB domain phosphotyrosine-binding domain, Pr proline-rich, pY phosphotyrosine, SOS son of sevenless, TRIM TCR-interacting molecule, ZAP70 [TCR] zeta chain-associated protein kinase

receptors directly phosphorylate IRSs, while members of the IL-4 receptor family (γc-chain-containing cytokine receptors, see Fig. 5.2) require Janus kinases (JAK) to complete the phosphorylation of YxxM motifs. IRS1 and Gab proteins display a pleckstrin homology (PH) domain that is selective for PtdIns(3,4,5)P_3, and can potentially provide amplification of the PI3K response. The importance of Gabs for PI3K signaling has been demonstrated in various systems, e.g. acquired immunity (Pratt et al. 2000), allergy (Gu et al. 2001) and transformation of myeloid cells by the $p190^{BCR/Abl}$ protein tyrosine kinase driving chronic myelogenousleukemia (CML; Sattler et al. 2002). The E3 ligase Cbl, has also been shown to recruit PI3K to growth factor and cytokine receptors. *In vivo*, however, c-Cbl and Cbl-b significantly reduces PI3K activity downstream of the T cell receptor. That the inhibitory action of Cbl is dominant in the long term was demonstrated in mice with disrupted c-Cbl, resulting in enhanced thymic positive selection (Murphy et al. 1998). Mice without Cbl-b displayed increased susceptibility for autoimmune diseases (Bachmaier et al. 2000).

Downstream of the T cell (TCR), B cell (BCR) and immunoglobulin receptors (FcRs) so-called immunoreceptor tyrosine-based activation motifs (ITAM) serve as initiation points for protein tyrosine kinase cascades. After the cross-linking of immunoreceptors, src-like membrane-anchored protein tyrosine kinases (Fyn, Blk, Lck) are concentrated, and locally phosphorylate ITAMs. ITAMs then recruit SH2 domain-containing protein tyrosine kinases including ZAP-70 and Syk. This enforced phosphorylation activity generates further PI3K docking sites on transmembrane adapter proteins (TRAPs) such as CD19, CD28, T cell receptor interacting molecule (TRIM), NTAL (also called LAB/Lat2) and linker for activation of T cells (LAT). Mice without LAT cannot generate T cells beyond the $CD4^-/CD8^-$ stage (Zhang et al. 1999; Roncagalli et al. 2010; Fuller et al. 2011). As for the growth factors mentioned above, soluble adapter proteins [Grb2, Gab and the B cell adaptor for phosphoinositide 3-kinase (BCAP)] contribute to PI3K activation. Deletions of BCAP (Yamazaki et al. 2002) or CD19 (Rickert et al. 1995) both lead to severe B cell phenotypes (Simeoni et al. 2004).

Toll-like receptor (TLR)-triggered responses are essential in host defense. The activation of TLRs by various ligands has been reported to activate PI3K. Elevated PI3K activity was mostly associated with an attenuation of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway and cytokine production at multiple levels (Fukao and Koyasu 2003; Hazeki et al. 2007). Depending on the stimuli and TLR receptor targeted, PI3K mediated pro- and anti-inflammatory effects (for a review see (Fukao and Koyasu 2003)). The activation of class I PI3Ks can take place via the interaction of p85 with aYxxM sequence on either TLR1, TLR2, or TLR6 (Li et al. 2010) or by p85 binding to Mal (MyD88-adaptor-like; also named Toll—IL-1 receptor domain-containing adaptor protein, TIRAP; Santos-Sierra et al. 2009). When the PI3K downstream target 3-phosphoinositide-dependent protein kinase-1 (PDK) was cell-specifically targeted in the myeloid cell linage, macrophages without PDK1 became more susceptible to lipopolysaccharide (LPS) stimulation via TLR4, and activation by Pam3CysSerLys4 (Pam3CSK4), a potent TLR2 agonist acting through TLR2/TLR1. This resulted in increased production

of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and a dramatically increased sensitivity to LPS-induced septic shock (Chaurasia et al. 2010).

5.1.5 Class IB PI3K: PI3Kγ

The PIK3cg gene encodes the only PI3K class IB member, PI3Kγ (Stoyanov et al. 1995; Stephens et al. 1997), which associates with a p101 (PIKr5; Stephens et al. 1997) or a p84/p87^{PIKAP} adapter protein (PIKr6; Suire et al. 2005; Voigt et al. 2006; Bohnacker et al. 2009). PI3Kγ and its adapter subunits are highly expressed in leukocytes, and at lower levels in smooth muscle cells, endothelia and cardiomyocytes (Wymann and Marone 2005; Wymann et al. 2003b; Patrucco et al. 2004; Vecchione et al. 2005; Alloatti et al. 2005; Okkenhaug and Vanhaesebroeck 2003; Ghigo et al. 2010). The PI3Kγ complex is translocated and activated by βγ subunits (Gβγ) of trimeric G proteins. PI3Kγ is expressed at high levels in white blood cells throughout the hematopoietic system, and mainly relays signals downstream of G protein-coupled receptors (GPCRs). Thus linked to a plethora of chemokine and other receptors of inflammatory mediators, PI3Kγ mediates processes in inflammatory and allergic reactions (Wymann et al. 2003b; Ghigo et al. 2010; Deane and Fruman 2004). Although lower PI3Kγ expression was detected in cardiomyocytes, vascular smooth muscle and endothelia, PI3Kγ plays a role in the control of vascular tone (Vecchione et al. 2005), heart contractility (Patrucco et al. 2004; Crackower et al. 2002; Oudit et al. 2004) and progress of atherosclerosis (Fougerat et al. 2008). The role of the p101 and p84 adapter subunits has not yet been fully explored, but they are required for a functional relay of GPCR signals to PI3Kγ. In this respect, the p101 adapter subunit sensitizes the PI3Kγ complex to Gβγ subunits (Stephens et al. 1997; Krugmann et al. 1999; Maier et al. 1999; Brock et al. 2003; Kurig et al. 2009), while p84 does not fulfill this function. Interestingly, p84-p110γ complexes require the interaction with activated Ras (see below), while p101 functions even when Ras activation is blocked (Kurig et al. 2009). As p101-p110γ and p84-p110γ complexes were found to generate functionally distinct pools of PtdIns(3,4,5)P_3 (Bohnacker et al. 2009), it is likely that the two adapter subunits moderate input and output signals of the respective PI3Kγ complexes.

Alternatively, Gβγ subunits were also shown to activate p110β. Here early studies demonstrated that Gβγ subunits and phosphorylated Tyr peptides activated p85-p110β complexes synergistically (Maier et al. 1999; Kurosu et al. 1997; Tang and Downes 1997). Cellular studies using overexpression of GPCRs, p110β, genetic and pharmacological tools proposed that p110β was the main PI3K downstream of some GPCRs. While this makes sense for receptors that do not couple exclusively to B. Pertussis toxin-sensitive G proteins, for example the receptors for lysophosphatidic acid (LPA), thrombin, the bradykinine and A1 adenosine receptors, the sphingosine-1-phosphate receptor and more (Roche et al. 1998; Graness et al. 1998; Kubo et al. 2005; Guillermet-Guibert et al. 2008), the data for some other GPCRs has to be reviewed critically for receptor transactivation and co-operation with protein tyrosine

kinase activities (Guillermet-Guibert et al. 2008). As an example, there is conflicting data demonstrating one hand that complement fragment 5a (C5a) signals exclusively via p110γ in macrophages (Hirsch et al. 2000), while others claimed that p110β was required (Guillermet-Guibert et al. 2008).

5.1.6 Activation of Class I PI3Ks by Small GTPases

A potential common activator for class I PI3Ks is activated, GTP-loaded Ras, as all class I PI3Ks display a Ras-binding domain (Fig. 5.2). This interaction is well documented for p110α (Sjolander and Lapetina 1992; Sjolander et al. 1991; Rodriguez-Viciana et al. 1994, 1996) and p110γ (Rubio et al. 1997, 1999). For p110α, Ras-interactions were demonstrated to be relevant in Ras-driven tumor promotion (Gupta et al. 2007), and an intact Ras-binding domain was shown to be crucial for the activation of the NADPH oxidase in neutrophils by GPCR ligands (Suire et al. 2006). These findings were corroborated by the resolution of the crystal structure of a Ras-p110γ complex defining the interface of the two proteins in detail. Ras-induced conformational changes in p110γ show that Ras is not only a docking site for p110 at the membrane, but a potent activator (Pacold et al. 2000). The importance of Ras activation upstream of p110β is controversial (Kang et al. 2006; Rodriguez-Viciana et al. 2004; Marques et al. 2008), and p110β was suggested to interact with the small GTPase Rab5 localized on early endosomes (Christoforidis et al. 1999; Shin et al. 2005; Kurosu and Katada 2001; Ciraolo et al. 2008; Jia et al. 2008). The p110δ catalytic subunit interacts with TC21 (or RRas2 (Rodriguez-Viciana et al. 2004; Delgado et al. 2009)), and depends on the GTPase for a translocation to T- and B-cell receptors (Delgado et al. 2009).

5.1.7 Class II PI3Ks

Class II PI3Ks are large enzymes (170–200 kDa) and include the three family members PI3K-C2α, β, and γ, which all have a C-terminal extension containing a Phox homology (PX) and a C2 homology domain. The C-terminal C2 domain is Ca^{2+}-insensitive due to the lack of a conserved aspartate residue (Falasca and Maffucci 2007; for a new classification of C2 domains see (Zhang and Aravind 2010)). The founder of the class II PI3K family was the *drosophila* PI3K_68D (MacDougall et al. 1995).

In contrast to class I PI3Ks, no class II regulatory subunits were identified. Class II PI3Ks have been mapped to the trans-Golgi network and low-density microsomes, but their mode of action is still poorly defined. The PI3KC2α and β isoforms have been reported to associate with their N-terminal region with clathrin (Domin et al. 2000; Gaidarov et al. 2001, 2005; Wheeler and Domin 2006). PI3KC2α has been clearly attributed roles in clathrin assembly and clathrin-mediated, microtubule-dependent

vesicular trafficking (Domin et al. 2000; Gaidarov et al. 2001, 2005; Zhao et al. 2007). Recent reports identified PI3KC2α as an essential factor in dynamin-independent endocytosis and fluid-phase endocytosis. As PI3KC2α and the PtdIns-3-*P* -binding Early Endosomal Antigen (EEA1) were recruited to cargo vesicles, it is likely that the relevant *in vivo* product involved in the process was PtdIns-3-*P* (Krag et al. 2010).

