1 Introduction

The ability to perceive and process cues from their environment is vital to both organisms and cells. For this reason, the cells of prokaryotic and eukaryotic organisms alike are equipped with a host of receptors that sense light, mechanical stimuli, and diverse molecules ranging from ions to complex macromolecules such as proteins. Within multicellular organisms, the cellular environment is largely composed of neighboring cells, which may contact a cell *via* adhesion molecules or which may be the source of neurotransmitters or hormones, acting on other cells even if they are spatially separated. Downstream of these receptors, a complex network of proteins transduces, amplifies, and integrates these manifold inputs, culminating in an organized cellular response that adapts the cell to the conditions prevailing in its environment. All of these processes are summarized under the term 'signal transduction'.

1.1 The family of phosphoinositide 3-kinases

Phosphoinositide 3-kinases (PI 3-kinases; PI3Ks) represent a family of lipid kinases whose members affect several aspects of cellular signal transduction. The PI3K family comprises three classes of enzymes that can be distinguished and grouped on the basis of their sequence similarity, substrate specificity, and mode of activation (Fig. 1.1).

1.1.1 Class II and III PI3Ks

The class III PI3K Vps34 (vacuolar protein-sorting defective 34) is the only PI3K that is present in all eukaryotes including yeast and plants, thus representing the most ancient PI3K (Engelman *et al.*, 2006). Both *in vivo* and *in vitro* it phosphorylates phosphatidylinositol (PtdIns; PI) to phosphatidylinositol 3-phosphate (PI(3)P). The highly conserved role of Vps34 lies in membrane trafficking, where it labels certain endosomal membranes (see Lindmo & Stenmark, 2006, for review). Vps34 associates with a p150 regulatory subunit that has serine/threonine kinase activity and interacts with the GTP-bound (active) form of the early-endosomal small GTP hydrolase (GTPase) Rab5 (Volinia *et al.*, 1995; Christoforidis *et al.*, 1999). Thereby, Vps34 is recruited to Rab5-containing membranes, leading to a spatially restricted formation of PI(3)P. PI(3)P is recognized by two lipid-binding domains, the zinc finger FYVE domain, which is named after the first four proteins where it was found (Fab1, YOTB, Vac1, and EEA1, see Stenmark *et al.*, 2002), and the PX domain (Phox-homologous domain, see Ellson *et al.*, 2002).

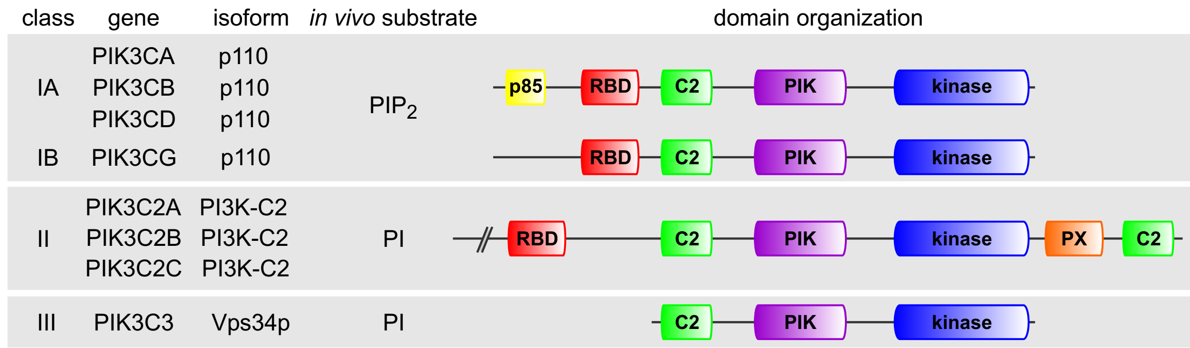

Figure 1.1: The family of PI3Ks. The classification of the PI3K family members reflects their substrate specificity and domain organization, which is schematically drawn based on SMART (simple modular architecture research tool, Schultz *et al.*, 1998). Abbreviations of domain names are as follows: p85, p85 binding domain; C2, C2 domain; RBD; Ras binding domain; PIK, PIK domain; PX, Phox-homologous domain. Figure adapted from Hawkins *et al.* (2006).

Most of these effector proteins also bind to active Rab5, rendering them coincidence detectors for PI(3)P and GTP-bound Rab5. They are critical for various processes related to endosomal membrane fusion (Lindmo & Stenmark, 2006). Vps34 is generally considered to be constitutively active. However, recent data indicate that Vps34 is stimulated to some extent by amino acid (aa)-rich medium, resulting in a Vps34-dependent activation of the kinase mammalian target of rapamycin (mTOR) in complex with raptor (see below; Byfield *et al.*, 2005; Nobukuni *et al.*, 2005).

The function of class II PI3Ks is less well understood. Although they are able to phosphorylate both PI and phosphatidylinositol 4-phosphate (PI(4)P) *in vitro*, they are thought to use PI as their predominant *in vivo* substrate (Lindmo & Stenmark, 2006). In contrast to the other PI3Ks, class II PI3Ks are monomeric enzymes. Their catalytic subunits are distinguished by an extended N terminus and additional C-terminal PX and C2 domains (see Fig. 1.1). Therefore they are named PI3K-C2α and so on. As for Vps34, the product of class II PI3K is recognized by FYVE and PX domain-containing proteins. However, class II PI3Ks generate PI(3)P at other membranes than Vps34 and may thereby address different effectors, most likely in an receptor-regulated fashion (Lindmo & Stenmark, 2006; Falasca & Mafucci, 2007). The ubiquitously expressed PI3K-C2α localizes to low-density microsomes and the trans-Golgi network independently of its PX and C2 domains (Domin *et al.*, 2000). It interacts with clathrin, indicating a possible role in clathrin-mediated membrane trafficking (Gaidarov *et al.*, 2001). PI3K-C2α has also been shown to be essential for ATP-dependent priming of neurosecretory granule exocytosis (Meunier *et al.*, 2005). Upon stimulation of cells with lysophosphatidic acid, PI3K-C2β translocates to the plasma membrane, where it might play a role in cell migration (Mafucci *et al.*, 2005). Although several stimuli including insulin, epidermal growth factor (EGF), and chemokines have been shown to activate class II PI3Ks (Turner *et al.*, 1998; Brown *et al.*, 1999;

Arcaro *et al.*, 2000), the precise mechanism and the physiological consequences of class II PI3K activation remain largely elusive.

1.1.2 Class I PI3Ks

Class I PI3Ks transduce signals from two major classes of cell surface receptors – G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) – to a set of common effectors, which regulate a plethora of cellular events. Like the class III PI3K, class I PI3Ks are heterodimeric enzymes composed of a p110 catalytic subunit and a regulatory subunit (Vanhaesebroeck *et al.*, 2001). Depending on the nature of the regulatory subunit, class I PI3Ks are further subdivided into classes IA and IB. The class IA PI3Ks p110α (Hiles *et al.*, 1992), p110β (Hu *et al.*, 1993), and p110δ (Vanhaesebroeck *et al.*, 1997) associate with regulatory subunits of the p85 family, which mediate activation downstream of RTKs (Fig. 1.2). The class IA regulatory subunits are encoded by three genes, giving rise to at least five different isoforms by alternative splicing (see Fig. 1.2 and Vanhaesebroeck *et al.*, 2001, for review), of which p85α is most abundant (Koyasu, 2003). Because the catalytic subunits do not display apparent preferences towards certain regulatory subunits (Hawkins *et al.*, 2006), class IA PI3K holoenzymes are referred to as the catalytic subunits. Whereas p110α and p110β are ubiquitously expressed, expression of p110δ is largely confined to leukocytes (Vanhaesebroeck *et al.*, 2001). p110γ represents the only class IB PI3K (Stoyanov *et al.*, 1995). It interacts with a p101 regulatory subunit that is involved in the activation of p110γ downstream of GPCRs and unrelated to the p85 adapters (Stephens *et al.*, 1997). p110γ is mainly expressed in leukocytes, but also in heart, liver, skeletal muscle, and pancreas (Stoyanov *et al.*, 1995).

