

Chapter 4

Phosphoinositide 3-Kinases—A Historical Perspective

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Abstract The phosphoinositide 3-kinase (PI 3-K) signal relay pathway represents arguably one of the most intensely studied mechanisms by which extracellular signals elicit cellular responses through the generation of second messengers that are associated with cell growth and transformation. This chapter reviews the many landmark discoveries in the PI 3-K signaling pathway in biology and disease, from the identification of a novel phosphoinositide kinase activity associated with transforming oncogenes in the 1980s, to the identification of oncogenic mutations in the catalytic subunit of PI 3-K in the mid 2000s. Two and a half decades of intense research have provided clear evidence that the PI 3-K pathway controls virtually all aspects of normal cellular physiology, and that deregulation of one or more proteins that regulate or transduce the PI 3-K signal ultimately leads to human pathology. The most recent efforts have focused on the development of specific PI 3-K inhibitors that are currently being evaluated in clinical trials for a range of disease states.

This chapter is devoted to a historical review of the landmark findings in the PI 3-K from its relatively humble beginnings in the early to mid 1980s up until the present day. When considering the key findings in the history of PI 3-K, it is essential to recognize the landmark studies by Lowell and Mabel Hokin in the 1950s who were the first to describe that extracellular agonists such as acetylcholine could stimulate the incorporation of radiolabeled phosphate into phospholipids (Hokin and Hokin 1953). Their work initiated an entirely new field of lipid signaling, and subsequent studies in the 1970s by Michell and Lapetina who linked phosphoinositide turnover to membrane-associated receptors that initiate intracellular calcium mobilization (Lapetina and Michell 1973). Later studies revealed that the phospholipase-mediated breakdown of the same minor membrane phospholipids such as PtdIns-4,5-P₂ (phosphatidylinositol-4,5-bisphosphate) is responsible for the release of two additional key second messengers, diacylglycerol (DG) and IP₃ (inositol-1,4,5-trisphosphate) (Kirk et al. 1981; Berridge 1983; Berridge et al. 1983).

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Berridge, Irvine and Schulz then revealed that one of the byproducts of this lipid signal relay pathway is the release of calcium from intracellular stores such as the endoplasmic reticulum (Streb et al. 1983). Finally, pioneering studies by Nishizuka in the late 1970s identified PKC (protein kinase C) as a phospholipid and diacylglycerol-activated serine/threonine protein kinase (Inoue et al. 1977; Takai et al. 1977). At this point, it probably seemed to most at the time that the story was complete, such that hydrolysis of phosphoinositides such as PtdIns-4,5-P₂ and PtdIns-4-P would account for the major mechanisms of agonist-stimulated lipid signaling leading to physiological responses. On the contrary, the story was far from complete and was about to become a lot more complex.

Keywords Phosphorylation · Phosphoinositide 3-kinase · Pleckstrin homology · Akt · PTEN

4.1 The Discovery of PtdIns 3-OH Phosphorylation and PI 3-Kinases

The discovery of an enzymatic activity that could phosphorylate the 3-OH position of the inositol head ring of phosphoinositides has its roots in initial work by Macara and Balduzzi who showed that a phosphatidylinositol kinase activity was found associated with an avian sarcoma virus when expressed in cells (Macara et al. 1984). Simultaneously, Erickson and Cantley, working on the recently-discovered oncogenic tyrosine kinase Src, showed that it could phosphorylate phosphatidylinositol and phosphatidylglycerol in immunoprecipitates (Sugimoto et al. 1984). This suggested to them that Src itself has lipid kinase activity, yet as it turned out later it was the Src-associated PI 3-K activity that was responsible for this reaction. This result prompted Cantley to extend these findings, and in collaboration with Roberts, Schaffhausen and Kaplan they showed that the phosphatidylinositol kinase activity was due to an 85 kDa phosphoprotein bound to the middle T antigen of the polyoma tumor DNA virus (Kaplan et al. 1987). Despite these exciting new findings demonstrating an interaction between lipid-derived signals and transforming oncoproteins, PI 3-K had not yet formally been discovered. It was Whitman, a graduate student in the laboratory of Cantley who realized that the product of the phosphatidylinositol activity in Src immunoprecipitates migrated slightly differently on thin layer chromatography plates from PtdIns-4-P, which was the presumed product of the reaction. A collaboration with Downes, an expert in inositol lipids, proved beyond doubt that the lipid product of the reaction was in fact PtdIns-3-P, the first new phosphoinositide identified in some 30 years (Whitman et al. 1988). Downes, Hawkins and Stephens working independently also provided evidence of the existence of PtdIns-3-P in astrocytoma cells (Stephens et al. 1989). As became evident later, that PtdIns-3-P transduces the PI 3-K signal in response to growth factor was somewhat of a red herring, since the primary activity of class I PI 3-Ks that are activated by both receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCR) is to interconvert PtdIns-4,5-P₂ into PtdIns-3,4,5-P₃. PtdIns-3-P, as it turned out later, is generated by