Several extracellular stimuli activate class II PI3Ks, such as growth factors like EGF, PDGF, insulin and SCF, chemokines (MCP-1), cytokines (leptin, TNF-α), and lysophosphatidic acid (LPA; Arcaro et al. 2000; Maffucci et al. 2005). Proline-rich regions in the N-terminus, and interactions with signaling adapters like Grb2 were proposed to mediate interaction with growth factor receptors (Wheeler and Domin 2006). For PI3KC2β it was shown that this enzyme can promote LPA-induced cell migration of ovarian and cervical cancer cells (Maffucci et al. 2005; for a review on class II PI3K in cancer see (Traer et al. 2006)). Other results point to a role of class II PI3Ks in insulin signaling (Cui et al. 2011; Falasca et al. 2007; Dominguez et al. 2011), which is supported by the finding that class II regulates exocytosis of insulin granules in pancreatic beta cells (Dominguez et al. 2011). Interestingly, a polymorphism in the PIK3C2G gene (encoding PI3KC2γ could be associated with type 2 diabetes in a Japanese population (Daimon et al. 2008)), and the nematode *C. elegans* accumulates fat when its only class II PI3K gene (*F39B1.1*) product is down-regulated. So far, these findings were not duplicated in mice where the *PIK3C2A* (PI3KC2α) and *PIK3C2B* (PI3KC2β) loci were targeted: in the latter mice, fat and body mass were actually significantly reduced. It must be said, however, that the mice lacking functional PI3KC2β were mainly investigated for epithelial differentiation (Harada et al. 2005), and that the mice with a modified *PIK3C2A* locus displayed a defect in renal function and still expressed a trace of a truncated PI3KC2α protein lacking the C-terminal PX and C2 domains (Harris et al. 2011).

The available data connects class II PI3Ks to multiple signaling events like the activation of MAPK (but not PKB/Akt (Cui et al. 2011)), activation of Ca^{2+}-triggered potassium channels (KCa3.1; Srivastava et al. 2009), the regulation of Rho (Wang et al. 2006), clathrin coated vesicular movement on microtubules (Zhao et al. 2007), exocytosis (Meunier et al. 2005) and endocytotic events mentioned above. For class II PI3Ks it is presently not possible to delineate connected signaling pathways, and to define a predictive network linked to general signaling outputs.

5.1.8 Class III PI3Ks

Class III PI3K: The *Saccharomyces cerevisiae* Vps34 protein (Vps34p, vacuolar protein sorting mutant 34) is the prototype of class III PI3K and plays an essential role in vesicular and protein trafficking from the Golgi to the yeast vacuole, which is the yeast equivalent to lysosomes in mammals (Schu et al. 1993). In yeast, Vps34p binds to the N-terminally myristoylatedSer/Thrkinase Vps15p. It has been shown that a functional Vps15p kinase is needed for the activation and recruitment of Vps34p to Golgi membranes (Herman et al. 1992). The Vps15p orthologue in mammals is

p150 (also referred to as hVps15), which translocates hVps34p to Rab5-positive early endosomes and Rab-7 positive late endosomes. Therefore, hVps34p is also central to endocytosis, vesicular trafficking (Christoforidis et al. 1999; Murray et al. 2002), and phagocytic uptake of bacteria (Sun et al. 2008).

Moreover, Vps34p interacts directly with Beclin-1 (yeast Atg6/Vps30p) and Atg14L to form a complex I, and alternative complexes containing UVRAG or UVRAG and Rubicon at the place of Atg14L (Funderburk et al. 2010; Matsunaga et al. 2010), which all play an important role in autophagy (Simonsen and Tooze 2009). Recently, it was proposed that the Vps34p-hVps15p-Beclin-1-Atg14L complex would also involve p110β as an element to regulate the autophagy (Dou et al. 2010). In yeast, deletion of Atg14 leads to defects in autophagy, while own regulation of cellular Beclin-1 affects autophagy and vesicular trafficking minimally (Kihara et al. 2001). In contrast, mice heterozygous for Beclin-1 display a decreased rate of autophagy and enhanced tumor formation (Qu et al. 2003; Yue et al. 2003). Recently, Vps34p was linked to the induction of autophagy in nutrient, amino acid, as well as energy (glucose) -deprived cells, and a role for Vps34p in the amino acid-induced activation of mTOR was proposed (Dann and Thomas 2006; Gulati and Thomas 2007; Nobukuni et al. 2007). The latter connection is controversial, and the deletion of *VPS34* in fruit flies did not affect TOR activity (Juhasz et al. 2008). In mice, Vps34 (encoded by PIK3C3) is required in early embryogenesis, and effects on mTOR activation were documented (Zhou and Wang 2010). Further in-depth reviews on class III PI3Ks can found in (Backer 2008; Simonsen and Tooze 2009; Funderburk et al. 2010; Backer 2010).

5.1.9 Downstream of Class I PI3Ks

The class I PI3K product PtdIns(3,4,5)P_3 is produced at the plasma membrane, where it serves as a docking site for proteins with PtdIns(3,4,5)P_3-specific PH domains (Ferguson et al. 2000). Members of the protein kinase B family (PKBα,β,γ/Akt1,2,3) are the most prominent representants of these signaling molecules, and link PI3Ks to the control metabolic activity, growth, proliferation and cellular survival pathways (Fig. 5.4). When PKB/Akt is recruited the plasma membrane, it is phosphorylated on Thr308 (numbers refer to PKBα/Akt1) by PDK1. In this process, binding of PDK1 itself to PtdIns(3,4,5)P_3 is crucial, as cells harboring a PDK1 with a non-functional PH domain cannot efficiently trigger PKB/Aktphosphorylation (McManus et al. 2004). To gain full activity, a second phosphorylation in the C-terminal, hydrophobic motif of PKB/Akt (Ser 473) by activities classified as "PDK2s" is required (Yang et al. 2002; Biondi and Nebreda 2003). A number of kinases have been shown to classify as PDK2 activities, such as mitogen-activated protein kinase-activated kinase 2 (MAPKAP-2), integrin-linked kinase (ILK), DNA-dependent protein kinase (DNA-PK_{cs}; Feng et al. 2004; Hanada et al. 2004; Bozulic and Hemmings 2009) and protein kinase Cβ (PKCβ; Kawakami et al. 2004). Finally it has been

shown that the TOR complex 2 (TORC2, the [Rictor]-TOR complex [rapamycin-insensitive companion of TOR]) displays major PDK2 activity (Sarbassov et al. 2005, for reviews see (Bozulic and Hemmings 2009; Polak and Hall 2006; Manning and Cantley 2007; Zoncu et al. 2011)). PKB/Akt-mediated phosphorylations regulate many downstream targets, both positively and negatively (see Fig. 5.4). Besides the modulation of PKB/Akt activity, the phosphorylation of Ser 473 seems also to be required for an efficient phosphorylation of N-terminal residues of the PKB/Akt substrates FOXO1/3A/4 (Jacinto et al. 2006). The hydrophobic motif phosphorylation might thus direct the PKB/Akt substrate selectivity (for a review see (Manning and Cantley 2007)).

The activation of the PI3K/PKB pathway is counteracted by two phosphoinositide phosphatases: (i) the 3-phosphoinositide phosphatase and tensin homolog deleted in chromosome ten (PTEN) regenerates PtdIns(4,5)P_2 from PtdIns(3,4,5)P_3 (Stambolic et al. 1998). PTEN is often mutated in tumors, leading to the accumulation of PtdIns(3,4,5)P_3 and a constitutive activation of the PI3K pathway (Liaw et al. 1997; Sansal and Sellers 2004; Hobert and Eng 2009; Farooq et al. 2010; Vivanco and Sawyers 2002; Wymann and Marone 2005; Cully et al. 2006). (ii) the SH2-domain-containing inositol phosphatase (SHIP), which has a 5-phosphoinositide phosphatase activity, generates PtdIns(3,4)P_2 from PtdIns(3,4,5)P_3 (Majerus et al. 1999; Kisseleva et al. 2000; Rohrschneider et al. 2000; Kalesnikoff et al. 2003). Besides the above lipid phosphatases, the protein phosphatase PHLPP can dampen PKB/Akt activation by the removal of the phosphate group at Ser 473 (Brognard et al. 2007; Gao et al. 2005; Mendoza and Blenis 2007).

5.1.10 Control of Cellular Growth, Transcription, and Translation

PKB/Akt controls cell growth via the nutrient sensor mTOR: PKB/Akt phosphorylates and thus inhibits TSC2 (tuberin), which constitutes together with TSC1 (hamartin) the tuberous sclerosis complex (Pan et al. 2004). In its active form, the TSC1/2 complex prevents the exchange of GDP for GTP on the GTPaseRheb. As the activation of TOR requires GTP-loaded Rheb, the PKB/Akt-mediated phosphorylation of TSC2 therefore initiates the activation of the TOR complex 1 (TORC1, or [Raptor]-TOR complex [regulatory-associated protein of TOR]). Active TORC1 phosphorylates and blocks 4E-BP1, which releases translation of mRNAs with 5′-polypyrimidin regions, which is supported by the parallel, TORC1-mediated phosphorylation of $p70^{S6K}$ (p70 S6 kinase) on Thr 389. S6K targets the ribosomal protein S6 (Dufner and Thomas 1999; Garami et al. 2003; Dann et al. 2007). Furthermore, TORC1 can be negatively regulated by PRAS40—controlling TORC1 substrate access (Van der Haar et al. 2007; Wang et al. 2007; Fonseca et al. 2007), or by an allosteric inhibition caused by the binding of the macrolide Rapamycin/FKBP12 complex to the FRB (FKBP/Rapamycin-binding) domain of TOR (Choi et al. 1996). When TORC1 signaling is permitted, transcription and translation are elevated, and