All class I PI3Ks preferentially phosphorylate phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) *in vivo*, although their *in vitro* set of substrates includes PI and PI(4)P as well. Their lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP_3) is recognized by certain PH (pleckstrin homology) domains. PH domains are characterized by a common fold rather than a specific sequence motif and are one of the most prominent protein domains (Lemmon, 2004). Although a generic function for PH domains has not been identified so far, some of them are able to bind PI lipids with high affinity and specificity (Hurley & Misra, 2000). Such PH domains feature basic residues at certain positions that make up a loose consensus sequence (Vanhaesebroeck *et al.*, 2001). The class I PI3K effectors bear PH domains that bind PIP_3 or phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$). The latter is generated by the SH2-containing inositol 5-phosphatase (SHIP). SHIP1 is predominantly expressed in hematopoietic cells, where it functions as a negative regulator of immune signaling (Huber *et al.*, 1998; Krystal, 2000). In contrast, the SHIP2 isoform is broadly expressed (Rohrschneider *et al.*, 2000), but appears to lack a significant physiological role, because SHIP2 knockout mice display only a very mild phenotype (Sleeman *et al.*, 2005). PI3K signals are terminated by the 3' phosphatase PTEN

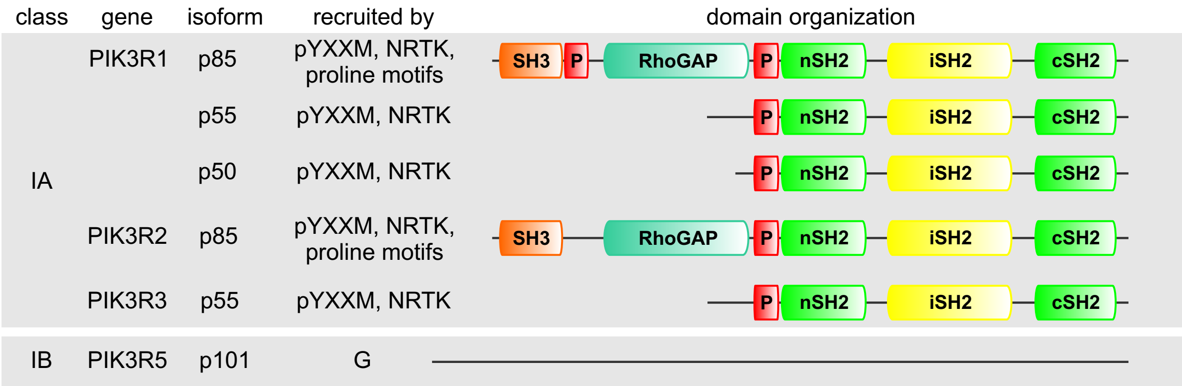

Figure 1.2: Regulatory subunits of class I PI3Ks. Gene and protein names of the regulatory PI3K subunits, recognized motifs and molecules, as well as schematic domain structures are displayed. p85-type regulatory subunits can interact with phosphorylated YXXM motifs (pYXXM) of activated RTKs or adapter proteins *via* their N- and C-terminal SH2 domains (nSH2 and cSH2), with nonreceptor tyrosine kinases (NRTKs) *via* their Pro-rich sequences (P), or with Pro-rich motifs of adapter molecules *via* their SH3 domain. Within the primary sequence of p101, SMART does not recognize any known protein domains. Figure modified from Hawkins *et al.* (2006).

(phosphatase and tensin homolog deleted on chromosome 10, see Leslie & Downes, 2002, for review), which dephosphorylates both PIP_3 and $PI(3,4)P_2$. PTEN was initially identified as a tumor suppressor gene, and its crucial role in regulating growth and proliferation was later ascribed to its phosphatase activity (Myers *et al.*, 1998). In addition, PTEN is essential for directional movement in *Dictyostelium discoideum* (Funamoto *et al.*, 2002; Iijima & Devreotes, 2002), whereas recent data indicate that mouse neutrophils rely on SHIP1 rather than PTEN to establish chemotactic movement (Nishio *et al.*, 2007). As the activity of both class IA and IB PI3Ks depends on extracellular stimuli, basal cellular levels of PIP_3 and $PI(3,4)P_2$ are very low.

Activation of class IA PI3Ks

Activation of class IA PI3Ks depends on their p85 regulatory subunits. Their inter-SH2 domains bind to the p85 binding domains of the catalytic subunits (Klippel *et al.*, 1993; Dhand *et al.*, 1994a). This interaction stabilizes the otherwise unstable class IA PI3K catalytic subunits and also inhibits their catalytic activity under resting conditions (Yu *et al.*, 1998b). The activation of class IA PI3Ks is schematically shown in Fig. 1.3. The nSH2 and cSH2 domains of the regulatory subunits recognize the sequence motif pYXXM (*i.e.* a phosphorylated Tyr followed by two arbitrary aa and Met; Klippel *et al.*, 1992; Songyang *et al.*, 1993). YXXM motifs are present in the cytoplasmic tails of RTKs and in adapter molecules such as insulin receptor substrate 1 (IRS-1) or T cell receptor-interacting molecule (TRIM). Tyr residues within these motifs become phosphorylated after activation of RTKs, which dimerize upon ligand binding and undergo trans-autophosphorylation at several sites within their cytosolic tails (see Schlessinger,

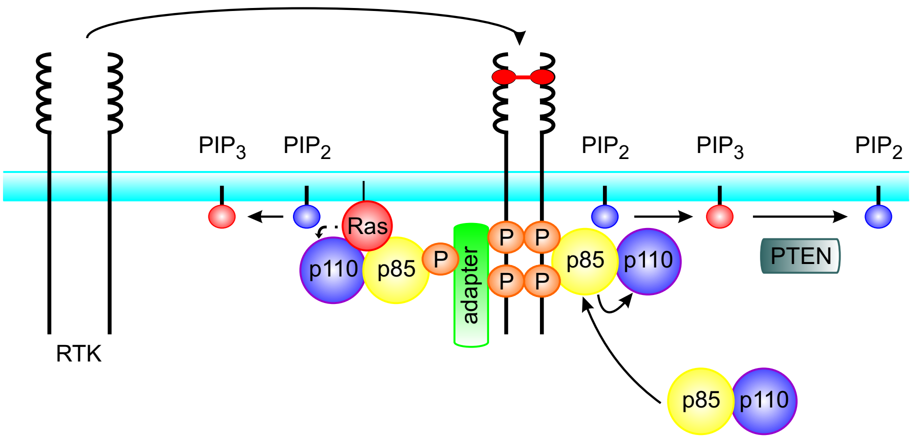

Figure 1.3: Activation of class IA PI3Ks. Class IA PI3Ks are activated downstream of RTKs. The p85 regulatory subunit binds to certain phosphorylated Tyr residues present in activated RTKs or adapter molecules. Thereby, it recruits the catalytic subunit to the plasma membrane. Binding to these motifs also relieves the p85-mediated inhibition of the catalytic subunit. Ras may further contribute to p110 activation. Activation *via* direct interaction with NRTKs and other, not Tyr-based activation pathways are likely to play minor roles and are thus omitted for clarity. PTEN reverses the effects of PI3K stimulation by dephosphorylating PIP_3 back to $PI(4,5)P_2$ (PIP_2). Figure adapted from Engelman *et al.* (2006).

2000, for review). By binding to these motifs, the p85 regulatory subunits mediate translocation of the cytosolic heterodimer to the plasma membrane (see *e.g.* Gillham *et al.*, 1999), where the catalytic subunit has access to its substrate. In addition, binding of pYXXM motifs to the N-terminal SH2 domain of the p85 adapters relieves the inhibition of the p110 subunits (Yu *et al.*, 1998a), probably through a conformational change at the nSH2-iSH2 domain interface (Shekar *et al.*, 2005).

At the plasma membrane, the GTP-bound form of the small GTPase Ras may further stimulate kinase activity through interaction with the Ras binding domain (RBD) present in all catalytic subunits (see Fig. 1.1, Rodriguez-Vicania *et al.*, 1994, 1996). Binding of phosphotyrosine motifs at the SH2 domains of the adapter is a prerequisite for Ras-induced activation, because the unligated cSH2 domain interferes with activation of p110 by Ras (Jiménez *et al.*, 2002). Strong evidence that stimulation by Ras is indispensable for full activation of class I PI3Ks has come from the characterization of *Drosophila melanogaster* flies expressing a Dp110 mutant that is unable to interact with Ras due to point mutations in its RBD (see also section 1.4.3). These flies are viable and fertile, but show severely reduced insulin responses, size, and egg numbers (Orme *et al.*, 2006). Dp110 is the only class I PI3K present in *Drosophila*, and it is homologous to the mammalian class IA PI3Ks.

Pathways leading to activation of class IA PI3Ks *via* the other protein-protein interaction domains of the p85 adapters (see Fig. 1.2) have also been described, but are probably less common and mostly await confirmation under physiological conditions (Okkenhaug *et al.*, 2007). However, signaling in immune cells downstream of antigen receptors such as the B and T cell receptor complexes likely involves such interactions (Koyasu, 2003; Okkenhaug *et al.*, 2007). For example, phosphorylated Tyr residues in a non-pYXXM motif within the nonreceptor tyrosine kinase (NRTK) Syk have been shown to interact with the SH2 domains of p85 proteins in a similar way as the canonical Tyr motifs (Moon *et al.*, 2005). Moreover, the SH3 domains of the p85 adapters may interact with adapter proteins such as Cbl in a Tyr-independent fashion *via* Pro-rich motifs (Soltoff & Cantley, 1996). The Pro-rich motifs of the p85 subunits, on the other hand, are recognized by SH3 domains of NRTKs such as Fyn and Lyn, also resulting in PI3K activity (Pleiman *et al.*, 1994; Kapeller *et al.*, 1994). Finally, the RhoGAP domain has been shown to interact with Cdc42 (Zheng *et al.*, 1994) and Rac (Bokoch *et al.*, 1996).

p110β bears some resemblance to the class IB PI3K p110γ in that it can also be activated by G$\beta\gamma$ (Kurosu *et al.*, 1997; Maier *et al.*, 1999). However, this G$\beta\gamma$-mediated activation is synergistic with phosphotyrosine-dependent activation pathways and thus different from the solely G$\beta\gamma$-dependent activation of p110γ. The regulatory p85 subunit of p110β is dispensable for G$\beta\gamma$-mediated activation of p110β (Maier *et al.*, 1999). In contrast to PI3Kγ, the physiological relevance of p110β activation by G$\beta\gamma$ remains to be validated. p110β is also insensitive to Ras proteins (Rodriguez-Viciana *et al.*, 2004).