class III PI 3-Ks, the prototype being the yeast ortholog *vps34p*. In this context, it is often overlooked that PtdIns-3,4,5-P₃ was actually discovered by Traynor-Kaplan and Sklar, who identified an inositol tetrakisphosphate (IP₄)-containing phospholipid in stimulated neutrophils (Traynor-Kaplan et al. 1988). They showed that PtdIns-3,4,5-P₃ is a new phosphoinositide that contains four phosphates, making it the most negatively charged lipid in the cell. Both the Cantley, Stephens and Sklar papers were published within a few months of each other in a period between 1988 and 1989, representing the birth of the PI 3-K field.

There then followed a flurry of activity by the laboratories highlighted above, though arguably the rest of the signaling world did not take much notice at first. At the time most were heavily invested in understanding the molecular mechanisms by which oncogenes such as Src and Ras transduce mitogenic signals, and delineating the mechanisms of activation of ERK (extracellular-regulated kinase). In the PI 3-K world, it was shown that exposure of a variety of cells with mitogenic growth factors such as PDGF (platelet-derived growth factor) (Auger et al. 1989) and GPCR (G protein coupled receptor) agonists (such as fMLP, formylmethionyl leucyl phenylalanine) (Stephens et al. 1991) stimulated the rapid accumulation of PtdIns-3,4,5-P₃, and also PtdIns-3,4-P₂, the latter primarily due to the activity of class II PI 3-Ks and/or breakdown of PtdIns-3,4,5-P₃ by lipid phosphatases. In contrast, the levels of PtdIns-3-P remained relatively constant in most cells studied. The conclusion was therefore that PtdIns-3,4-P₂, and primarily PtdIns-3,4,5-P₃, are true second messengers that transduce the PI 3-K signal. The initial identification of the 85 kDa phosphoprotein associated with Src and polyoma middle T led to its biochemical purification and subsequent cloning by several laboratories (Escobedo et al. 1991; Otsu et al. 1991; Skolnik et al. 1991). During these efforts, Carpenter and Cantley recognized that p85, that we now know represents one of the regulatory subunits of class I PI 3-Ks, was found tightly associated with a p110 protein (Carpenter et al. 1990). Protein microsequencing of purified p110 allowed for the design of degenerate oligonucleotide probes, and using this information Waterfield's laboratory was the first to clone and characterize the catalytic subunit of PI 3-K, in their case p110 α (Hiles et al. 1992). Analysis of the p110 sequence revealed significant homology with the yeast gene *vps34p*, which in 1990 Herman and Emr showed is required for vacuolar protein sorting and segregation in budding yeast (Herman and Emr 1990). Subsequent pioneering studies by Emr's laboratory showed that yeast *vps34p* and its mammalian ortholog are class III PI 3-Ks with a unique substrate specificity, incapable of generating PtdIns-3,4-P₂ or PtdIns-3,4,5-P₃, and exclusively responsible for PtdIns-3-P synthesis (Schu et al. 1993; Brown et al. 1995; De Camilli et al. 1996).