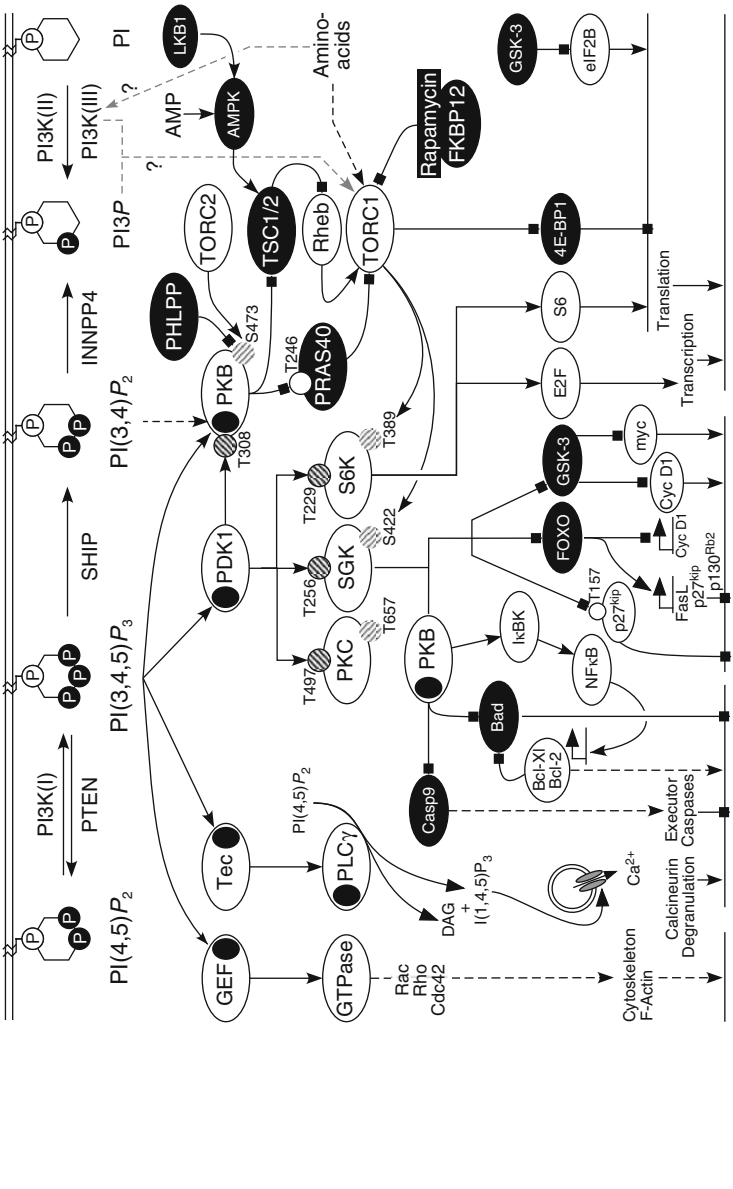

Fig. 5.4 Roadmap from PtdIns(3,4,5)P_3 to cellular outputs relevant in cancer and inflammation. PtdIns(3,4,5)P_3 is produced from ATP and PtdIns(4,5)P_2 by an activated class I PI3K (see Fig. 5.3) at the plasma membrane. Signaling of PI3K is completely reversed by the 3-phosphoinositide phosphatase PTEN, and the 5-inositol phosphatase SHIP produces PtdIns(3,4)P_2 with a limited cell activating capacity. *Pointed arrows* and *white protein symbols* denote activating pathways, *squared arrows* and *black proteins* represent inhibitory outputs. Pleckstrin homology (PH) domains sensitive to PtdIns(3,4,5)P_3, are shown as *black*

Fig. 5.4 (Continued) *ovals*. PH domains dock enzymes like phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB) at the plasma membrane (Franke et al. 1995; Burgering and Coffer 1995; Stokoe et al. 1997). PDK1 then phosphorylates protein kinase B (PKB/Akt) on Thr308 (Alessi et al. 1997; the residue numbers here refer to the human α isoforms). PDK1 has been also reported to phosphorylate a number of other AGC (protein kinases A, G, and C) Ser/Thr kinases within a conserved activation loop sequence (*hatched*, *outlined circles*), such as Serum- and glucocorticoid-induced protein kinase (SGK), protein kinase C (PKC; Dutil et al. 1998), cAMP-dependent kinase (PKA), Ribosomal S6 kinase (RSK), PKC-related protein kinase 2 (PRK2), and $p70^{S6K}$ (S6K; Pullen et al. 1998; for a recent review on AGC kinases see (Pearce et al. 2010)). Activity of PKB is further increased by a phosphorylation within the C-terminal hydrophobic motif (Ser473 in PKBα/Akt1) by so-called PDK2 activities (Yang et al. 2002; Biondi and Nebreda 2003). A prominent example is the mTOR complex 2 (TORC2; Sarbassov et al. 2005). Other reported PDK2 activities like the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs, Feng et al. 2004), mitogen-activated protein kinase-activated kinase 2 (MAPKAP-2), integrin-linked kinase (ILK), and protein kinase Cβ (PKCβ; Kawakami et al. 2004) are not depicted for clarity. Interestingly, it was recently found that the site on SGK1 (Ser422) that corresponds to Ser473 on PKB is phosphorylated by TORC1, and SGK1 can exert similar actions as PKB (Hong et al. 2008; Toker 2008). When fully activated, PKB signals to a plethora of downstream targets modulating multiple cellular outputs. PKB-mediated phosphorylation and inactivation of the GTPase activating protein (GAP) activity of TSC2 (tuberin) in the TSC1 (hamartin)/TSC2 complex (TSC1/2; for a review see (Pan et al. 2004)), leads to GTP loading of the small GTPaseRheb and the subsequent activation of the mTOR complex 1 (TORC1; Garami et al. 2003; Zhang et al. 2003; Tee et al. 2003; Inoki et al. 2003). Important negative regulators in the area are the PHdomain leucine-rich repeat protein phosphatase (PHLPP; Brognard et al. 2007; Gao et al. 2005; Mendoza and Blenis 2007) dephosphorylating phospho-Ser473 of PKB; PRAS40 (proline-rich Akt substrate of 40 kDa or Akt1S1) that interferes with TORC1 substrate-binding when not phosphorylated by PKB (Van der Haar et al. 2007; Wang et al. 2007; Fonseca et al. 2007); and the FKBP12-rapamycin complex exclusively inhibiting with TORC1 (Choi et al. 1996; Polak and Hall 2009; Sengupta et al. 2010). When cellular energy levels are low, AMP concentrations rise and activate AMPK (trimeric AMP-activated protein kinase), which enhances the inhibitory effect of the TSC complex on Rheb (Hardie 2005; Martin and Hall 2005). PDK1 and TORC1 activate p70 S6 kinase ($p70^{S6K}$, S6K), and when GSK-3β and 4EBP1 are inhibited by PKB and TORC1, respectively, transcriptional and translation restrictions are released to promote cellular growth. Class O forkhead transcription factors (FOXOs) are phosphorylated by PKB and serum and glucocorticoid-inducible kinase (SGK), and then interact with 14.3.3 proteins trapping them in the cytosol. In a coordinated fashion, GSK-3β is inhibited by phosphorylation, and stops the phosphorylation of cyclin D1 and its degradation by the proteasome. The entry into cell cycle is tightly controlled by CDK inhibitor $p27^{kip}$, tumor suppressors like $p130^{Rb2}$, cyclin D1 (Cyc D1), myc. The expression levels (where indicated with *transcription arrows*) and activity status of these molecules is controlled by forkhead transcription factors (mainly FOXO1, 3A, 4) and the indicated upstream kinases. Anti-apoptotic signals are triggered downstream of PKB/Akt, which phosphorylates and inactivates caspase 9 (Casp9) and the cell death inducer Bad. A route via NFκB leads to the upregulation of Bcl-XL and Bcl-2, which neutralize Bad and promote cell survival. In lymphocytes, a Tec family kinase/PLCγ axis controls the sustained elevation of Ca^{2+} levels. Ca^{2+}-calmodulin then activates calcineurin, a protein phosphatase activating NF-AT (nuclear factor of activated T cells; not shown). Via PtdIns(3,4,5)P_3-sensitive GEFs (guanine nucleotide exchange factors) including Tiam (T cell lymphoma invasion and metastasis), Vav, PIX (PAK-associated guanine nucleotide exchange factor) and P-Rex (also stimulated by Gβγ subunits), small the small GTPasesRac, Rho and and Cdc42 are stimulated and promote cytoskeletal changes and cell migration

protein and lipid biosynthesis drive an increase in cell mass required for the entry in cell cycle progression.

5.1.11 PI3K-mediated Control of Cell Cycle Progression

Entry into cell cycle progression is tightly controlled by the alternating expression of cyclins and their interaction with cyclin-dependent kinases (CDKs) or CDKs inhibitors. Activated PKB/Akt phosphorylates forkhead transcription factors of the class O (FOXOs) on three different sites (for a review see (Manning and Cantley 2007)). When phosphorylated, FOXOs bind to 14-3-3 proteins, which act as phospho-Ser and phosphor-Thr "receptors" and retain FOXOs in the cytosol (Burgering and Kops 2002). When in the nucleus, FOXOs repress many genes required for cell cycle entry, and a cytosolic retention of FOXOs thus releases the transcription of cyclin D1, while the transcription of the CDK inhibitor $p27^{Kip1}$ is attenuated (Alvarez et al. 2001; Burgering and Medema 2003). Glycogen synthase kinase 3β (GSK3β) phosphorylates cyclin D1, triggering its targeting and degradation to the proteasome. When phosphorylated by PKB/Akt, GSK3β is inhibited, and cyclin D1 accumulates (Liang and Slingerland 2003). The resulting increase in cyclin D1 levels combined with the concerted reduction in $p27^{Kip1}$ allows cells to transit from G1 to the S phase (Liang and Slingerland 2003; Foijer and te Riele 2006).

5.1.12 PI3Ks—Driving Cell Survival and Anti-apoptotic Signaling

The stimulation of the PI3K/PKB pathway branches into many anti-apoptotic events: PKB/Akt directly phosphorylates and inhibits caspase 9, a protease crucial for the initiation of the apoptotic cascade (Cardone et al. 1998; for a cross-species comparison see (Datta et al. 1999)). PKB/Akt also inactivates the *Bcl-2*-associated death promoter (BAD) by a phosphorylation at Ser 136, which then liberates the anti-apoptotic proteins Bcl-2 and Bcl-XL (del Peso et al. 1997; Datta et al. 1997). In another branch PKB/Akt phosphorylates IκB kinase (IκBK), which blocks the action of the inhibitor I-κB to release the transcription factor NF-κB. NF-κB is now free to translocate to the nucleus where it activates transcription of cell survival proteins such as Bcl-2 and Bcl-XL (Li and Verma 2002; Ozes et al. 1999; for reviews see (Schinzel et al. 2004; Kaufmann et al. 2004)). As mentioned above, FOXOs are retained in the cytosol by the action of PKB/Akt. As FOXOs promote transcription of FasL, PKB/Akt prevents ligand-induced apoptosis (Brunet et al. 1999). The connections of these pathways are further detailed in Fig. 5.4.

In the above signaling scenarios, the PtdIns(3,4,5)P_3 → PDK1 → PKB/Akt axis seems to play a central role in the control of cellular growth, the entry into the cell cycle and the initiation of survival pathways. When PtdIns(3,4,5)P_3 is elevated due to loss of PTEN, this disrupts embryonic development in mice (Di Cristofano et al.