Activation of class IB PI3K

In contrast to the class IA PI3Ks, PI 3-kinase γ (PI3Kγ) is activated by GPCRs *via* heterotrimeric G proteins. Agonist-bound GPCRs stimulate the exchange of GDP to GTP at the GTPase Gα subunit, leading to dissociation of the G$\beta\gamma$ dimer from the Gα subunit (reviewed in Sprang, 1997). Both the GTP-bound Gα and the G$\beta\gamma$ subunits are capable of binding and activating several intracellular effectors (Hamm, 1998). The Gα subunits are grouped into four classes according to their similarity and main effectors of the founding member of each group, *i.e.* G proteins that stimulate (G_s) or inhibit ($G_{i/o}$) adenylyl cyclases, G proteins coupling to phospholipase C (PLC) β enzymes *via* their Gα subunits ($G_{q/11}$), and G proteins of the $G_{12/13}$ class, which *e.g.* activate guanine nucleotide exchange factors (GEFs) of Rho GTPases (Cabrera-Vera *et al.*, 2003). Multiple Gβ and Gγ subunits exist, resulting in different G$\beta\gamma$ complexes with varying specificity towards effectors. However, all β subunits share the so-called β propeller structure, on top of which the effector binding site is located (Sprang, 1997; Hamm, 1998). Most of them localize to the plasma membrane or intracellular membranes by virtue of the short α-helical Gγ subunit, which bears a lipid modification at its C terminus (Clapham & Neer, 1999). Among the effectors of G$\beta\gamma$ are the PLC isoenzymes PLCβ_2 and PLCβ_3, adenylyl cyclases, serine/threonine kinases, tyrosine kinases, and ion channels (Clapham & Neer, 1999; Hamm, 1998). Because the subclass of $G_{i/o}$ proteins is most abundant in cells, mainly stimulation of G_i-coupled GPCRs elicits activity of G$\beta\gamma$ effectors (Wettschureck & Offermanns, 2005). Of the class I PI3Ks, p110γ is chiefly activated by G$\beta\gamma$ subunits, whereas phosphotyrosine-containing

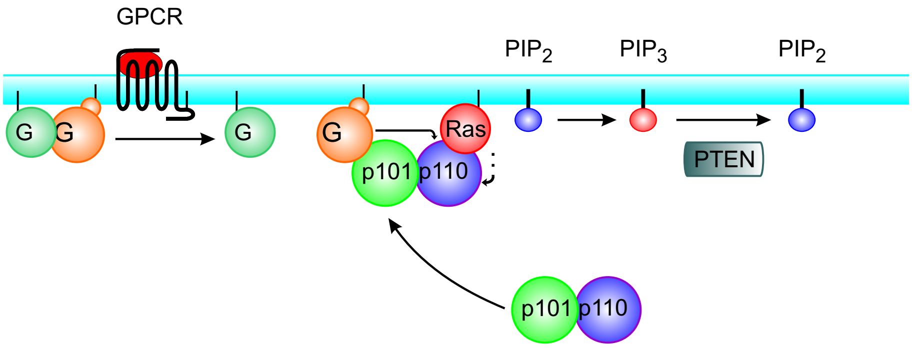

Figure 1.4: Activation of PI3Kγ. In contrast to class IA PI3Ks, the class IB PI3K is activated downstream of GPCR stimulation. G$\beta\gamma$ subunits from heterotrimeric G_i proteins are recognized by p101, leading to translocation of the p110γ/p101 heterodimer to the plasma membrane. G$\beta\gamma$ is capable of further allosteric activation of p110γ. Ras proteins may also activate p110γ. Figure adapted from Engelman *et al.* (2006).

peptides are ineffective in stimulating PI3Kγ (Stoyanov *et al.*, 1995; Stephens *et al.*, 1997).

The activation of PI3Kγ by G$\beta\gamma$ subunits involves the p101 regulatory subunit (Stephens *et al.*, 1997). Although monomeric p110γ can be stimulated to some extent by G$\beta\gamma$ dimers *in vitro*, p101 appears to be necessary for optimal stimulation of PI3Kγ by G$\beta\gamma$ (see section 1.4.2). Studies by Brock *et al.* (2003) significantly contributed to the currently established model of PI3Kγ activation. Accordingly, G$\beta\gamma$ subunits released upon stimulation of G_i-coupled GPCRs are bound by p101, whereby the p110γ/p101 heterodimer translocates to the plasma membrane (Fig. 1.4). There, p110γ has access to its substrate PI(4,5)P_2, and G$\beta\gamma$ further enhances its catalytic activity by an allosteric mechanism *via* a binding site on p110γ itself. As is the case for class IA PI3Ks, Ras proteins can further activate p110γ at the site of the membrane (Pacold *et al.*, 2000; Suire *et al.*, 2002).

1.2 Effector systems of class I PI3Ks

Because class IA and IB PI3Ks produce the same lipid second messengers, they address the same set of effectors. Still, for some effectors, preferential activation by a specific PI3K may sometimes be inferred from the cell type-specific expression of some effectors and PI3K isoforms. All known class I PI3K effectors are characterized by PIP_3 or PI(3,4)P_2-binding PH domains (see above). Most PI3K effectors can be grouped as serine/threonine kinases, tyrosine kinases, GEFs and GTPase-activating proteins (GAPs) for small GTPases, or as scaffold proteins (Fig. 1.5). The number of PH domain-bearing effectors is estimated to exceed 20 in most cell types (Krugmann *et al.*, 2002a). Activation of these effectors mainly involves two often complementary mechanisms. In most cases, binding of PIP_3 to the PH domain leads to a translocation of the effector from the cytosol to the plasma membrane, which is often accompanied by a derepression of an intramolecular inhibition mediated by the unliganded PH domain.

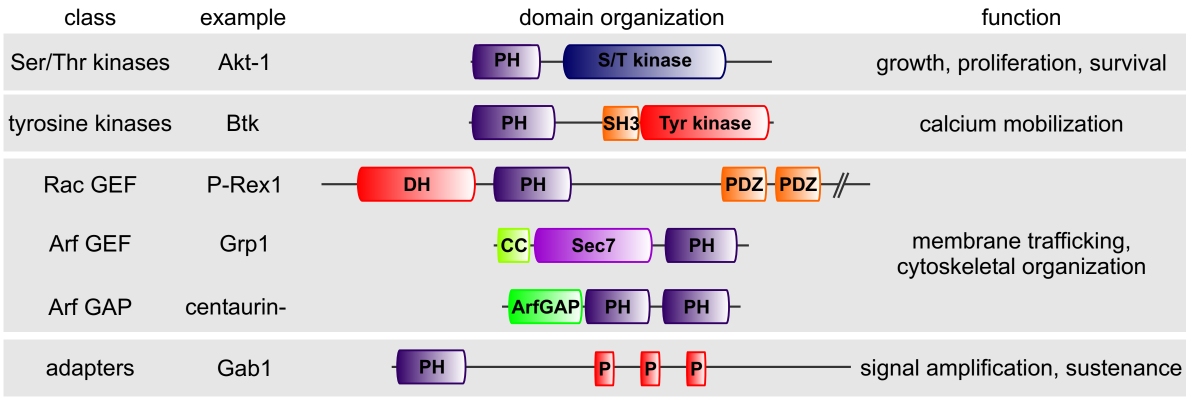

Figure 1.5: Effectors of class I PI3Ks. Major effectors of class I PI3Ks can be grouped according to their relation to certain protein families. Almost all of these effectors contain PIP_3 or $PI(3,4)P_2$-binding PH domains. General functions for effector classes are given alongside the domain structure of a major representative. Abbreviations of domain names are as follows: ArfGAP, catalytic domain of Arf-specific GAPs; CC, coiled-coil domain; DH, Dbl-homologous catalytic domain of Rho GEFs; P, Pro-rich motifs; PDZ, domain found in PSD-95, Dlg, and ZO-1/2; PH, PH domain; Sec7, Sec7-homologous domain of Arf-specific GEFs; SH3, Src homology 3 domain; S/T kinase, catalytic domain of serine/threonine kinases; Tyr kinase, catalytic domain of tyrosine kinases. In a few cases, overlapping and infrequent domains are omitted for clarity.