4.2 Expansion of the Family of PI 3-Kinases and Their Regulators

In the years that followed the cloning of p85 α and p110 α , several laboratories described additional PI 3-K isoforms in both mammals, flies, nematodes, yeasts and even plants, and this required reclassification of the PI 3-K family into distinct classes.

We now know there are eight mammalian PI 3-kinases: class Ia enzymes (p110 α , p110 β , p110 δ) and class Ib (p110 γ); class II enzymes (PI 3-K-C2 α , PI 3-K-C2 β , PI 3-K-C γ); and class III enzymes (*vps34p*). Note that the nomenclature refers to the catalytic subunits, yet distinct regulatory subunits for each class exist: (class Ia, p85 α , p55 α , p50 α , p85 β , p55 γ); class Ib, p101 and p84; a p150 regulatory subunit for *vps34p* has also been identified. For more detail, see the following reviews (Vanhaesebroeck et al. 2010; Engelman et al. 2006; Hawkins et al. 2006) and Vol. I, Chap. 5 by Wymann.

The protein sequencing and cloning of the p85 α regulatory subunit coincided with the discovery of SH2 (Src homology 2) domains in pioneering studies by the Hanafusa and Pawson laboratories (Moran et al. 1990; DeClue et al. 1987; O'Brien et al. 1990; Matsuda et al. 1990). Recognizing that p85 subunits have two copies of SH2 domains provided the mechanism by which PI 3-K is relocalized to sites of activated, phosphorylated receptor tyrosine kinases. Zhou and Cantley then used this information to decode the specificity of SH2 domains, including that of p85 α , towards phosphotyrosine motifs in RTKs (Zhou et al. 1993). At the same time, Kazlauskas and Cooper developed an approach in which individual tyrosines in the PDGF-receptor are individually mutated to prevent binding to SH2 domains in proteins including PI 3-K (Kazlauskas and Cooper 1989). This showed that both PI 3-K and PLC γ -1 (phospholipase C- γ) are required to transduce the PDGF signal to DNA synthesis and cell cycle progression (Valius and Kazlauskas 1993). These PDGF-R mutants were then subsequently used by many other laboratories to probe the mechanisms by which PI 3-K engages the cell cycle, and also activates secondary effectors such as S6K1 (p70 ribosomal protein S6 kinase-1) (Chung et al. 1994). Importantly, Kazlauskas later went to show that receptors such as PDGF-R stimulate two waves of PI 3-K activity, one immediately after ligand stimulation lasting about an hour, and a second much larger wave typically 4–8 h post stimulation (Jones et al. 1999). Using PDGF-R mutants as well as PI 3-K inhibitors, his laboratory showed that the first wave is actually dispensable for cell cycle progression to S phase, instead it is the second wave that is responsible.

The next major discovery in the field was wortmannin. In 1993, two laboratories, Wymann and Arcaro as well as Matsuda's group showed that the furanosteroid metabolite isolated from the fungus *Penicillium funiculosum*, known as wortmannin, potently inhibits fMLP-stimulated PtdIns-3,4,5-P₃ synthesis in neutrophils at low nanomolar doses (Arcaro and Wymann 1993; Yano et al. 1993). Other laboratories subsequently confirmed the inhibitory activity of wortmannin on PI 3-K. A year later, Vlahos at Ely Lilly published a structurally distinct compound termed LY294002, that also directly inhibits PI 3-K (Vlahos et al. 1994). Remarkably, both compounds are still widely used in the field, considering that they have since been shown to have off-target effects on other kinases. Indeed much more specific PI 3-K inhibitors have since been described, to the extent that isoform-specific compounds are now available, many of them in phase I and II clinical trials. Regardless, the availability of wortmannin and LY294002 stimulated much progress in the still nascent PI 3-K signaling field.