1998) and fruit flies (Stocker et al. 2002). Viability could be restored in PTEN null flies by the manipulation of the *drosophila* PKB/Akt PH domain: when flies expressed PKB/Akt with a PH domain with a low affinity for PtdIns(3,4,5)P_3, "lethal levels of PtdIns(3,4,5)P_3" were tolerated, suggesting that PKB/Akt was the major hub for PtdIns(3,4,5)P_3-sensing in the fly (Stocker et al. 2002). In the human genome there are, however, > 275 PH domain-containing proteins encoded (source: SMART database at smart.embl-heidelberg.de). These are complemented with 48 proteins with PX domains (a domain first found in phagocyte NADPH oxidase cytosolic factors), 27 with FYVE (Zinc finger domain first found in Fab1, YOTB, Vac1 and EEA1) domains, 20 with ENTH (Epsin*N*-terminal homology) domains, ANTH, FERM and other lipid-binding domains (Varnai et al. 2005; Balla 2005; Takenawa and Itoh 2006). For many of these proteins interactions with poly-phosphorylated phosphoinositides have been reported, and some prominent signaling molecules bind PtdIns(3,4,5)P_3 through PH domains (see also Fig. 5.4): TEC family protein tyrosine kinases including Btk (Brutons's tyrosine kinase; Readinger et al. 2009; Mohamed et al. 2009), and PLCγ1 (phospholipase Cγ1; Rebecchi and Pentyala 2000; Maroun et al. 2003; Ji et al. 1997) play an important role in innate and acquired immunity; and β-ARK1/GRK2 (β-adrenergic receptor kinase1/G protein-coupled receptor kinase; Takenawa and Itoh 2006; Jaber et al. 1996) modulates GPCR signaling and cardiovascular functions. Structural data for PtdIns(3,4,5)P_3-bound PH domains is available (Ferguson et al. 2000; Milburn et al. 2003), and the PH domain protein families have been thoroughly analyzed across various species (Park et al. 2008). In spite of the existing diversity of PtdIns(3,4,5)P_3 selective PH domain containing proteins, PKB research is with > one third of all articles overrepresented in the PI3K literature (for a recent in-depth review on phospholipid-binding domains see (Lemmon 2008)).

5.1.13 PI3Ks—A Connection to Migration and Polarization

Small Rho family GTPases including Rac, Cdc42, TC10 and Rho play important roles in cell migration, polarization and cytoskeletal rearrangements. The activity of these Rho GTPases is regulated by GAPs (GTPase activating proteins), GDIs (guanine nucleotide dissociation inhibitors) and guanine nucleotide exchange factors (GEFs). While GAPs increase the rate of hydrolysis of the GTP bound to activatedGTPases to GDP, GDIs retain Rho GTPases in the cytosol. Activated GEFs promote the reloading of small GTPases with GTP, and thus enable their subsequent interaction with downstream effectors (Heasman and Ridley 2008). Rho GEFs can be divided in (i) Dbl-like proteins (> 70 members), which contain the catalytic Dbl homology (DH) domain and a pleckstrin homology domain, and (ii) Dock family proteins (11 members) with the active Dock homology region (DHR-2; also named Docker-ZH2 domain) and a DHR-1 mediating translocations (Cote and Vuori 2007).

A number of Dbl-like GEFs contain PtdIns(3,4,5)P_3-binding PH domains. Many of these GEFs, like Tiam (T-lymphoma invasion and metastasis inducing protein; a

GEF for Rac), PIX (PAK-associated guanine nucleotide exchange factor; reloading Rac and Cdc42), Vav (targeting RhoA, Rac, and Cdc42), and ARNO (Arf nucleotide binding site opener; activating Arf1,6) integrate the PI3K signal with the input of an upstream protein kinase, before they are fully active (for a review see (Wymann and Marone 2005)). In contrast, P-Rex binds to Gβγ subunits released from trimeric G proteins after GPCR activation and PtdIns(3,4,5)P_3 before it activates Rac (Welch et al. 2002). For Dock family proteins, Dock2 and Dock180 were shown to bind PtdIns(3,4,5)P_3 via their DHR-1 domain (Cote and Vuori 2007).

The regulation of Rho GTPases by GEFs is crucial in many physiological processes and disease contexts. While Ras proteins are activated by mutations (Diaz-Flores and Shannon 2007), deviations in Rho family GTPasesignaling is often caused by the overexpression of corresponding GEFs (Ellenbroek and Collard 2007; Vega and Ridley 2008). Rho GTPase activation promotes the dissemination of cancer cells and immune cells throughout tissues, driving metastasis and chronic inflammation. A subset of small GTPases such as RhoA, Rac and Cdc42 also activate transcriptional events (for a review see (Benitah et al. 2004)).

5.2 PI3Ks in Physiology and Disease

As outlined above, and illustrated in Figs. 5.3 and 5.4, PI3K relays growth factor, cytokine and G protein receptor-coupled signaling to a network balancing a cell's activities and cellular energy consumption. Downstream of PI3Ks, the TOR complexes 1 and 2 are important hubs integrating hormonal input, energy and nutrient availability. PtdIns(3,4,5)P_3-dependent kinase cascades originating for example from PDK1, PKB/Akt, TEC family kinases or the activation of PtdIns(3,4,5)P_3-sensitive GEFs contribute to the control of growth, cell cycle, survival and migration. In chronic inflammatory and autoimmune disease, an overshooting cytokine network triggers the activation of immune cells, while in cancer oncogenes are activated by mutations and epigenetic effects. Many PI3K-dependent pathways are shared in cancer and inflammation, but operate in different contexts and yield cell-specific outputs. Various cell types use specific class I PI3K isoforms, and an understanding of PI3K isoform selective signaling is a prerequisite to develop refined targeted therapies in cancer and inflammation.

5.2.1 PI3Ks in Innate and Acquired Immunity

In acquired immunity, T lymphocytes and B lymphocytes are activated by specific antigens exposed to them by antigen-presenting cells, such as dendritic cells (DCs) and activated macrophages. The development of T cell subsets (Th1, Th2, Th17, Treg, $CD8^+$ cytotoxic T cells, etc.) is fine tuned by cytokine signals and regulated by

PI3Ks (Deane and Fruman 2004; Okkenhaug and Fruman 2010). T helper cells (Th) are then required for B cell development and to raise a humoral immune response.

Mice lacking functional PI3Kγ (Patrucco et al. 2004; Hirsch et al. 2000; Li et al. 2000; Sasaki et al. 2000) and/or PI3Kδ (Okkenhaug et al. 2002; Clayton et al. 2002; Jou et al. 2002) are viable and fertile, and were extensively studied in inflammatory disease models. Mutant mice without the catalytic subunit of PI3Kδ (p110δ; Clayton et al. 2002; Jou et al. 2002), or mice expressing a catalytically inactive p110δ (with a D910A mutation; Okkenhaug et al. 2002) display impaired development of marginal zone B-cells and peritoneal B1-cells, and signals emerging from the B-cell receptor (BCR) are attenuated. In mice lacking the p110δ protein completely, T cell maturation in the thymus was normal, while mice with the catalytically inactive p110δ produced more naïve peripheral T-cells. Later it was reported that mice with inactive PI3Kδ have elevated counts of $Foxp3^+$ regulatory T-cells (Treg) in the thymus, but $Foxp3^+$ cell numbers were reduced in peripheral organs, likely modulated by a PI3K-FOXO1/3a connection. Interestingly, in spite of impaired BCR signaling and reduced IgM and IgG responses, mice with inactive p110δ increase IgE production, and have a tendency to develop autoimmunity (Oak et al. 2006; Ji et al. 2007; Patton et al. 2006; Merkenschlager and von Boehmer 2010). As Th2 responses are also reduced in mice without functional p110δ, elevated IgE levels are best explained by the negative regulatory effect of p110δ on the IgE class switch (Zhang et al. 2008; Omori et al. 2006), or mechanisms of IgE production that do not require cognate T cell help (McCoy et al. 2006).

In T cells without functional PI3Kγ, initial TCR signaling is not affected directly (Sasaki et al. 2000), but secondary signals, and the accumulation of 3-phosphorylated phosphoinositides at the immune synapse is impaired (Alcazar et al. 2007). As a result, T-cells of mice without functional PI3Kγ display significant developmental and signaling defects, yielding impaired thymocyte selection, reduced numbers of double-positive ($CD4^+$ $CD8^+$) cells and an altered CD4 to CD8 ratio (Rodriguez-Borlado et al. 2003), as shortened $CD4^+$ memory T-cell survival (Barber et al. 2006). When PI3Kγ and PI3Kδ were genetically targeted, double mutant mice displayed severe defects in thymocyte development, loss of thymus structure reducing the number of $CD4^+$/$CD8^+$ double positive cells, and a dramatic shift towards Th2 immune responses resulting in highly elevated IgE levels (Ji et al. 2007; Webb et al. 2005).

PI3Kγ has been shown to be instrumental in migration of neutrophils, macrophages (Hirsch et al. 2000; Li et al. 2000; Sasaki et al. 2000; Wymann et al. 2000; Jones et al. 2003) and dendritic cells (Del Prete et al. 2004) towards chemokines and other GPCR ligands. PI3Kγ-derived PtdIns(3,4,5)P_3 was thus dubbed "the compass of leukocytes" (Rickert et al. 2000; Servant et al. 2000; Wang et al. 2002). Detailed investigations of neutrophil migratory processes confirmed that PI3Kγ is key for migration, but rather for cell polarization and "stop and go" decisions than for path finding (Ferguson et al. 2007). That a PtdIns(3,4,5)P_3 gradient is required in the process was nicely demonstrated in neutrophils from mice lacking the lipid 5′-phosphatase SHIP (Nishio et al. 2007), or from mice expressing constitutively membrane targeted PI3Kγ (Costa et al. 2007). While loss of PTEN did not affect

neutrophil migration (Nishio et al. 2007), PTEN was essential for the formation of gradients in PtdIns(3,4,5)P_3 in the amoebic form of *Dictyostelium*discoideum (Chen et al. 2007; Funamoto et al. 2002). In T cells is has been shown that the guanine nucleotide exchange factor DOCK2 (dedicator of cytokinesis 2) mediates the activation of Rac largely independent of PI3K (Nombela-Arrieta et al. 2004, 2007). In neutrophils DOCK2 translocation to the leading edge is PtdIns(3,4,5)P_3-dependent, but was recently suggested to be supported by phosphatidic acid generated by ligand-stimulated phospholipase D activity (Nishikimi et al. 2009; Kunisaki et al. 2006).

Simplified, one could deduce from the above that resting cells of the myeloid linage depend on PI3K for migration and adhesion, while manipulations of PI3Ks in lymphocytes modulate linage development. Loss of a single class PI3K modulates the output of both the innate and the acquired immune system, but did not lead to severe immune deficiencies in mice. PI3Kγ and PI3Kδ are thus considered as valuable targets in inflammatory, allergic and autoimmune disease.