The PDK-1/Akt system Owing to its ubiquitous expression, multitude of targets, and connection to various signaling pathways, the serine/threonine kinase Akt conveys a plethora of class I PI3K-dependent responses (Fig. 1.6). Like most protein kinases of the AGC family (termed after the three prominent members PK<u>A</u>, PK<u>G</u>, and PK<u>C</u>), full activity of Akt relies on two phosphorylation events that order and stabilize the active conformation of the catalytic site. They occur within the activation loop (or T-loop) and a hydrophobic motif (HM) at their C terminus (Yang *et al.*, 2002a, 2002b). By virtue of their PH domains, 3-phosphoinositide-dependent kinase-1 (PDK-1) and Akt colocalize at the plasma membrane, where the T-loop site of Akt (Thr 308) is phosphorylated by PDK-1 (Fig. 1.6, Mora *et al.*, 2004). Binding of PIP_3 to the PH domain of Akt is associated with a conformational change that enhances access of PDK-1 to the T-loop site (Thomas *et al.*, 2002; Milburn *et al.*, 2003). PDK-1 is constitutively active by virtue of trans-autophosphorylation at its T-loop site (Casamayor *et al.*, 1999; Wick *et al.*, 2003). Akt is the only substrate of PDK-1 that is phosphorylated in an PIP_3-dependent manner. PDK-1 also acts as the T-loop kinase for other AGC kinases such as p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinases (RSK), serum- and glucocorticoid-induced kinase (SGK), PKCζ, and probably other PKC isoforms (Parekh *et al.*, 2000; Williams *et al.*, 2000; Mora *et al.*, 2004). The phosphorylated (or Glu/Asp-replaced) HM sites of theses kinases are recognized by a hydrophobic pocket (PIF-pocket) on PDK-1, mediating docking between PDK-1 and its PIP_3-independent substrates (Biondi *et al.*, 2001).

Although several kinases can phosphorylate Akt at Ser 473 *in vitro* (Woodgett, 2005), the *in vivo* HM motif kinase has long been elusive. However, recent studies have convincingly

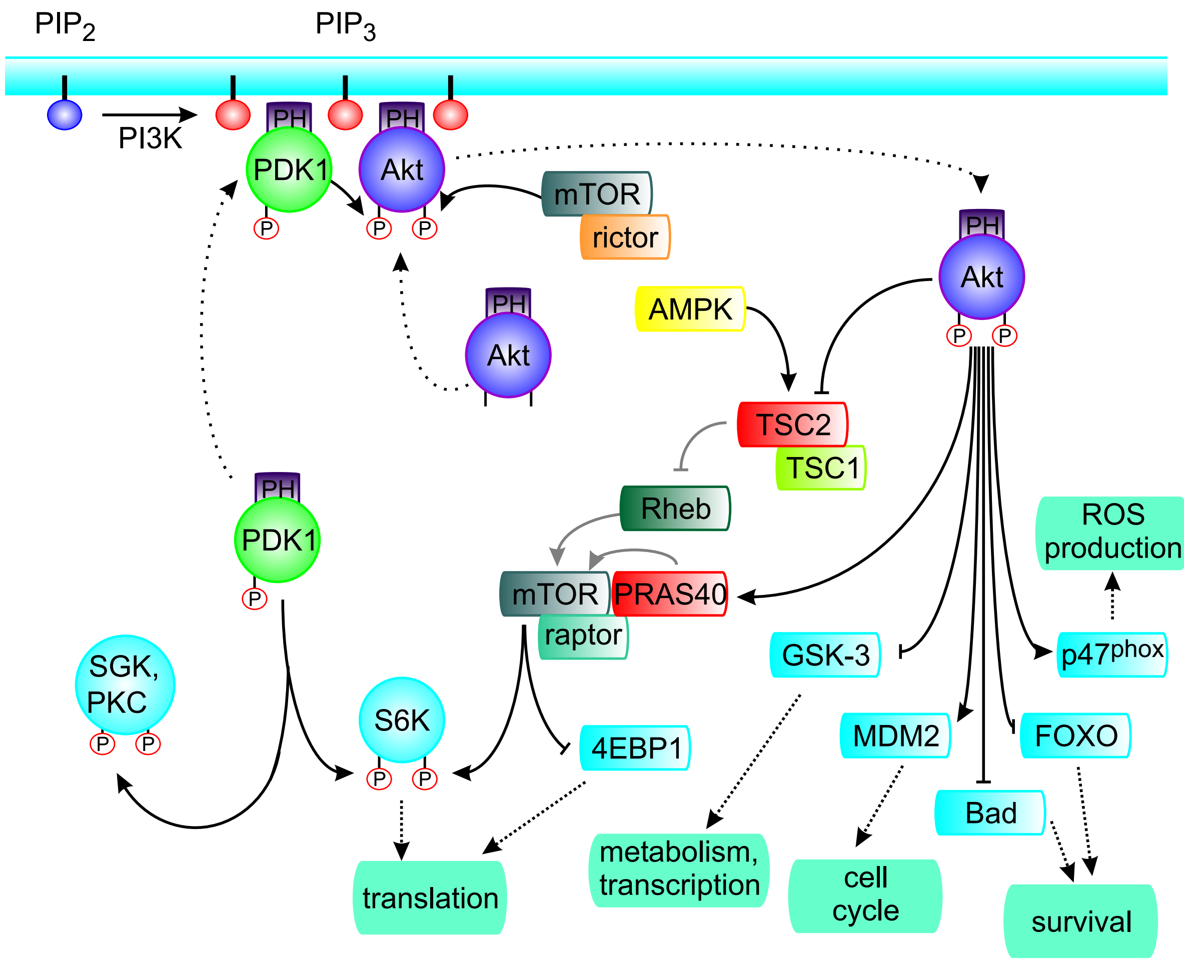

Figure 1.6: Interplay of PDK-1, Akt, and mTOR pathways. Schematic overview of the phosphorylation events and cellular responses mediated by PDK-1, Akt, and mTOR. The T-loop (left) and HM (right) phosphorylation sites of the AGC kinases PDK-1, Akt, SGK, and S6K are symbolized by short protrusions. Activating and inhibitory phosphorylation events are denoted by black arrows and black lines, respectively. Other activating and inhibitory actions are indicated by the respective gray counterparts. Dashed arrows represent PH domain and PIP_3-dependent translocation events. For definitions of abbreviations and further information see main text.

ascribed this role to the rictor/mTOR complex (Sarbassov *et al.*, 2005b; Guertin *et al.*, 2006; Jacinto *et al.*, 2006). The kinase mTOR exists either as a rapamycin-sensitive complex (mTORC1) containing regulatory-associated protein of mTOR (raptor) or as a complex (mTORC2) with rapamycin-insensitive companion of mTOR (rictor), together with the common subunit LST8 and possibly others (Bhaskar & Hay, 2007). mTORC1 is an integrator of growth factor and nutrient signals, whereas mTORC2 primarily functions as the HM motif kinase of Akt and PKCα (Bhaskar & Hay, 2007; Shaw & Cantley, 2006; Wullschleger *et al.*, 2006). It is still controversial whether PDK-1-mediated phosphorylation depends on phosphorylation of Akt at Ser 473. Several lines of evidence indicate that both phosphorylations occur independently (Alessi *et al.*, 1996; Williams *et al.*, 2000; Biondi *et al.*, 2001; Guertin *et al.*, 2006). Both phosphorylation events are PIP_3-dependent, probably due to a dual requirement for plasma membrane localization of Akt and relief of PH domain-controlled blockade of the phosphorylation sites (Hresko & Mueckler, 2005; Woodgett, 2005).

Through its vast spectrum of substrates, Akt influences many aspects of cellular function. One of the most notable targets is the tumor suppressor protein tuberin, also called tuberous sclerosis complex 2 (TSC2; Inoki *et al.*, 2002; Manning *et al.*, 2002). In complex with its binding partner hamartin (TSC1), it functions as a GAP for the Ras-like GTPase Rheb (reviewed in Bhaskar & Hay, 2007). GTP-bound Rheb, however, is required for the activation of mTORC1 by an as yet not fully elucidated mechanism. The GAP activity of TSC is modulated by various kinases. Phosphorylation of TSC2 by Akt inhibits GAP activity and thus translates into activation of mTORC1. Akt may also activate mTORC1 by direct phosphorylation of PRAS40, a newly identified complex member (Haar *et al.*, 2007). Conversely, the adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), which is activated under conditions of low energy levels, activates TSC2 and inhibits mTORC1. Among others, the main targets of mTORC1 are S6K, for which it is the HM motif kinase, and 4EBP1, an inhibitor of cap-dependent translation. Thereby, mTORC1 – and thus Akt – constitute a major control point for overall translational activity and especially cap-dependent translation of *e.g.* specific cell-growth regulators *via* 4EBP1 and eIF4e (Holz *et al.*, 2005; Richter & Sonenberg, 2005; Sarbassov *et al.*, 2005a; Shaw & Cantley, 2006).