The discovery that PI 3-K functions a direct effector of GTP-loaded Ras by Rodriguez-Viciana and Downward added further credence to the model that the PI 3-K pathway contributes to cancer etiology, considering the numerous human solid tumors that harbor oncogenic activating mutations in the Ras GTPase (Rodriguez et al. 1994). Although largely overlooked, it was initially Lapetina who first showed that p21 Ras can associate with PI 3-K in cells (Sjolander et al. 1991). This was later confirmed in the seminal paper by Downward's group who showed that GTP-Ras is required for efficient PI 3-K activation by growth factors, and they further identified a direct PI 3-K binding site on Ras (Rodriguez et al. 1994). Thus PI 3-K was added to the list of Ras effectors which now encompass the Ser/Thr kinase Raf, the nucleotide dissociation factor RalGDS and the phospholipase PLC ϵ . It is also important to note that as first recognized by Hawkins and Stephens (Wennstrom et al. 1994; Welch et al. 2003), PI 3-K and PtdIns-3,4,5-P₃ also contribute to the regulation of other GTPases, most notably Rac, and this in turn has profound effects on the regulation of the actin cytoskeleton and cell motility in virtually all cells. This is best illustrated in both neutrophils and *Dictyostelium* amoeba where a gradient of PtdIns-3,4,5-P₃ and Rac activation is rate-limiting for actin assembly and directed cell migration (Funamoto et al. 2001) (also see Vol. II, Chap. 6 by Yin and Vol. II, Chap. 7 by Parent).

4.3 How Do PI 3-Kinase Lipid Products Affect Cell Function?

In the mid 1990s, the field of PI 3-K was firmly established, but was missing a critical piece of the puzzle. How do the PI 3-K lipid products, particularly PtdIns-3,4,5-P₃, transduce the signal to immediate effectors and then onto cellular responses? The first clue came from Fesik and Harlan, who showed that PH (pleckstrin homology) domains bind with high affinity to phosphoinositides, in their case, PtdIns-4,5-P₂ (Harlan et al. 1994). One year later, Tsichlis working with Kaplan and Franke published a seminal finding identifying the first *bona-fide* effector of PI 3-K, the Ser/Thr kinase Akt (Franke et al. 1995). Akt had been originally identified by Tsichlis as the cellular homolog of the transforming retroviral oncogene *v-Akt* (Bellacosa et al. 1991). It was independently identified by Woodgett and termed PKB (protein kinase B), as it was sequence-related to PKA (protein kinase A) and PKC (protein kinase C) (Coffer and Woodgett 1991), and also by Hemmings as Rac (related to A and C kinases (Jones et al. 1991)). Akt contains a PH domain, and this prompted Tsichlis and colleagues to investigate whether Akt is PI 3-K target, and indeed they showed that PDGF stimulates Akt activation in a PI 3-K-dependent manner (Franke et al. 1995). Burgering and Coffer came to the same conclusion independently (Burgering and Coffer 1995). Initially suggested to be mediated by PtdIns-3-P, several groups subsequently showed that the PH domain of Akt has high affinity for PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ (Frech et al. 1997; Andjelkovic et al. 1997; Franke et al. 1997). Although several groups showed that PI 3-K lipids, particularly PtdIns-3,4-P₂, could weakly activate Akt *in vitro*, it was not until two groups independently

discovered PDK-1 (phosphoinositide-dependent kinase-1), the enzyme that is responsible for full Akt activation. Working with Hemmings and Cohen, Alessi had originally shown that Akt is phosphorylated at two key residues, Thr308 in the T loop segment of the kinase domain, and Ser473 in a hydrophobic region at the carboxyl-terminus (Alessi et al. 1996). Later, working in the lab of Cohen, Alessi set about to purify the enzymatic activity capable of phosphorylating and activating Akt. This led to the identification of PDK-1 as the Thr308 upstream kinase (Alessi et al. 1997). Once again, Hawkins and Stephens working with Stokoe independently identified the same activity, and they further showed that PDK-1 phosphorylates Akt at Thr308 in a manner that depends on PtdIns-3,4,5-P₃ binding to the Akt PH domain (Stokoe et al. 1997). Subsequent studies by Alessi and other laboratories demonstrated that PDK-1 is the master upstream T loop kinase for many AGC kinases, such as S6K, PKCs, SGKs and several others (Alessi 2001; Mora et al. 2004). As for Ser473, whose phosphorylation contributes to full Akt activation, several upstream kinases were proposed over the years, and while some of these may function in specific settings, it wasn't until 2005 when Sabatini's group showed that the mTORC2 (mammalian target of rapamycin complex 2) complex represents the physiologically-relevant Ser473 kinase, at least in most cells and tissues (Sarbasov et al. 2005). It is also important to note that PH domains with very discrete phosphoinositide-binding specificities are now known to exist (Lemmon 2008). Moreover, since the discovery of PH domains, other domains in signaling proteins have been described that bind other 3 phosphoinositides, such as the PX and FYVE domains, providing the mechanistic basis for the regulation of specific cellular responses to individual PI 3-K lipid products.