5.2.2 PI3K in Inflammation and Allergy

Tissue resident cells including macrophages and mast cells initiate inflammation and allergy when triggered by pathogens or antigens. Cytokines and chemokines released by these cells activate endothelia in close-by blood vessels to recruit neutrophils, monocytes or lymphocytes to the site of inflammation. PI3Kγ is required for chemokine-dependent recruitment of neutrophils to tissues, and macrophages require PI3Kγ to fight peritoneal infections (Hirsch et al. 2000). In allergy, tissue mast cell concentrations are elevated, and migration of mast cells also depends on PI3Kγ (Kitaura et al. 2005). Invading pathogens are opsonized by triggering the complement cascade, and are decorated with specific antibodies, or interact with Toll-like receptors (TLRs). All these actions can lead to PI3K activation and the promotion of cytokine production (Ghigo et al. 2010; Wymann et al. 2000, Fig. 5.3).

5.2.3 Atherosclerosis and Cardiovascular Disease

Atherosclerosis is initiated by the excessive uptake of oxidized low-density lipoproteins by (LDL) by macrophages. These macrophages accumulate in the intima of blood vessels, and chronic lipid uptake turns them into foam cells. The disintegration of foam cells leads to the formation of fatty streaks and atherosclerotic plaques. Finally, rupture and repair of atherosclerotic plaques leads to stenosis and eventually to the closure of arteries by thrombosis, culminating in myocardial infarction and stroke (Lusis 2000; Glass and Witztum 2001). Atherosclerosis is an inflammatory disease (Hansson and Hermansson 2011), and mouse genetic data demonstrates that chemokine receptor signaling selectively recruits monocytes (Boring et al. 1998) and T cells (Heller et al. 2006; Braunersreuther et al. 2007a, 2007b; Damas et al. 2007)

during the onset of atherosclerosis. These findings have initiated the search for novel drugs to treat atherosclerosis beyond statins (Opar 2007).

Oxidized LDL activates PI3K in macrophages, and the release of granulocyte/macrophage colony-stimulating factor (GM-CSF) promotes the on-site proliferation of macrophages (Biwa et al. 2000a, 2000b). Interestingly, oxidized LDL did not activate PI3K in macrophages derived from PI3Kγ null mice (Chang et al. 2007). Mice devoid of apo-lipoprotein E (apoE; Zhang et al. 1992; Plump et al. 1992; Nakashima et al. 1994) or the LDL receptor (LDLR; Ishibashi et al. 1993) rapidly develop atherosclerotic plaques, which were significantly reduced in mice without PI3Kγ (Fougerat et al. 2008; Chang et al. 2007). The attenuation of plaque formation observed in PI3Kγ null mice was also reproduced using AS605240 (Fougerat et al. 2008; Chang et al. 2007), a selective PI3Kγ inhibitor (Camps et al. 2005). It has been reported that the lack of PI3Kγ attenuates E-selectin-dependent neutrophil adhesion to endothelial cells, and a role of PI3Kγ in endothelial cells was proposed to control cell recruitment significantly (Puri et al. 2005). Bone marrow transplantation experiments could, however, demonstrate that the main role of PI3Kγ in atherosclerosis is associated with the hematopoietic compartment (Fougerat et al. 2008, 2009). Macrophages are without doubt the executers of atherosclerosis, but a role for type 1 helper T cells (Th1) in the acceleration of atherosclerotic lesions has been proposed (Song et al. 2001), while regulatory T cells counteract the formation of plaques (Ait-Oufella et al. 2006; Nilsson et al. 2009).

The formation of atherosclerotic lesions remains non-symptomatic for a long time, and only stenosis and complete occlusion of critical blood vessels is noticed. The frequency of plaque rupture is enhanced in patients with hypertension due to increase shear forces. Interestingly, PI3Kγ null mice are protected against angiotensin II-induced hypertension (Vecchione et al. 2005). Moreover, loss of PI3Kγ also protected mice from ADP-induced thromboembolic vascular occlusion, which is initiated by micro-coagulation of blood platelets (Hirsch et al. 2001). Pharmacologic experiments using p110β selective compounds (TGX-221) demonstrated subsequently the importance of PI3Kβ in platelet-mediated thrombosis triggered by ADP, collagen and integrin-dependent stimulation (Jackson et al. 2005). These data were confirmed in mice expressing a catalytically inactive form of the p110β catalytic subunit of PI3Kβ (Canobbio et al. 2009). PI3Kβ downstream of alpha(IIb)beta3 integrins, and PI3Kγ and PI3Kβ downstream of the ADP-binding P2Y12 receptor thus cooperate to maintain stable platelet aggregates (Cosemans et al. 2006).

PI3Ks also promote cardiac hypertrophy, which is a consequence of chronic hypertension in humans: when specifically expressed in the heart, constitutively activated PI3Kα caused an increase in heart and cardiomyocyte size. In these mutant mice, cardiac function and architecture as determined by echocardiography was not affected (Shioi et al. 2000). A more dramatic increase in heart size could even be achieved by the targeted expression of a permanently activated form of PKB/Akt. Interestingly, this phenotype could be counteracted by treatment with rapamycin, demonstrating that the PI3K/PKB/TOR pathway is an important regulator of cell and organ size (Shioi et al. 2002). Similar results were obtained by the inactivation of PTEN in cardiomyocytes: heart size was increased due to an increase cell size

of cardiomyocytes, but additionally a reduced cardiac contractility was observed in hearts lacking PTEN. Surprisingly it was found that the combination of targeting PTEN and elimination of PI3Kγ protein in cardiomyocytes reconstituted contractility without reverting heart size (Crackower et al. 2002). Class I PI3Ks, and likely PI3Kα (Luo et al. 2005), seem therefore to control cardiomyocyte size, while PI3Kγ is linked to the regulation of contractile force. Unchallenged PI3Kγ knock-out mice do not display a cardiovascular phenotype, but when subjected to pressure overload by transverse aortic constriction (TAC), they rapidly suffered from fatal heart failure. As mice expressing a catalytically inactive PI3Kγ protein had no signs of apoptosis and fibrosis in the heart, it became clear that PI3Kγ had a function in the heart that is not linked to its lipid kinase activity. Finally it was determined that PI3Kγ functions as a scaffold for cAMPsignaling, as it interacts with phosphodiesterase 3B. If PI3Kγ protein is absent in the heart, cAMP rises and contractility under stress increases (Patrucco et al. 2004). Recently, it was found that cAMP regulation works in both directions, because cAMP-dependent kinase (PKA) can phosphorylate and inactivate PI3Kγ in the heart (Perino et al. 2011).

In summary, inhibition of PI3K in hypertension, hypertrophy and atherosclerosis appears to be beneficial. Recent pharmacological studies indeed demonstrated that the inhibition of PI3Kγ and PI3Kδ reduced infarct size caused by inflammatory processes initiated after ischemia/reperfusion damage. Mice treated with the dual-specific PI3Kγ/δ inhibitor TG100-115 displayed reduced inflammation and edema at infarct sites. Tissue repair processes and endothelial cell mitogenesis, which are required after myocardial infarction, were not affected by the compounds (Doukas et al. 2006, 2007). Clinical trials in patients suffering from acute myocardial infarction were concluded (Table 5.1; refer to Vol. 1, Chap. 6).

5.2.4 Allergic and Hypersensitivity Responses

Mast cell are primary effector cells in inflammation, allergic disease such as, asthma, rhinitis and atopic dermatitis. Mast cells bind IgE with a high affinity receptor (FcεRI). Crosslinking of FcεRI receptors tips the balance towards mast activation, as src-like protein tyrosine kinases (e.g. Lyn) phosphorylate immunoreceptor tyrosine-based motifs (ITAMs) on the FcεRI receptor's β and γ chains. Subsequently, the SH2-containing Syk protein tyrosine is recruited and promotes the phosphorylation of multiple tyrosines on membrane-anchored adapters such as LAT, NTAL/LAB (Rivera 2005), and Grb2-associated binder 2 (Gab2; Gu et al. 2001). Class IA PI3Ks are then translocated and activated to trigger Bruton's tyrosine kinase (Btk) and phospholipase Cγ (PLCγ) by providing PtdIns(3,4,5)P_3 as a docking site for the PH domains of Btk and PLCγ. Activation of PLCγ leads eventually to the release of Ca^{2+} from intracellular stores, which trigger store operated Ca^{2+} channels to finally cause the release of histamine-containing granules and the production of inflammatory mediators (Kraft and Kinet 2007; Kim et al. 2008).

Surprisingly, it has been found that mice lacking functional PI3Kγ (knock-outs or catalytically inactive K833R mutants) are protected in models of passive systemic or cutaneous anaphylaxis. This finding was corroborated in bone marrow-derived mast cells (BMMCs), which depend on PI3Kγ for a full-scale degranulation response when exposed to IgE/antigen complexes. It was then established that a release of adenosine triggered PI3Kγ activation, which synergizes with the protein tyrosine kinase cascade downstream of FcεRI receptors (Laffargue et al. 2002; Wymann et al. 2003a). Mice harboring a catalytically inactive PI3Kδ also displayed a partially attenuated response to IgE/antigen complexes, while the relay of stem cell factor signaling to PI3K was completely abrogated, suggesting that in mast cells PI3Kδ is the only class IA PI3K associating with the c-kit receptor (Ali et al. 2004, 2008). PI3Kγ and PI3Kδ are thus currently evaluated as therapeutic targets in allergic disease: although asthma models in the mouse have a somewhat limited predictive value for the clinical outcome in man (Stevenson and Birrell 2011), studies using the PI3Kδ-selective inhibitor IC87114 in ovalbumin challenged BALB/c mice attenuated a number of disease parameters like leukocyte recruitment, mucus secretion, and Th2-derived release of cytokines and IgE into lung cavities (Lee et al. 2006a, 2006b). Paradoxically, other studies showed that unspecific and ovalbumin-specific IgE levels increase due to PI3Kδ inhibition (Zhang et al. 2008; Omori et al. 2006). Dual inhibition of PI3Kγ and PI3Kγ was achieved using aerosols of TG100-115 in ovalbumin-challenged mice, and was efficiently reducing airway hyper-responsiveness (AHR) even in a semi-therapeutic setting where the drug was applied after the ovalbumin challenge (Doukas et al. 2009). Other studies pointed to a role of PI3Kγ in the chemokine-mediated and ovalbumin-induced leukocyte recruitment to the lung in response to ovalbumin sensitization (Thomas et al. 2005, 2009).