The constitutively active glycogen synthase kinase 3 (GSK-3) is inhibited by Akt phosphorylation (Cross *et al.*, 1995), generally leading to derepression of proteins otherwise inactivated by GSK-3 (Woodgett, 2001). Besides metabolic enzymes such as glycogen synthase and ATP-citrate lyase, GSK-3 phosphorylates and inactivates transcription factors like c-Jun and c-Myc, the translation initiation factor eIF-2B, and regulators of cell cycle progression (Cyclin D1), among others (Doble & Woodgett, 2003). Thus, *via* inactivation of GSK-3, Akt promotes metabolic and transcriptional activity. Akt also augments cell cycle progression through phosphorylation of the p53 ubiquitin ligase MDM2 and the cell cycle inhibitor $p27^{Kip}$ (Mayo & Donner, 2002). Akt is further capable of phosphorylating Raf at Ser 259, resulting in inhibition of Raf and its downstream effectors of the mitogen-activated protein kinase (MAPK) cascade (Zimmermann & Moelling, 1999). As a result, proliferative signals are enhanced. Akt mediates cell survival by phosphorylation of Bad, thereby preventing it from binding and inhibiting anti-apoptotic Bcl proteins (Datta *et al.*, 1997). Caspase 9 may also be directly phosphorylated and inactivated by Akt (Cardone *et al.*, 1998). Moreover, Akt is involved in the activation of the nuclear factor κB pathway by phosphorylating IKKα (Ozes *et al.*, 1999). Members of the FOXO family of forkhead transcription factors are also phosphorylated by Akt, resulting in their inactivation through 14-3-3 protein-mediated sequestration in the cytoplasm (Brunet *et al.*, 1999). Among the genes positively regulated by FOXO proteins are those encoding the pro-apoptotic proteins Fas ligand and Bim (Burgering & Kops, 2002). The generation of reactive oxygen species (ROS) is elicited by PI3K activity, partly due to Akt-mediated phosphorylation of the p47^{phox} subunit of the NAPDH oxidase complex (Chen *et al.*, 2003; Perisic *et al.*, 2004).

Tyrosine kinases Members of the Tec kinase family such as Btk (Bruton's tyrosine kinase), Etk, and Itk are NRTKs that possess an N-terminal PIP$_3$-binding PH domain unique among tyrosine kinases. After PI3K-dependent translocation to the plasma membrane, Tec kinases are phosphorylated and activated by Src family kinases. G$\beta\gamma$ is able to support PIP$_3$-mediated membrane localization of Btk (Lowry & Huang, 2002). Expression of Tec kinases is mostly restricted to hematopoietic cells such as B, T, and mast cells (Finkelstein & Schwartzberg, 2004). There they mediate calcium mobilization after stimulation of antigen receptors through phosphorylation of PLCγ, which requires tyrosine phosphorylation for optimal activity (Takesono *et al.*, 2002).

GEFs and GAPs for small GTPases GTPases of the Rac family are crucially involved in cytoskeletal reorganization during cell migration and chemotaxis, primarily by regulating localized actin turnover (Raftopoulou & Hall, 2004). They also contribute to activation of the NAPDH oxidase complex (Perisic *et al.*, 2004). Rac proteins become activated downstream of class I PI3Ks by PH domain-containing GEFs (reviewed in Welch *et al.*, 2003). In addition to mediating colocalization with the lipid-tethered GTPases at the plasma membrane, PIP$_3$ binding probably relieves an PH domain-dependent inhibition of the catalytic Dbl domain (Rossman *et al.*, 2005). The Rac GEFs most effectively stimulated (~20-fold) by PIP$_3$ are P-Rex1 and SWAP-70. P-Rex1 is highly expressed in neutrophils and synergistically activated by both PIP$_3$ and G$\beta\gamma$ (Welch *et al.*, 2002). SWAP-70 is enriched in B cells and mast cells, where it relays signals from antigen receptors (Shinohara *et al.*, 2002; Pearce *et al.*, 2006). The activity of Tiam 1, a Rac-specific GEF, is increased ~2-fold by PIP$_3$ and can also be stimulated by Ras (Fleming *et al.*, 2000; Lambert *et al.*, 2002). PIP$_3$ enhances the activity of Vav proteins, a family of Rac GEFs that are expressed in hematopoietic cells and mainly activated by tyrosine phosphorylation (Han *et al.*, 1998). The DOCK180/ELMO complex is an atypical GEF for Rac that stimulates GTP binding of Rac although it does not contain a Dbl domain (Brugnera *et al.*, 2002). The DOCK homology region-1 domain of DOCK180 is a novel PIP$_3$ binding domain, which renders the subcellular localization of the DOCK180/ELMO complex class I PI3K-dependent (Côté *et al.*, 2005).

Both GEFs and GAPs with PH domains can be found for small GTPases of the Arf family. Arf GTPases participate in the regulation of vesicle trafficking, organelle structure, and membrane-related actin structures (D'Souza-Schorey & Chavrier, 2006). Especially Arf6 is thought to be involved in plasma membrane and actin remodeling during pseudopod and membrane ruffle formation in cell migration and phagocytosis, in part *via* recruitment of the DOCK180/ELMO complex and subsequent activation of Rac (Santy *et al.*, 2005). Arf GEFs of the cytohesin family contain a PH domain as their characteristic feature (Jackson *et al.*, 2000). Among them are the general receptor for phosphoinositides 1 (Grp1), whose PH domain is highly specific for PIP_3 (Klarlund *et al.*, 1997, 1998), cytohesin-1 (Kolanus *et al.*, 1996), ARNO (Arf nucleotide binding site opener, Chardin *et al.*, 1996), and cytohesin-4 (Ogasawara *et al.*, 2000). PI3Ks also control the deactivation of Arf6 *via* Arf GAPs of the centaurin family. Many of its members feature PH domains and are thus often lipid-regulated. PIP_3-dependent activity is mainly observed for the ARAP (Arf GAP with a Rho GAP domain and PH domains) subfamily consisting of ARAP1–3 (Randazzo & Hirsch, 2004). ARAP1 and ARAP3 are also functional Rho GAPs and may thus provide a link between Arf and Rho signaling (Krugmann *et al.*, 2002a; Miura *et al.*, 2002). Despite the knowledge concerning PIP_3-dependent Arf modulators, the precise role of PI3Ks in the regulation of Arf function is still not fully understood.

Adapter proteins Scaffold proteins containing PIP_3-binding PH domains have also been identified. Members of the Gab (Grb2-associated binder)/Dos subfamily of adapter proteins feature multiple Tyr phosphorylation sites and Pro-rich motifs that are involved in interactions with proteins like the class IA p85 adapters and Grb2, respectively (Gu & Neel, 2003). The PH domain of Gab1 is necessary for sustained signaling downstream of the EGF receptor (Rodrigues *et al.*, 2000) and for its participation in B cell receptor signaling, where Gab1 contributes to signal amplification (Ingham *et al.*, 2001). The Gab2 isoform plays an essential role in mast cell degranulation (Gu *et al.*, 2001). Another group of adapter proteins comprises Bam32 (B lymphocyte adapter molecule of 32 kDa) as well as TAPP1 (tandem PH domain-containing protein 1) and TAPP2 (Allam & Marshall, 2005). The C-terminal PH domain of these adapters, especially of the homologous TAPP proteins, prefer $PI(3,4)P_2$ over PIP_3, rendering their translocation to the plasma membrane more slowly and sustained than that of PIP_3-specific effectors. Bam32 functions downstream of the B cell receptor in relaying signals to the MAPK pathway (Han *et al.*, 2003). It may also be involved in the internalization of RTKs (Anderson *et al.*, 2000). TAPP proteins interact with PDZ domain-containing proteins, but the functional significance of these interactions and the specific roles of TAPP proteins are still largely elusive (Allam & Marshall, 2005).

Protein kinase-mediated effects of class I PI3Ks In addition to their ability to phosphorylate lipids, all class I PI3Ks exhibit protein kinase activity as well that is mainly evident as auto- and trans-phosphorylation. The mechanisms involved are slightly different for each isoform. Whereas p110α only phosphorylates bound p85α at Ser 608 (Dhand *et al.*, 1994b), p110β autophosphorylates at Ser 1070 within its catalytic domain and less efficiently phosphorylates associated p85 subunits (Beeton *et al.*, 2000; Czupalla *et al.*, 2003). Likewise, p110δ mainly autophosphorylates at Ser 1039 (Vanhaesebroeck *et al.*, 1999). For all class IA PI3Ks, autophosphorylation or adapter trans-phosphorylation is accompanied by a down-regulation of the lipid kinase activity (Dhand *et al.*, 1994b; Beeton *et al.*, 2000; Czupalla *et al.*, 2003). p110γ has also been shown to undergo autophosphorylation, which, however, does not influence its activity (Stoyanova *et al.*, 1997; Czupalla *et al.*, 2003). The regulation of these protein kinase activities remains largely elusive, although studies have provided evidence that p110α-mediated phosphorylation of p85 is enhanced upon platelet-derived growth factor stimulation (Foukas *et al.*, 2004) and that p110δ autophosphorylation is increased in CD28-stimulated Jurkat cells (Vanhaesebroeck *et al.*, 1999). For p110γ, inconsistent results have been obtained regarding a G$\beta\gamma$-dependent increase in autophosphorylation (Bondev *et al.*, 1999; Czupalla *et al.*, 2003). Moreover, autophosphorylation of p110γ has not been detected in a cellular context yet.