Once Akt was identified as a direct effector of PI 3-K and PtdIns-3,4,5-P₃, the field moved quickly to identify the immediate substrates of Akt that would transduce the signal to a specific response. Cohen identified the first Akt substrate, GSK-3 β , a rate-limiting enzyme in the regulation of glycogen synthase in insulin-responsive cells and tissues (Cross et al. 1995). Because Cooper initially showed that PI 3-K comprises a key cellular survival pathway (Yao and Cooper 1995), this prompted Greenberg and colleagues to subsequently show that indeed Akt is one of the PI 3-K effectors in cellular survival mechanisms (Dudek et al. 1997). His laboratory also showed that the pro-apoptotic protein BAD is an Akt substrate whereby phosphorylation attenuates its apoptotic function (Datta et al. 1997). Secondly, Brunet also working in Greenberg's lab showed that the pro-apoptotic FOXO3a transcription factor is phosphorylated by Akt, and in turn this blocks its apoptotic function (Brunet et al. 1999). Burgering's lab published the same finding for AFX (Kops et al. 1999; Morris et al. 1996). It is important to note that much of the work on the importance of PI 3-K and Akt signaling in cellular survival was pioneered by Ruvkun and colleagues, who provided irrefutable genetic evidence for a linear pathway in *Caenorhabditis elegans* comprising PI 3-K (*age-1*) (Morris et al. 1996), PDK-1 (*pdk-1*) (Paradis et al. 1999), Akt (*akt-1* and *akt-2*) (Paradis and Ruvkun 1998), and FOXO (*daf-16*) (Ogg et al. 1997) that controls survival and lifespan in the nematode worm. Similar findings using *Drosophila* genetics also corroborated the importance of PI 3-K signaling to cellular growth and survival (Leever et al. 1996).

At the start of the new millennium, the PI 3-K field was firmly established as one of the central mechanisms by which extracellular stimuli initiate a lipid signaling pathway that culminates in the synthesis of PtdIns-3,4,5-P₃, activation of downstream effectors such as Akt, and in turn alterations in cell growth, proliferation, survival, migration and gene transcription. Much of this had been achieved using biochemistry and cell-based assays with overexpression, and inhibitors such as wortmannin. What was missing was a genetic approach to begin to probe the contributions of individual regulatory p85 and catalytic p110 subunits in normal physiology and disease. In 1999, Kadowaki's group published the first p85 α (*PIK3RI*) knockout mouse which displayed increased insulin sensitivity and hypoglycemia (Terauchi et al. 1999). Shortly thereafter, Fruman and Cantley reported that ablation of all three splice variants encoded by p85 α (p85 α , p55 α and p50 α) results in perinatal lethality due to hepatocyte necrosis and chylous ascites (Fruman et al. 2000). Similar to Kadowaki's finding, they also reported that loss of p85 α alone results in hypoglycemia and increased insulin sensitivity. Knockout of p85 β has also been reported, with phenotypes similar to p85 α null mice (Ueki et al. 2002). At about the same time reports began to emerge on the knockouts of the p110 catalytic subunits. Knockout of p110 α and p110 β results in early embryonic lethality, and this has precluded a detailed analysis of the role of these isoforms in adult tissues (Bi et al. 1999, 2002). On the other hand, considerable information on p110 isoform-specific signaling has been gained by analysis of the p110 γ and p110 δ isoforms, which are preferentially, although by no means exclusively, expressed in the immune system. Hirsch and Wymann showed that p110 γ knockout mice are viable, yet their neutrophils show an impaired respiratory burst and migration phenotype (Hirsch et al. 2000). Macrophages from these mice reveal impaired motility, and defective accumulation in a sepsis model. Interestingly, both Hirsch, Wymann and Penninger also reported effects on cardiomyocyte contractility that at least in one case was attributed to a non-kinase scaffolding function of p110 γ that functions through a cyclic AMP (cAMP) and phosphodiesterase (PDE3B) pathway (Crackower et al. 2002; Patrucco et al. 2004). Two groups also independently deleted p110 δ , and also generated 'knock-in' mice, and although the mice are viable, they reveal impaired immune cell development and function, much like the p110 γ null mice (Clayton et al. 2002; Jou et al. 2002). In combination, these studies have provided compelling evidence that p110 γ and p110 δ are viable targets for immunity and cardiac dysfunctions. To this end, specific p110 γ and p110 δ inhibitors are currently undergoing clinical trials for a range of immunological pathologies (see below and Vol. I, Chap. 6 by Hirsch for more details).