While patients with allergic asthma often respond to treatment with corticosteroids or β_2-adrenergic agonists acting as bronchodilators, chronic obstructive pulmonary disease (COPD) patients suffer from a progressive disease refractive to current treatment (Barnes 2008; Hansel and Barnes 2009). COPD is induced by smoking in >90% of all cases, and exposure to cigarette smoke or LPS are used in animal models to mimic the human disease driven by type 1 helper T cells (Th1). In a smoke exposure model, TG100-115 successfully attenuated inflammatory readouts and reversed the steroid resistance observed in these settings (Doukas et al. 2009). As similar results were obtained by genetic inactivation of PI3Kδ (Marwick et al. 2009), and using PI3Kδ-specific inhibitors (IC87114; To et al. 2010), resolution of COPD parameters might have been mediated by the inhibition of PI3Kδ even in the case of TG100-115. Because ARH and COPD models are currently discussed controversially (Stevenson and Birrell 2011), and reference molecules for COPD models are missing, definitive conclusions concerning the best PI3K isoform profiles require further studies.

5.2.5 Autoimmune Diseases: Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease and affects about 1% of the world's population. RA has a gradual onset inflicting initially a limited number of

synovial joints, where inflammation progresses to cause cartilage and bone erosion, culminating in joint destruction. RA can involve other tissues like skin, blood vessels, heart, lungs and muscles. The disease is initiated by $CD4^+$ memory T cells, which cross the synovial membrane. Subsequently, they release cytokines including IL-2 and interferon γ (IFN-γ), which in turn activate macrophages and fibroblasts, and trigger monocyte recruitment. A wave of pro-inflammatory cytokines such has TNF-α, IL-1 and IL-6 isthen released to set off chronic inflammation (Firestein 2003, 2006; Steiner 2007). In the final stage of the disease, T- and B-cells, dendritic cells, macrophages, mast cells, and hyperplastic synovial fibroblasts collaborate to maintain inflammation. A constant influx of high numbers of neutrophils into the joint endorses cartilage and bone destruction, and tissue repair and neovascularization in the synovial membrane promote the process. As mentioned above, PI3Kγ and PI3Kδ have non-redundant roles in T- and B-cell differentiation and function, and the lipid kinases are key to leukocyte and mast cell migration, and mast cell degranulation.

Mouse models for RA include active immunization models like collagen-induced arthritis (CIA, Stuart et al. 1984) and passive models utilizing auto-antibodies from immunized or auto-immune mice (anti-collagen II-IA: Terato et al. 1992; K/BxN serum model: Korganow et al. 1999; Schaller et al. 2001). CIA is initiated by the intradermal injection of type II collagen. Subsequently, features of the human disease like cell-infiltration into the synovial space, hyperplasia, pannus formation, and cartilage and bone erosion, can be observed. CIA needs functional T- and B-cells, whereas in the passive models full T- and B-cell function is dispensable. In contrary to mouse models, no specific auto-antigen has been identified in the human disease.

When PI3Kγ null mice were challenged in a passive (αCII-IA) RA model, mutant mice were protected from RA development, and showed minimal paw swelling, and bone and cartilage erosion. An orally available salt of the PI3Kγ-specific inhibitor AS605240 was effective in the CIA and αCII-IA model, and even had a therapeutic effect when added after the onset of the disease. Both, the genetic ablation of PI3Kγ and the pharmacological inhibition of the enzyme suppressed the recruitment of neutrophils to the joints, which is a hallmark of RA (Camps et al. 2005).

5.2.6 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a complex autoimmune disease correlating with polygenic genetic disposition (Harley et al. 2008; Fairhurst et al. 2006). The disease is initiated by autoreactive $CD4^+$ memory T-cells, which trigger polyclonal B-cell expansion, leading to hyper-gammaglobulimia. Anti-nuclear autoantibodies (ANAs) often precede the clinical manifestation of the disease by years. In the late stage of the disease, patients eventually develop glomerulonephritis due deposition of autoantibody complexes in the kidney, culminating in renal failure.

Inbred mice of the MRL strain, which are homozygous for the lymphoproliferation (lpr) mutation (the MRL-*lpr/lpr* model; Cohen and Eisenberg 1991; Singer et al.

1994) progress spontaneously towards a SLE-like autoimmune disease. The MRL-*lpr/lpr* mice show clinical features of the human disease like the generation of ANAs, increased numbers of autoreactive $CD4^+$ T-cells, and finally the accumulation of immunoglobulin complexes in kidney and salivary glands (Liu and Wakeland 2001).

That PI3K signaling could be relevant in the progress of SLE emerged when it was observed that the heterozygous deletion of PTEN, or the constitutive activation of class IA PI3K by the transgenic expression of a truncated regulatory subunit (Lck promoter—p65(PI3K) transgene), in T lymphocytes led to a SLE-related disease (Di Cristofano et al. 1999; Borlado et al. 2000). When PI3Kγ was deleted from Lck-p65 mice, these animals showed an attenuated form of SLE. Lymphoproliferation and T-cell infiltration was still imminent (Barber et al. 2006), but the survival of $CD4^+$ T-cells was impaired. This resulted in a reduced progress of nephritis and longer life span (Barber et al. 2005). The importance of PI3Kγ in the SLE progression was further underlined by the action of the PI3Kγ inhibitor AS605240 in MRL-*lpr/lpr* mice, where it reduced $CD4^+$ T-cells, autoantibody concentrations and kidney failure, thus increasing live span. In mice, PI3Kγ inhibition was better tolerated than dexamethasone used as a reference drug. It must be noted that glucocorticoids lead to a dramatic immunosuppression in rodents, which makes them very susceptible to infections (Chatham and Kimberly 2001). The comparison of dexamethasone and AS605240 indicated that PI3Kγ inhibitors might have a decent therapeutic window in SLE, without causing too severe side effects.

5.2.7 PI3Ks in Chronic Inflammation and Allergy—Preliminary Conclusions

Combining mechanistic, cellular and mouse model data, we have a good validation that PI3Ks are valuable drug targets in chronic inflammation, allergy and autoimmune disease. As mentioned above, PI3Kγ and PI3Kδ are required for B- and T-cell development and function, and PI3Kγ has a prominent role modulating chemotaxis and recruitment of myeloid cells. PI3Kβ and PI3Kγ are involved in platelet aggregation. A role for PI3Kγ has also been demonstrated in pancreatitis (Lupia et al. 2004). Other diseases like psoriasis (Lowes et al. 2007; Schon and Boehncke 2005), and multiple sclerosis (Hauser and Oksenberg 2006; Hemmer et al. 2002) rely on cellular networks that should also respond to PI3K inhibition. The effects of PI3Kγ on cardiovascular tone open avenues for preventive treatments in hypertension and cardiovascular disease (refer to Vol. 1, Chap. 6).

In spite of all these findings, there are not too many isoform-specific PI3K inhibitors available (see Table 5.1), and only one was transiently in clinical trials for anti-inflammatory actions (TG100-115). One reason for this might be that PI3K inhibitors in non-fatal disease have to meet higher safety standards, another that they have to be better and cheaper than existing medication, as they compete with steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and recently developed biological targeted therapies (e.g. anti-TNF-α, IgE, IL neutralizing antibodies). Data of biotech

companies has been presented in recent scientific meetings, demonstrating that there is a pipeline for isoform specific inhibitors.

5.3 The PI3K/mTOR Pathway in Cancer

Patients with metastatic solid tumors have often a very bad prognosis, and even under best possible standard care the survival after diagnosis is short. The development of imatinib/Gleevec and its success as BCR-abl kinase inhibitor for the treatment of chronic myelogenousleukemia raised hopes that targeted therapies with limited adverse effects could be achieved for other cancers. As shown in Fig. 5.3, many oncogenes activated or amplified in cancer feed into the PI3K pathway: ErbB2/Her2 is amplified in breast cancer, c-kit is mutated in gastrointestinal stromal tumors (GIST), VEGFR promotes angiogenesis in growing tumors, EGFR drives proliferation of non-small cell lung cancer of non-smokers, mutated Ras signals in lung cancer of smokers, and last but not least, antagonists of the PI3K pathway such as lipid phosphatases are frequently lost in cancer.

5.3.1 PtdIns(3,4,5)P_3 Rising—Loss of PTEN

The tumor suppressor PTEN counteracts and balances the action of PI3K by hydrolysis of PtdIns(3,4,5)P_3. In normal cells, PTEN levels and activity are tightly controlled by transcription factors, methylation, oxidation, phosphorylation, ubiquitination, micro RNAs and more (for reviews see (Leslie et al. 2008; Carracedo et al. 2011)). The importance of levelingPtdIns(3,4,5)P_3 was convincingly demonstrated in mice with a targeted PTEN locus: even *Pten* heterozygous mice developed multiple forms cancers, manifesting in the prostate, breast, uterus, and other organs (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999).

Many tumors attenuate expression of PTEN, which often occurs by methylation of the *PTEN* promoter, or by a process called “loss of heterozygosity”. This involves the deletion of both PTEN alleles, and results in a complete loss of PTEN protein expression. Downregulation of PTEN by promoter methylation has been frequently detected in melanoma, prostate, breast, endometrial and colorectal cancer, as well as and leukemia (Khan et al. 2004; Goel et al. 2004; Stahl et al. 2004; Mirmohammadsadegh et al. 2006). Spontaneous mutations in *PTEN* have been identified in more than half of all melanoma, glioma, prostate, endometrial and ovarian cancers, while attenuation of PTEN is less frequent in breast cancer (Mirmohammadsadegh et al. 2006; Li et al. 1997; Cairns et al. 1997; Wu et al. 2003). Mutation of *PTEN* is a late step in tumor progression (discussed in more detail in (Wymann and Marone 2005)), and is usually detected in late stage or metastatic tumors (for reviews see (Vivanco and Sawyers 2002; Wymann and Marone 2005; Cully et al. 2006; Abraham 2004)). A reason for the late appearance of changes in PTEN in tumors is likely to be

"oncogene-inducible senescence" (for a short review see (Braig and Schmitt 2006)). Oncogene-inducible senescence was observed before for Ras and BRaf, and has been described as a fail-safe mechanism preventing tumor growth after single oncogene mutations. For PTEN, oncogene-inducible senescence has been demonstrated in a conditional *PTEN*-deficient mouse model: deletion of both PTEN alleles in the prostate is surprisingly slow to generate prostate cancer. When p53 was targeted at the same time, however, aggressive and fatal prostate cancer developed rapidly (Chen et al. 2005), demonstrating that loss of PTEN is opposed by p53 tumor suppressor genes. Tumors thus profit from loss of PTEN only at a late stage and as "second hit" mutagenesis.