Since many ATP-binding proteins distinct from protein kinases undergo autophosphorylation *in vitro*, a validation of class I PI3K protein kinase activity depends on the identification of physiological substrates (Hunter, 1995). Bondeva *et al.* (1998) have shown that an engineered variant of p110γ, which is unable to phosphorylate lipids but retains its protein kinase activity, still activates the extracellular signal-regulated kinases (ERK1/2). Activation of the MAPK ERK1/2 is thought to result from direct phosphorylation of their upstream kinase MEK-1 by p110γ, which, however, is not performed by the p110γ/p101 complex (Bondev *et al.*, 1999). For class IA PI3Ks, phosphorylation of insulin receptor substrate 1 has been demonstrated both *in vitro* and in insulin-stimulated primary adipocytes (Lam *et al.*, 1994; Rondinone *et al.*, 2000). In the same context, a direct phosphorylation of phosphodiesterase 3B (PDE3B, see also below) has been proposed as well (Rondinone *et al.*, 2000). Recently, *in vitro* assays have revealed a phosphorylation of 4EBP1 and H-Ras by p110α and p110γ (Foukas & Shepherd, 2004). Due to the still incomplete understanding of protein kinase-mediated effects, the concept of protein kinase-dependent PI3K signaling is not generally accepted (Hawkins *et al.*, 2006). In addition, PI3K lipid signaling is presumably far more important, given the plethora of PIP_3-dependent PI3K effectors.

1.3 Physiological roles of PI3Kγ

Although the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced generation of PIP_3 was among the first PI3K-dependent effects observed (Traynor-Kaplan *et al.*, 1988; Stephens *et al.*, 1993), knowledge concerning the physiological functions of PI3Kγ largely stems from three independent lines of p110γ knockout mice that have been generated and characterized in recent years (Hirsch *et al.*, 2000; Li *et al.*, 2000; Sasaki *et al.*, 2000). p110$\gamma^{-/-}$ mice are viable, fertile, but exhibit alterations in various physiological and pathophysiological contexts. According to its expression pattern, p110γ performs its functions mainly in the hematopoietic system, but also in heart and certain other tissues.

1.3.1 Leukocyte systems

Neutrophils of p110γ knockout mice are defective in the production of PIP_3 upon stimulation with fMLP, C5a, and other chemokines addressing G_i-coupled GPCRs. In consequence of this deficiency, they exhibit markedly diminished responses in downstream effects, such as phosphorylation of Akt, ROS production, and migration towards chemotactic stimuli (Hirsch *et al.*, 2000; Li *et al.*, 2000; Sasaki *et al.*, 2000). Similar ramifications of p110γ ablation have also been observed for macrophages (Hirsch *et al.*, 2000; Jones *et al.*, 2003), dendritic cells (DC, Del Prete *et al.*, 2004), T cells (Sasaki *et al.*, 2000; Reif *et al.*, 2004), and eosinophils (Pinho *et al.*, 2005). All of these cell types display impeded responsiveness to various chemokines, resulting in chemotactic defects. Reduced recruitment was also observable in *in vivo* models of inflammatory conditions such as septic peritonitis (neutrophils and macrophages, Hirsch *et al.*, 2000) and allergic pleurisy (eosinophils, Pinho *et al.*, 2005). Genetic ablation of p110γ does not affect B and T cell antigen receptor signaling (Sasaki *et al.*, 2000), which chiefly involves class IA PI3Ks (Okkenhaug *et al.*, 2007). However, co-stimulatory inputs from GPCRs are reduced in T cells. Moreover, proliferation of T cells is diminished, probably due to decreased GPCR-dependent anti-apoptotic signaling normally relayed by p110γ and Akt (Sasaki *et al.*, 2000). p110$\gamma^{-/-}$ mice further exhibit a smaller thymus gland and a decreased $CD4^+/CD8^+$ T cell differentiation ratio (Rodríguez-Borlado *et al.*, 2003).

The exact involvement of p110γ and other PI3Ks in chemotaxis is still largely unresolved in mechanistical terms. Genetic ablation of p110γ as well as inhibition with wortmannin diminishes polarized accumulation of F-actin at the leading edge (Ferguson *et al.*, 2007), which is a hallmark of chemotaxis (see *e.g.* Weiner, 2002), although presumably not essential for this process according to recent data (Nishio *et al.*, 2007). In contrast to a previous study that reported a loss of directional movement for p110$\gamma^{-/-}$ neutrophils (Hannigan *et al.*, 2002), Nishio *et al.* (2007) have shown that p110$\gamma^{-/-}$ neutrophils retain directionality but exhibit a reduced speed if assayed on albumin-coated glass. Moreover, Ferguson *et al.* (2007) observed that p110$\gamma^{-/-}$ neutrophils move normally in terms of direction and speed once movement has commenced. However, depending on the properties of the surface, a proportion of cells remains immobile. Assessment of the adhesive properties of neutrophils has revealed an enhanced surface expression of the integrin Mac-1 upon stimulation with chemoattractants (Sengeløv *et al.*, 1993). This response is defective in p110$\gamma^{-/-}$ cells (Ferguson *et al.*, 2007). Thus, disregulation of adhesion rather than chemotactic sensing may be the major cause for the reduced chemotactic activity of p110γ-deficient cells.

Previously it has been assumed that PI3Kγ and its product PIP_3 constitute the chemotactic 'compass', mostly based on the colocalization of PI3Kγ and PIP_3 with the leading edge of chemotacting cells (Servant *et al.*, 2000; Wang *et al.*, 2002), although this view had been questioned (Ward, 2004). Instead, PI3Kγ appears to be involved in the movement itself and in the amplification and stabilization of polarization rather than in the initiation of directionality (Ferguson *et al.*, 2007; Chen *et al.*, 2007). Positive feedback loops *via* PIP_3-sensitive small GTPases that further increase PIP_3 accumulation at the leading edge contribute to the PI3K-dependent maintenance of polarity (reviewed in Charest & Firtel, 2006, 2007). Recent data on the slime mold *Dictyostelium discoideum*, an organism well-studied with regard to chemotaxis, indicate that the products of phospholipase A_2 may be responsible for initiation of chemotaxis, probably acting in parallel to PI3K-mediated signals (Chen *et al.*, 2007). Only combined interference with both pathways strongly reduced chemotactic responses (Chen *et al.*, 2007). An involvement of phospholipase A_2 in neutrophil chemotaxis towards some chemokines has been suggested as well (Locati *et al.*, 1996; Carnevale & Cathcart, 2001). Likewise, leukocyte recruitment to inflammatory sites is significantly but not completely abrogated in p110$\gamma^{-/-}$ mice, pointing to the presence of additional factors in chemotaxis (Hirsch *et al.*, 2000; Del Prete *et al.*, 2004; Pinho *et al.*, 2005).

In mast cells, degranulation is elicited by clustering of antigen receptors of the FcϵRI class (Gilfillan & Tkaczyk, 2006), resulting in activation of PI3Kδ (Ali *et al.*, 2004). How PI3K activity translates to degranulation is not completely resolved, but probably involves recruitment of Btk to the plasma membrane, where it activates PLCγ_1, contributing to the calcium mobilization necessary for degranulation (Gilfillan & Tkaczyk, 2006). Like p110$\delta^{-/-}$ mice, p110$\gamma^{-/-}$ mice show reduced responses in passive systemic anaphylaxis tests, and their mast cells also exhibit diminished degranulation in response to antigen–IgE stimulation (Laffargue *et al.*, 2002). It could be demonstrated that mast cells feature an p110γ-dependent autocrine feedback loop, in which released allergic mediators such as adenosine stimulate G_i-coupled receptors, leading to activation of PI3Kγ and thus enhanced degranulation (Laffargue *et al.*, 2002). Although the precise etiology of rheumatoid arthritis is largely unresolved, it is known to involve recruitment of neutrophils and mast cells to inflamed joints, probably explaining why genetic ablation of p110γ also attenuates the progression of modeled rheumatoid arthritis in mice (Camps *et al.*, 2005).

Platelets of p110γ knockout mice show decreased aggregation after stimulation with ADP but respond normally to thrombin and thromboxane (Hirsch *et al.*, 2001). Presumably, ADP acts *via* the G_i-coupled P_2Y_{12} ADP receptor, whereas the other stimuli mainly activate G_q-coupled receptors that do not elicit PI3Kγ activity. Moreover, p110$\gamma^{-/-}$ platelets exhibit increased levels of the soluble second messenger adenosine 3',5'-cyclic monophosphate (cAMP) both under basal and prostaglandin E_1-stimulated conditions (Hirsch *et al.*, 2001). p110γ knockout mice exhibit normal bleeding times but a reduced mortality in a model of ADP-induced thromboembolism (Hirsch *et al.*, 2001).

1.3.2 Heart and vascular system

In addition to its many duties in leukocytes, PI3Kγ is significantly involved in heart function. Whereas p110α is crucial for regulation of cardiomyocyte cell size, PI3Kγ is a negative regulator of cardiac contractility (Crackower *et al.*, 2002). Hearts of p110γ knockout mice show enhanced contractility, and, conservely, hearts of PTEN-deficient mice exhibit a decreased contractility (Crackower *et al.*, 2002). Cardiac contractility is regulated to a great extent by cAMP and subsequent activation of PKA (reviewed in Bers, 2002). p110$\gamma^{-/-}$ cardiomyocytes contain increased levels of cAMP (Crackower *et al.*, 2002), accounting for their enhanced contractility. Recent studies on mice expressing a catalytically inactive (kinase-dead; KD) variant of p110γ have revealed that the impact on cAMP levels is not mediated by the catalytic activity of PI3Kγ but results from a scaffolding interaction that activates PDE3B (Patrucco *et al.*, 2004). Although the phenotype of p110$\gamma^{KD/KD}$ mice matches that of p110$\gamma^{-/-}$ mice in leukocytes, cardiac contractility is comparable to wild-type in p110$\gamma^{KD/KD}$ mice. p110$\gamma^{KD/KD}$ cardiac myocytes exhibit diminished levels of Akt and ERK1/2 phosphorylation but unchanged cAMP levels (Patrucco *et al.*, 2004).