4.4 Linking PtdIns-3,4,5-P₃ to Cancer

A major leap forward in our understanding of the importance of PI 3-K signaling in pathophysiology was made by the discovery of the PtdIns-3,4,5-P₃ phosphatase PTEN. Parsons group had been studying a region on chromosome 10q23 known to be frequently subjected to loss of heterozygosity (LOH), particularly in prostate

cancer. This led to speculation that a yet-to-be-discovered tumor suppressor gene resides in this area whose loss would predispose to malignancy in several cancer types. Li, Parsons and colleagues used positional cloning to identify an open reading frame that encoded a protein with homologies to protein tyrosine phosphatases, chicken tensin and bovine auxilin (Li et al. 1997). They therefore named the gene *PTEN*, for phosphatase and tensin homolog deleted on chromosome 10. The same year Steck and colleagues also independently cloned the same gene and named it *MMAC1*, for mutated in multiple advanced cancers (Steck et al. 1997). Both groups recognized the striking homology to the catalytic domain of protein tyrosine phosphatases. However, the discovery that *PTEN* is a PtdIns-3,4,5-P₃ phosphatase is attributed to Dixon and Maehama (Maehama and Dixon 1998). Dixon, an expert in protein tyrosine phosphatases, realized that *in vitro* *PTEN* has a much higher specific activity towards acidic and thus negatively-charged phosphopeptides compared to uncharged peptides. He then recognized that *PTEN* might utilize lipid rather than proteinaceous substrates, and recalled that PtdIns-3,4,5-P₃ is the most negatively charged lipid in the cell. Sure enough, when PtdIns-3,4,5-P₃ was used as substrate, *PTEN* efficiently dephosphorylated it back to PtdIns-4,5-P₂, providing evidence that *PTEN* is the first identified 3 phosphoinositide phosphatase (Maehama and Dixon 1998). Given that *PTEN* LOH is a frequent event in various human solid tumors, this once again bolstered the link between PI 3-K, PtdIns-3,4,5-P₃ and tumorigenesis. We now know that inactivating mutations, deletions and LOH are frequent events in the *PTEN* locus, to the extent that over 30% of glioblastoma patients have *PTEN* loss leading to hyperactivation of PI 3-K and Akt signaling (Keniry and Parsons 2008).