5.3.2 Mutations in PIK3CA (p110α)

The key role for PI3Ks in cancer progression was further underlined by frequently occurring mutations in the gene coding for the catalytic subunit of PI3Kα (PIK3CA) in human tumors. *PIK3CA* mutations cluster in two hotspots coding for the helical (see PI3Ka in Fig. 5.2; exon 9) and the catalytic domain (PI3Kc; exon 20; Samuels et al. 2004; Thomas et al. 2007; TCGA study 2008; Parsons et al. 2008; Stemke-Hale et al. 2008; for a review see (Bader et al. 2005)). When mutated, both sites yield a constitutive active enzyme with transforming capacity in fibroblasts. Cells expressing mutant p110α display also an increased invasive capacity. This implies that *PIK3CA* mutations promote tumor cell survival and metastasis (Kang et al. 2005; Ikenoue et al. 2005; Samuels et al. 2005). In human cancers, the helical domain residues Glu542 and Glu545 are usually mutated to Lysine, and the C-terminal kinase domain residue His1047 is converted to Arginine. A rational for the increased activity of mutant p110α was provided by crystallographic data obtained from a complex of p85 fragments bound to the N-terminus of p110α (Miled et al. 2007; Huang et al. 2007). As described above, the p85 regulatory subunit interacts tightly with p110α, stabilizes the p110α protein and inhibits PI3K activity at the same time. In normal wild type p110α, the N-terminal SH2 domain of p85 (nSH2) mediates its inhibition via contacts within the helical domain. When negative charges in the helical domain are inverted by the mutation to Lys (Glu545Lys), charge-charge interactions are disrupted and full PI3K activity is released. The activation of p110α by C-terminal mutations (His1047Arg) can be best understood in the context of the concept of the "regulatory square" recently proposed for the regulation of p110β activity (Zhang et al. 2011; Vogt 2011). Of a set of three α-helices forming a square around the catalytic groove of p110s, the "elbow" at the start of the last α-helix is in contact with the C-terminal SH2 domain of p85 (cSH2). In the presence of the cSH2, the C-terminal helix in p110 seems to be clamped into a conformation that constrains residues in the catalytic loop into an inactive conformation. In wild type p110β, a Leucine (Leu1043 in p110β) at the elbow position allows for high basal activity, and p110β activity could indeed be restrained when Leu1043 was exchanged for a Histidine (Zhang et al. 2011). The His1047 in the elbow region seems thus to restrict

p110α activity inherently, while p110β and p110δ are constrained by the cSH2 of p85 (for reviews see (Backer 2010; Vogt 2011; Vadas et al. 2011)).

PIK3CA is amplified in various tumors, frequently in ovarian, cervix and lung cancers (Shayesteh et al. 1999; Zhang et al. 2002; Racz et al. 1999). Activating *PIK3CA* mutations were detected in solid tumors in breast, endometrial, colorectal, upper digestive tract, gastric, pancreas, brain, lung and hepatocellular carcinomas (Bader et al. 2005; Samuels and Ericson 2006; for a list of mutation frequencies see (Liu et al. 2009)).

5.3.3 Mutations in p85 Regulating Class IA PI3Ks

Truncated forms of p85 regulatory subunits have been shown earlier to constitutively activate class IA PI3Ks. A truncation mutant containing amino acids 1-571 of p85α fused to a fragment of the eph tyrosine kinase family (p65-PI3K) has been isolated from a mouse lymphoma model (Borlado et al. 2000; Chan et al. 2002; Jimenez et al. 1998; for mechanistic investigations see (Shekar et al. 2005, #40429; Backer 2010, #62337; Huang et al. 2008, #56273; Huang et al. 2007, #46673; Miled et al. 2007, #38727)), but was not identified in human cancers. A somewhat longer truncation mutant was isolated from a human lymphoma cell line (p76-PI3K; Jucker et al. 2002), and an infrequent incidence of p85α (*PIK3R1*) mutations were reported in ovarian and colon tumors (Philp et al. 2001), and breast cancer (Wood et al. 2007). The highest frequency of p85α mutations was detected in glioblastoma (TCGA study 2008; Parsons et al. 2008). Until recently, little was known concerning their capacity to activate p110α, and their relevance in tumor progression. A meta-analysis of p85α mutations was performed in (Jaiswal et al. 2009), where a number of mutants modulating contacts between the p85α-iSH2 and the p110 C2 domain were investigated. In particular Asn564Asp and Asp560Tyr mutants of p85α were effectively promoting fatal tumorigenesis in a BaF3 cell mouse model (Jaiswal et al. 2009). *PI3KR1* was also recently identified as a colon cancer oncogene in a transposon insertion screen (Starr et al. 2009). The attenuation of lipid kinase activity by p85-p110 interactions is therefore crucial in cellular hemostasis, and the discussed structural studies are key for the understanding how mutations in the class IA heterodimer releases constraints on lipid kinase in disease.

5.3.4 Downstream of PI3K: TOR

Cellular growth is an important parameter in tumor progression, and is regulated by the availability of energy and nutrients. A central hub to integrate nutrient, energy, but also hormonal inputs are the target of rapamycin (TOR) complexes. There are two target of rapamycin (TOR) complexes: (i) TORC1, where the TOR protein kinase is associated with Raptor and (ii) TORC2 is bound to Rictor. TORC1 is

activated by downstream of PKB/Akt (schematically shown in Fig. 5.4, for a in depth review on TOR signaling see (Zoncu et al. 2011)) and the Ras/MAPK cascades (not discussed here), which phosphorylate and inactivate TSC2. An energy sensing pathway acting via AMPK (AMP-dependent protein kinase) can shut off TORC1 when AMP accumulates. Finally, amino acids regulate TORC1 association with RAG proteins, re-localizing the complex to late endosomes (Sancak et al. 2010; Sancak and Sabatini 2009). Activation of TORC1 initiates cap-dependent translation via phosphorylation of 4E-BP1 (eIF4E-binding protein 1), and phosphorylation of $p70^{S6K}$ promotes translation of ribosomal proteins (Dufner and Thomas 1999) and ribosome biogenesis (Wullschleger et al. 2006).

Mutations or loss of heterozygosity in TSC components gives rise to the initially benign, autosomal dominant TSC syndrome (van Slegtenhorst et al. 1997), manifested by hamartomas in a variety of organs (Cheadle et al. 2000), and an elevated risk to develop renal carcinoma. The serine-threonine protein kinase LKB1 upstream of AMPK normally balances TORC1 activity (Woods et al. 2003). Loss of function of LKB1 causes the Peutz-Jeghers syndrome, a familial colorectal polyp disorder. Peutz-Jeghers syndrome patients have a high risk for cancers in various tissues (Boudeau et al. 2003). Some signaling molecules downstream of TOR were also used as diagnostic markers: elevation of eIF4E correlates with a bad prognosis in a variety of cancers (Bjornsti and Houghton 2004).

5.4 Pharmacological Targeting of PI3K/TOR Signaling

Drugs to inhibit signals emerging from mutated or up-regulated growth factor receptors are already on the market or in clinical trails, and include neutralizing antibodies and protein tyrosine kinase receptor inhibitors. Knowledge from targeted therapies interfering with for example EGFR, ErbB2/Her2, and VEGFR, have validated two strategies to attack tumor cells: a first one aiming to reverse tumor autonomous signaling, and a second one targeting tumor-induced angiogenesis. As it turns out, targeting PI3K and TOR contributes to both approaches.

5.4.1 Targeting TOR—Rapamycin and Derivatives

Besides their effects on immune cells, Rapamycin derivatives (rapalogs; see Table 5.1) act as anti-angiogenic drugs. Rapamycin considerably reduces the production of VEGF, and more importantly intercepts the action of this growth factor on vascular endothelia (Guba et al. 2002). The latter has been shown to be mediated by the inhibition of hypoxia-inducible factor 1α (HIF1α) expression (Lane et al. 2009). RAD001, CCI-779, AP23573 and other rapalogs are highly specific, allosteric inhibitors of TORC1. Rapalogs bind to FK506-binding protein 12 (FKBP12) with a K_d in the sub-nanomolar range (Banaszynski et al. 2005), and this rapalog/FKBP12

complex then tightly interacts with the FRB domain of the TOR kinase. The activity of TORC2 is not affected by rapalogs.

Presently, there are >700 clinical trials registered exploring the actions of these compounds in proliferative disease, mostly designed as studies combining rapalogs and best standard of care, chemotherapy, or other targeted therapies. RAD001/Everolimus and CCI-779/Temsirolimus made it recently to market, and are approved for the treatment of organ rejection and renal cell carcinoma (Motzer et al. 2008; Atkins et al. 2009; Dancey 2010; for more references see Table 5.1). The endpoint of the clinical studies was progression-free survival (PFS). Renal cell carcinoma patients receiving Everolimus had a median PFS of 4.9 month, while the PFS of patients receiving placebo was 1.9 month (Motzer et al. 2008; Atkins et al. 2009; FDA documentation at http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/022334lbl.pdf). Encouraging results with rapalogs were also obtained in mantle cell lymphoma (Johnston et al. 2010b; see also the approval of Temsirolimus (Dancey 2010)), soft tissue and bone sarcoma (Blay 2011), and endometrial cancer (for reviews see (Hay 2005; Faivre et al. 2006; Guertin and Sabatini 2007)).

In a subset of tumor-derived cell lines and patient biopsies elevated levels of PKB/Akt Ser473 phosphorylation were detected after the inhibition of TORC1 using rapalogs (Sun et al. 2005; O'Reilly et al. 2006; O'Donnell et al. 2008; Tabernero et al. 2008). It has been established, that $p70^{S6K}$, activated downstream of TORC1, phosphorylates insulin receptor substrate-1 (IRS1), and blocks its interaction with the insulin receptor. This leads to reduced phosphorylation of IRS on YxxM motifs, and thus attenuates recruitment of p85/p110 complexes and class IA PI3K (Shaw and Cantley 2006; Harrington et al. 2005; Manning 2004). When the activity of $p70^{S6K}$ is blocked due to TORC1 inhibition, PKB/Akt is hyper-phosphorylated and eventually promotes cell survival and metastasis (see Fig. 5.4). Others have reported that the targeting of TORC1 can trigger a PI3K-dependent feedback loop activating MAPK in human cancer (Carracedo et al. 2008). All this raised concerns that feedback activation of these stimulatory pathways would diminish the success of rapalog-based therapies (Hay 2005; Shaw and Cantley 2006; Rosen and She 2006). In contrast, it has been recently demonstrated that high basal levels of PKB/Akt Ser473 phosphorylation correlate with a sensitivity to RAD001, and that a subsequent RAD001-induced increase in phospho-PKB/Akt does not correlate with cell viability after rapalogs exposure (Breuleux et al. 2009). It has already been demonstrated earlier, that cells expressing constitutively activated PKB/Akt (myr-PKB/Akt; *N*-terminally myristoylated) were sensitive to CCI-779 (Neshat et al. 2001). Similarly, it was found that transgenic expression of myr-PKB/Akt in endothelial cells induced a pathological, tumor-like form of angiogenesis in non-tumoral tissues, which could be reversed by rapamycin (Phung et al. 2006). Early notions that loss of PTEN was an indicator for sensitivity to rapalogs (Neshat et al. 2001), have been disputed recently (Yang et al. 2008; for in-depth reviews see (Faivre et al. 2006; Hay 2005; Dancey 2010)). In the future it will be interesting to compare the efficiency of rapalogs with the performance of molecules targeting PI3K and mTOR kinase activities in clinical settings.