Although p110γ does not control cardiac cell growth under normal conditions, several studies point to p110γ-dependent signaling in pathological cardiac hypertrophy (Oudit *et al.*, 2004). Long-term stimulation with β-adrenergic receptor (βAR) agonists is known to cause hypertrophy (Salazar *et al.*, 2007). In p110$\gamma^{-/-}$ mice subjected to such a treatment, hypertrophy is less severe than in wild-type mice, and p110γ knockout mice are protected from subsequent fibrosis and cardiac dysfunction (Oudit *et al.*, 2003). Similarly, a chronic pressure overload induced *e.g.* by aortic constriction results in a left-ventricular hypertrophy in mice. During this process, activity of p110γ is enhanced (Naga Prasad *et al.*, 2000), and p110γ expression is upregulated as well (Patrucco *et al.*, 2004). p110$\gamma^{-/-}$ mice exhibit a markedly reduced hypertrophic response under chronic pressure overload, but develop hallmarks of heart failure such as dilated chambers and fibrosis, as well as necrotic lesions (Patrucco *et al.*, 2004). p110$\gamma^{KD/KD}$ mice maintain cardiac function and do not develop necrotic lesions, although they share the diminished hypertrophic response with p110$\gamma^{-/-}$ mice (Patrucco *et al.*, 2004). Thus, the kinase activity of p110γ is required for hypertrophic cardiac remodeling, probably involving stimulation of Akt and ERK1/2. Development of necrotic lesions and heart failure, however, depends on elevated cAMP levels, as has been shown also in other contexts (see *e.g.* Iwase *et al.*, 1996).

p110γ is expressed in vascular smooth muscle cells (VSMC), where it is involved in the regulation of vascular tone. In rat portal vein myocytes, a slow elevation in the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) is observed after stimulation with angiotensin II (Morel *et al.*, 1996). This response is mediated by the angiotensin AT_{1A} receptor and G$\beta\gamma$ subunits released from G proteins of the 12/13 family, which have been reported to activate L-type calcium channels (Macrez-Leprêtre *et al.*, 1997). As a result, contractility is enhanced. Several studies have shown that PI3Kγ is involved in this process (Viard *et al.*, 1999; Quignard *et al.*, 2001) by enabling Akt-dependent phosphorylation of calcium channel β subunits, which induces trafficking of the channels to the plasma membrane (Viard *et al.*, 2004). Correspondingly, angiotensin II-induced phosphorylation of Akt and subsequent vasoconstriction is abolished in vessels derived from p110γ knockout mice (Vecchione *et al.*, 2005). Both p110$\gamma^{-/-}$ and p110$\gamma^{KD/KD}$ mice do not develop hypertension upon chronic stimulation with angiotensin II (Vecchione *et al.*, 2005). In addition to controlling calcium currents, PI3Kγ is also involved in Rac-mediated production of ROS in angiotensin-stimulated VSMC (Vecchione *et al.*, 2005).

1.3.3 Other tissues

The pancreas contains different specialized cell types that secrete hormones and digestive enzymes. Secretion of insulin from pancreatic β-cells is markedly reduced in p110γ knockout mice upon glucose injection, but the underlying mechanism is unresolved (MacDonald *et al.*, 2004). Acinar cells of p110$\gamma^{-/-}$ mice show decreased $[Ca^{2+}]_i$ signals, trypsinogen activation, and stimulation of inflammatory pathways in response to the peptide hormone cholecystokinin, a key regulator of enzyme secretion acting *via* GPCR (Gukovsky *et al.*, 2004). Pancreatitis is a condition that arises from intra-acinar cell conversion of inactive zymogens to active digestive enzymes, leading to injury of acinar cells, disregulated enzyme secretion, and inflammatory responses. In models of acute pancreatitis, p110$\gamma^{-/-}$ mice exhibit diminished acinar cell injury, necrosis, and neutrophil recruitment, resulting in reduced lethality (Lupia *et al.*, 2004). Thus, PI3Kγ is a regulator of secretion in pancreatic cells and also contributes to inflammatory aspects of pancreatic diseases both through its action in acinar cells and in neutrophils.

PI3Kγ has also been detected in endothelial cell systems. Before they egress to inflamed tissues by transmigration, neutrophils attach to endothel cells of vessel walls. This process is mediated by adhesion molecules like E-selectin and is stimulated by proinflammatory cytokines. An involvement of p110γ in the tethering and rolling of leukocytes on endothelial cells has been demonstrated using neutrophils from wild-type mice and vessels from p110$\gamma^{-/-}$ mice. In response to tumor necrosis factor α, attachment of the neutrophils is severely reduced, and rolling velocities are concomitantly increased (Puri *et al.*, 2005). Thereby, p110γ controls inflammatory responses by regulating both chemotaxis in leukocytes and their recruitment through endothelial cells.

1.4 Structure and interaction partners of PI3Kγ

In addition to the characterization of the physiological relevance and cellular effects of PI3Kγ, biochemical studies have provided insight into the molecular basis of the function of p110γ.

Several proteins are known to interact with p110γ, and the characteristics of these interactions are summarized below.

1.4.1 Structure of p110γ

p110γ is the only catalytic PI3K subunit whose structure has been resolved in atomic detail, although without the N-terminal 143 aa (Walker *et al.*, 1999, 2000). The conserved RBD, C2, and catalytic domains are organized around the central helical PIK domain. It is reminiscent of domains containing so-called HEAT repeats, which are often involved in protein-protein interactions. All but one surface of the PIK domain are employed in interdomain contacts. The other is solvent-exposed and may interact with other proteins. The RBD and C2 domains structurally resemble those found in other proteins. Although its *in vivo* role is still unclear, the C2 domain may participate in interaction with membrane phospholipids, because it can bind to multilamellar phospholipid vesicles *in vitro* (Walker *et al.*, 1999). Binding of the RBD to Ras resembles the binding mode of other Ras effectors (Pacold *et al.*, 2000). The catalytic domain of p110γ is composed of an N- and C-terminal lobe, which create the catalytic cleft at their interface. The overall fold and many details of the ATP binding site are similar to serine/threonine protein kinases. As mentioned above, class I PI3Ks including PI3Kγ also exhibit protein kinase activity. Co-crystals of p110γ with several inhibitors confirmed that the ATP binding site is the point of attack for PI3K inhibitors (Walker *et al.*, 2000). The binding cassette is highly conserved both within and beyond the PI3K family, explaining why common PI3K inhibitors are neither isoform-selective nor specific for PI3Ks. Wortmannin, which covalently modifies a conserved lysine within the ATP binding site (Arcaro & Wymann, 1993; Wymann *et al.*, 1996), and the competitive inhibitor LY-294002 (Vlahos *et al.*, 1994) are widely used PI3K inhibitors. Both inhibit all PI3Ks and also related protein kinases such as mTOR and DNA-dependent protein kinase (DNA-PK), although not PI3K-C2α.

Because p110γ lacks the N-terminal p85 binding domain of the class IA PI3Ks, it is commonly assumed that p110γ interacts with p101 *via* its unique N-terminal domain. Deletion of the N terminus (aa 1–122) of p110γ severely diminished binding to p101 in one study (Krugmann *et al.*, 1999), whereas Maier *et al.* (1999) found that the 97 N-terminal aa of p110γ are dispensable for interaction with p101. Unfortunately, the crystal structure of p110γ lacks the first 143 aa so that the structure of its N terminus is unknown. Although both N- and C-terminal elements of p110γ have been implicated in binding to G$\beta\gamma$ subunits (Leopoldt *et al.*, 1998), the G$\beta\gamma$ binding site(s) of p110γ have not been identified so far.

1.4.2 Properties of the p101 regulatory subunit

Although p101 is intimately linked to the function of p110γ, it is considerably less well understood. Even its role in the G$\beta\gamma$-mediated activation of p110γ has been widely debated until recently and is still not fully resolved. p101 is now generally thought to enable translocation of p110γ to the plasma membrane by binding to G$\beta\gamma$ with high affinity. Interaction between G$\beta\gamma$ and p110γ leads to an additional allosteric activation of p110γ, but is itself insufficient to drive translocation of p110γ to the plasma membrane. Both aspects of PI3Kγ activation have been demonstrated to be active in living cells (Brock *et al.*, 2003).

Initially, p101 has been identified in pig neutrophils as a protein that binds tightly to p110γ and facilitates its activation by G$\beta\gamma$ both *in vitro* and in cellular model systems (Stephens *et al.*, 1997). Stephens *et al.* (1997) reported that p101 has an about 5-fold higher affinity towards G$\beta\gamma$ than has p110γ, and p101 increases G$\beta\gamma$-stimulated activity of p110γ by about 100-fold. In contrast, p110γ alone has been shown to be activated by G$\beta\gamma$ in *in vitro* lipid kinase assays as well (Stoyanov *et al.*, 1995; Leopoldt *et al.*, 1998). The use of either PI (Leopoldt *et al.*, 1998) or PI(4,5)P_2 (Stephens *et al.*, 1997) as the substrate in these assays may, however, indicate a possible source of these divergent results. Indeed, Maier *et al.* (1999) could show that p101 considerably enhances the G$\beta\gamma$-stimulated catalytic activity of p110γ towards PI(4,5)P_2 but not PI, concluding that p101 sensitizes p110γ towards G$\beta\gamma$ in the presence of PI(4,5)P_2. p101 may, thus, influence the substrate specificity of p110γ. In the presence of PI(4,5)P_2, half-maximal stimulation of p110γ activity by G$\beta\gamma$ is observed at 5 nM for the p110γ/p101 heterodimer and at 100 nM for p110γ alone (Maier *et al.*, 1999). The observation that p101 but not p110γ is localized to the plasma membrane upon coexpression with G$\beta\gamma$ (Brock *et al.*, 2003) supports that p101 has a significantly higher affinity for G$\beta\gamma$ in living cells and that it thus represents the primary docking site for G$\beta\gamma$ within the heterodimeric complex. It is, however, unclear whether p101 and p110γ form a single G$\beta\gamma$ binding site within the heterodimer or whether they provide separate binding sites.

In contrast to these data, mainly two lines of evidence argue against an essential role of p101 in cellular systems. First, monomeric p110γ is sufficient to reconstitute fMLP-induced production of PIP_3 in permeabilized, cytosol-depleted neutrophils (Kular *et al.*, 1997). Second, stimulation of U937 cells with all-trans retinoic acid leads to an upregulation of p110γ alone, suggesting that p110γ functions as a monomer in certain cell types (Baier *et al.*, 1999). Related to this issue, the expression pattern of p101 has not been analyzed in detail yet. Interestingly, p101 appears to be stabilized by p110γ but not *vice versa* (Brock *et al.*, 2003). The opposite is observed for class IA PI3Ks (see above).

With respect to the mechanism of PI3Kγ activation, studies have been performed on lipid monolayers and vesicles, demonstrating that p110γ/p101 heterodimers are associated with vesicles independently of G$\beta\gamma$ (Krugmann *et al.*, 2002b). Moreover, an artificially lipid-anchored p110γ/p101 dimer could be stimulated by G$\beta\gamma$ to a similar extent as wild-type dimer (Krugmann *et al.*, 2002b). These data point to a G$\beta\gamma$-dependent allosteric modulation rather than membrane translocation as the chief activation mechanism. In agreement with the findings of Brock *et al.* (2003), however, p110γ is located within the cytosol of resting neutrophils and is found in the particulate fraction only upon chemokine stimulation (Naccache *et al.*, 2000). In living HEK293 cells, an artificially membrane-targeted p110γ can be stimulated by G$\beta\gamma$, but wild-type p110γ depends on p101 to reach the plasma membrane (Brock *et al.*, 2003).

The molecular structure of p101 has not been resolved so far. Strikingly, is does not show substantial similarity to any known protein. Moreover, domain identification algorithms such as SMART (Schultz *et al.*, 1998) fail to detect common protein domains within the p101 sequence. Still, some information is available concerning the structural organization of p101, although mostly in relation to p110γ. Fluorescence resonance energy transfer (FRET) studies using fluorescently tagged p101 and p110γ proteins have revealed that the N and the C termini of both subunits are closer to each other than to the opposite termini (Brock *et al.*, 2003). Based on a study of p101 deletion mutants, large areas of p101 have been implicated to contribute to interaction with p110γ, whereas the N terminus of p101 has been found to be indispensable for activation of heterodimers by G$\beta\gamma$ (Krugmann *et al.*, 1999). However, the proper binding sites for both p110γ and G$\beta\gamma$ on p101 are still elusive.

1.4.3 Ras as an activator of p110γ

Ras family proteins such as H-Ras, N-Ras, K-Ras, R-Ras, and Tc21 are capable of activating p110γ in their GTP-bound state in transfected cells and on neutrophil membranes (Suire *et al.*, 2002; Rodriguez-Viciana *et al.*, 2004). However, constitutively active Ras proteins are unable to relocate p110γ to the plasma membrane upon coexpression, pointing to an allosteric rather than a translocation-based activation mechanism (Suire *et al.*, 2002). Indeed, crystallographic analysis of a p110γ–N-Ras complex revealed conformational changes within p110γ compared to the uncomplexed structure that also alter the phospholipid headgroup binding site (Pacold *et al.*, 2000). The dissociation constant for the p110γ–N-Ras interaction is around 3 µM, which is considerably higher than that of the Ras–Raf-RBD complex (Pacold *et al.*, 2000). Both for the p110γ monomer and the p110γ/p101 dimer, activation by Ras is synergistic with G$\beta\gamma$-mediated activation, leading to an additional 8-fold increase in activity for the G$\beta\gamma$-stimulated heterodimer (Pacold *et al.*, 2000). Certain mutations within the RBD yield a variant of p110γ that is unable to bind to Ras. Mice with targeted mutations in the p110γ gene that lead to expression of a Ras binding-defective p110γ show severe reductions in various p110γ-mediated neutrophil responses (Suire *et al.*, 2006). These findings indicate that interaction with Ras is vital to the physiological requirements of PI3Kγ as well.

1.4.4 Interaction with GRK2 and PDE3B in heart

In the context of characterizing the cardiac function of PI3Kγ, two novel interaction partners for p110γ have been identified. The first of them is the G protein-coupled receptor kinase (GRK) 2 (also called β-adrenergic receptor kinase 1, βARK1, see Pitcher *et al.*, 1998; Penn *et al.*, 2000, for review), which phosphorylates ligand-occupied βARs, thereby inducing their desensitization and endocytosis. βARs are crucial enhancers of cardiac contractility that act *via* the G_s–adenylyl cyclase–cAMP–PKA pathway, leading to phosphorylation of *e.g.* sarcolemmal L-type Ca^{2+} channels and phospholamban (Bers, 2002; Rockman *et al.*, 2002).

A series of studies revealed that PI3Kγ is involved in endocytosis of βARs by locally providing docking sites for PI-dependent endocytotic proteins (Naga Prasad *et al.*, 2001, 2002, 2005). p110γ forms a cytosolic complex with GRK2 that translocates to agonist-occupied receptors, which are then phosphorylated by GRK2. After binding of β-arrestin, clathrin adapter proteins such as AP-2 are recruited in a manner that is dependent on 3'-phosphorylated PI lipids (Gaidarov *et al.*, 1996; Gaidarov & Keen, 1999). Such lipids are generated by p110γ at the site of the receptor complex, targeting it to clathrin-coated pits (Naga Prasad *et al.*, 2001, 2002). The interaction between GRK2 and p110γ is mediated by the 200-aa helical PIK domain present in all PI3Ks (Naga Prasad *et al.*, 2002). Indeed, p110$\gamma^{-/-}$ mice show no alterations in βAR levels (Crackower *et al.*, 2002), suggesting that other PI3K isoforms such as p110α, which is prominently expressed in heart, may substitute for p110γ (Oudit *et al.*, 2003; Nienaber *et al.*, 2003). In mice, cardiac overexpression of a catalytically inactive, truncated p110γ or of its PIK domain prevents βAR downregulation upon chronic stimulation and improves cardiac function after infarction (Nienaber *et al.*, 2003; Perrino *et al.*, 2005). Recent findings indicate that the protein kinase activity of p110γ (or p110α) is likewise crucially involved in this process by phosphorylation of non-muscle tropomyosin (Naga Prasad *et al.*, 2005), which presumably leads to changes in actin bundling critical for endocytosis (Merrifield *et al.*, 1999).

PDE3B represents another important interaction partner for p110γ in heart. Characterization of mice carrying a mutation in the PIK3CG gene that renders p110γ catalytically inactive uncovered a scaffolding interaction between p110γ and PDE3B (Patrucco *et al.*, 2004). Interaction with p110γ stimulates PDE3B activity independently of the catalytic function of p110γ. Because p110γ was unable to stimulate PDE3B purified from hearts of p110$\gamma^{-/-}$ mice, it has been speculated that a regulatory complex must contain further proteins that are essential for PDE3B activation (Patrucco *et al.*, 2004). Intriguingly, the PDE3A isoform, which is most abundant in heart, does not interact with p110γ. Moreover, PDE3B is considered to be expressed almost exclusively in vasculature rather than myocytes in heart (Kerfant *et al.*, 2006). These observations may suggest additional layers of p110γ-mediated regulation of cardiac cAMP levels. As the assays revealing the interaction between PDE3B and p110γ were performed on whole-heart lysates, it is not possible to specify the cellular origin of the precipitated PDE3B-p110γ complexes. The phenotype of the p110$\gamma^{KD/KD}$ mice, however, suggests that the interaction with PDE3B appears to play a major role in the PI3K-dependent regulation of cAMP levels in cardiac myocytes. A detailed characterization of the p110γ-PDE3B interaction or the identification of further potential members of the complex regulating PDE3B in heart have not been reported yet.