5.4.2 Targeting PI3K and mTOR Kinase

Although wortmannin (IC_{50} = low nM, Arcaro and Wymann 1993; Yano et al. 1993; Wymann and Arcaro 1994) and LY294002 (IC_{50} = high μM, Vlahos et al. 1994) inhibit a broad range of PI3K and PIKK family enzymes, the two compounds were instrumental to dissect PI3K signaling and to initiate PI3K drug development (Marone et al. 2008; Wymann and Schneiter 2008). The development of drug-like PI3K inhibitors was complicated by the relative difficulty to set up relevant *in vitro* and cell-based high throughput assays. Phosphoinositides are (still) complicated substrates, and variations of assay conditions can influence PI3K activity considerably. Moreover, cellular PI3K activity is mostly detected indirectly by phosphorylated PKB/Akt, where the readout can be convoluted by feedback mechanisms. The direct detection of cellular PtdIns(3,4,5)P_3 is cumbersome (Dove and Michell 2009), and only recently non-radioactive, mass-spectroscopy-based methods became available to determine cellular PtdIns(3,4,5)P_3 (Clark et al. 2011; Kiefer et al. 2010; Pettitt et al. 2006; for a commentary see (Wymann and Wenk 2011)).

Early efforts to produce drug-like molecules from wortmannin have been initiated even before PI3K was identified as the inhibitor's target. Wander AG in Bern, Switzerland, was the first pharmaceutical company to unknowingly develop "PI3K inhibitors" as anti-inflammatory compounds (Baggiolini et al. 1987). Trials to separate toxicity and pharmacological action of wortmannin-derivatives were not successful before the target enzyme was identified (Arcaro and Wymann 1993; Yano et al. 1993; Wymann and Arcaro 1994; Thelen et al. 1994), but were taken up by others later: Ihle and colleagues (Ihle et al. 2004) modified the furan ring of wortmannin to slow down covalent reactions of wortmannin-derivatives (Wymann et al. 1996). The result of this work led to the development of the wortmannin-derivative PX-866, which displays reduced liver toxicity as compared to wortmannin, and is currently in clinical trials in solid tumors (see Table 5.1, Ihle et al. 2004; Williams et al. 2006).

Semaphore Pharmaceuticals modified LY294002, and linked it to a RGD peptide to yield SF1126. SF1126 displays an increased solubility and targets the LY294002-derivative to integrins on cancers cells (Garlich et al. 2008; Ozbay et al. 2010).

Recently, the design of small molecules targeting PI3K has been facilitated by the availability of an extensive collection of inhibitor/PI3K structures. The release of the first class I PI3K structure—the catalytic PI3Kγ subunit p110γ (Walker et al. 1999)—was soon followed by p110γ bound to ATP and a variety of PI3K inhibitors such as wortmannin, LY294002, and the less specific kinase inhibitors staurosporine and quercetin (Walker et al. 2000). The elucidation of the PI3Kγ structure bound to Ras provided further insight into the regulation of the lipid kinase, and its putative orientation in respect to the plasma membrane (Pacold et al. 2000). The elucidation of structures of partial p110α bound to p85 fragments (Miled et al. 2007; Huang et al. 2007) and p110β (Zhang et al. 2011) provided insight into the regulation of class IA PI3K activities (see above, for a review see (Vadas et al. 2011)). Finally, the resolution of crystal structures for p110δ and bound inhibitors clarified the dynamics

of the various p110 isoform structures, and explained specific structural dynamics opening a specificity pocket in proximity to Met752 of p110δ, which is relevant for p110δ inhibitor isoform specificity (e.g. for the p110δ inhibitor IC87114 (Knight et al. 2006; Berndt et al. 2010)).

In the past years, PI3K inhibitors have been refined, and several molecules targeting all class I PI3K isoforms (pan-PI3K inhibitors) have entered clinical testing (see Table 5.1 for molecules and references). There is a plethora of data available documenting the preclinical action and efficacy of PI3K inhibitors, which was established in xenograft and in syngeneic mouse models (for reviews see (Marone et al. 2008; Liu et al. 2009; Engelman 2009)). In these models it was clearly documented that PI3K inhibition acts usually cytostatic, arrests the cell cycle of tumor cells in G1 and only exceptionally triggers apoptosis (in vitro). In the in vivo setting, pan-PI3K inhibition displays a strong anti-angiogenic effect, which results in PI3K-induced tumor cell death and impressive reduction in tumor size in mouse models. Importantly, the effects of pharmacological targeting of PI3Ks was matched in genetic models. In this respect, it was demonstrated that PI3Kα plays not only an important role in driving tumor cell growth (see mutations in p110α, above), but takes a central role in angiogenesis (Graupera et al. 2008). Interestingly, the ablation of PI3Kβ activity attenuated tumor growth in the mammary gland (Ciraolo et al. 2008) and prostate (Jia et al. 2008; in mutant mice. When prostate cancer tumor formation was induced by the loss of PTEN, is was only the inactivation of PI3Kβ that was efficiently preventing tumor growth, while the inactivation of PI3Kα remained without significant effect (Jia et al. 2008). These results were confirmed using inducible shRNA vectors targeting specific PI3K isoforms (Wee et al. 2008).

Although class I PI3Ks and their lipid product PtdIns(3,4,5)P_3 are central to metabolic control (Wymann et al. 2003b), PI3K inhibitors did not display excessive toxicity in mouse models. As constitutive, genetic inactivation of PI3Kα causes embryonic lethality (Graupera et al. 2008; Bi et al. 1999), and genetic inactivation of PI3Kβ effects male fertility (Ciraolo et al. 2010) and triggers late stage insulin resistance (Ciraolo et al. 2008; Jia et al. 2008), but pharmacological pan-PI3K inhibition only mildly lowers blood glucose levels, it is tempting to speculate that an intermediate restoration of PI3K signaling in non-tumor tissue due to a short half-life of PI3K inhibitors reduces toxicity.

Many of the early PI3K inhibitors and clinical candidates act on PI3K and TOR in parallel. This dual mode of action was predicted for wortmannin early on (Wymann et al. 1996), and is followed by compounds like PI-103 (Fan et al. 2006), BEZ235 (Maira et al. 2008; Marone et al. 2009), BGT226, PX-866 and other molecules listed in Table 5.1. As for the pan-PI3K inhibitors above, dual-PI3K/mTOR kinase inhibitors produce impressive tumor responses in vivo (Marone et al. 2009; Workman et al. 2010; Ihle and Powis 2010; Falasca 2010; Roock et al. 2011; for reviews see (Marone et al. 2008; Liu et al. 2009; Engelman 2009; Wong et al. 2010)). In some cases, strong, prolonged mTOR kinase inhibition induces a dose-dependent hyperphosphorylation of PKB/Akt (as observed for PI-103 in melanoma (Marone et al. 2009)). Like for the TORC1-mediated feedback loop discussed above for rapamycin,

it might be crucial to better elucidate feedbacks in the PI3K/mTOR pathway to optimize the action of dual-PI3K/mTOR inhibitors.

5.4.3 Plasticity of Tumorsignaling Pathways—Resistance

Cancer progression is a complex process and involves signaling pathways distinct from PI3K. Feedback loops and cross-talk between signaling pathways can provide escape routes for cancer cells and lead to adaptive resistance. A better knowledge of the plasticity and dynamics of signaling pathways in a given patient's tumor maximizes the chances of successful targeted therapies. Imatinib (Gleevec) inactivating the constitutively activated Bcr-Abl kinase in chronic myelogenousleukemia (CML) patients is initially very efficient, because Bcr-Abl is initially the exclusive driver of CML cell proliferation. Only when Bcr-Abl is further mutated to prevent imatinib binding, or when leukemia cells acquire further oncogenic mutations, resistance to imatinib occurs.

PI3K integrates plenty of input signals from upstream receptors (Fig. 5.3) that are currently targeted with neutralizing anti-bodies or protein tyrosine kinase inhibitors. As such, breast cancer patients with tumors depending on HER2/ErbB2 are treated with trastuzumab (Herceptin), while non-small cell lung cancer patients with EGF receptor amplifications or mutations are treated with gefitinib (Iressa) or erlotinib (Tarceva). In these settings, PI3K inhibition is expected to be beneficial. Cells with a loss of PTEN or activating mutations in p110α have been shown to be sensitive to PI3K inhibition. When Ras is mutated in a tumor, such as lung cancer, the MAPK pathway and PI3K signaling are activated in parallel. Here, mouse models have demonstrated that PI3K inhibitors meet resistance, but are efficient when combined with Raf or MEK inhibitors (Wee et al. 2009; Grant 2008; Engelman et al. 2008; Downward 2008). That tumor cells develop resistance to PI3K inhibitors by the mutation of gatekeeper residues is presently considered to be unlikely, as mutational screens to generate inhibitor resistant PI3K did not produce inhibitor-resistant enzyme with relevant activity (Zunder et al. 2008).

5.5 Closing Remarks

Looking back two decades when PI3K, wortmannin, TOR, and PKB/Akt (at that time called RAC1) entered the picture, we came a long way: a plethora of input signals for PI3Ks have been identified, and the map downstream of PI3K is well filled and connected to important hubs signaling through PKB/Akt and TOR. In some instances, the available literature focuses still too much on "topical" molecules, and we thus lack a deeper understanding if specific isoforms or relatives of lipid and protein kinases and phosphatases have non-redundant physiologic functions. The same is true for the PI3K regulatory and adaptor subunits and their splice variants, where we lack precise

mechanisms how they control PI3K isoform-specific signaling in time and space. The feedback of TOR and S6K attenuating the coupling of PI3K to the activation of the insulin receptor illustrates that the whole PI3K-PKB-TOR pathway is regulated in a highly complex manner. Present therapeutic strategies using ATP-binding site inhibitors to block PI3K and PIKK activities does not quite match the complexity of the signaling network. Allosteric inhibitors and compounds targeting specific signaling complexes could provide more specific tools. Future approaches will also require solid quantification and the establishment of phosphoinositide fluxes.

Acknowledgements I apologize for not citing numerous excellent original articles due to space restrictions.

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