It took almost 20 years since the discovery of PI 3-K for researchers to identify oncogenic activating mutations in *PIK3CA*, the gene that encodes p110 α . In spite of the findings that PI 3-K associated with transforming oncogenes, that it mediates growth factor signaling in response to mitogens, and the fact that the PtdIns-3,4,5-P₃ phosphatase *PTEN* is a tumor suppressor, the discovery in 2004 that *PIK3CA* is an oncogene frequently mutated in human solid tumors was the first real direct evidence linking this pathway to cancer (Samuels et al. 2004; Campbell et al. 2004). In certain tumors, for example estrogen receptor positive breast cancer, *PIK3CA* is the most frequently mutated oncogene, leading to hyperactivation of Akt and other PI 3-K effectors to drive cellular proliferation, survival and metastasis (reviewed by (Engelman 2009)). Surprisingly, no oncogenic activating mutations have been detected in other p110 isoforms, particularly *PIK3CB*, yet studies have clearly demonstrated that p110 β can promote tumorigenesis, particularly in settings on *PTEN* loss (Zhao et al. 2005; Jiang et al. 2010). On the other hand, mutations in PI 3-K regulatory subunits, for example *PIK3R1* are found in human tumors, albeit at relatively low frequencies, and thus may activate p110 β and p110 δ indirectly (Jaiswal et al. 2009). Similarly, activating oncogenic mutations in Akt isoforms have also been described in various human tumors, again at low frequency (Carpten et al. 2007). The frequency and spectrum of *PTEN* LOH and mutations, *PIK3CA* amplifications and mutations, *PIK3R1* and Akt oncogenic mutations render PI 3-K one of the most frequently mutated and druggable pathways in human cancer. It is not surprising therefore that numerous clinical trials for novel drugs are targeting one or more of these enzymes

for therapeutic benefit. Presently the compound in the most advanced trials is the dual mTOR and PI 3-K inhibitor NVP-BEZ235 from Novartis (Maira et al. 2008). Other dual TOR/PI 3-K inhibitors are also being evaluated for targeted therapy, and this is because the TORC1 pathway effects a negative feedback loop on PI 3-K, such that inhibition of TORC1 with rapamycin can actually enhance PI 3-K/Akt activity *in vivo* (O'Reilly et al. 2006). As already discussed, specific p110 β inhibitors are also in trials for treatment of thrombosis, and a p110 δ inhibitor is being evaluated for hematological malignancies, in addition to a range of next generation p110 α and p110 β inhibitors (reviewed by (Vanhaesebroeck et al. 2010)). While the outlook for the use of these inhibitors is promising, it is likely that they will be used in dual targeted therapies with drugs that block activated or amplified receptor tyrosine kinases (such as EGF-R or Met) or drugs that interfere with ERK activity. Moreover, while some or possibly even many of the PI 3-K inhibitors currently in trials will fail, this will be of significant benefit to the research community who will use these much more specific inhibitors for basic research. It is also reasonable to argue that the use of these inhibitors, coupled with global RNAi screening approaches in tumor cells as well as model organisms such as flies will result in the identification of previously unappreciated mechanisms by which PI 3-K promotes disease states.

In this context, much of the activity and function of PI 3-K in both normal physiology and human disease has been attributed to Akt, most likely because of the availability of numerous reagents for studying Akt biology. Yet it is clear that a significant proportion of PI 3-K and PtdIns-3,4,5-P₃ and PtdIns-3,4-P₂ signaling is mediated by non-Akt effectors. Indeed a recent RNAi screening experiment revealed that in certain breast cancer cells harboring oncogenic *PIK3CA*, Akt is actually dispensable for growth and proliferation, yet both PDK-1 and SGK3 (serum and glucocorticoid-regulated kinase-3) are required (Vasudevan et al. 2009). Because SGK3 has a PX domain that interacts primarily with PtdIns-3-P in endosomes, the suggestion is that *PIK3CA* may mediate oncogenic signaling in certain human tumors through PtdIns-3-P at the endosome. Moreover, other targets of PI 3-K likely function independently of Akt, for example the tyrosine kinase Btk which binds with high affinity to PtdIns-3,4,5-P₃ and PtdIns-3,4-P₂ (Isakoff et al. 1998; Fruman et al. 2002). A large family of PtdIns-3,4,5-P₃ binding proteins include guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) for members of the Rho superfamily of small GTPases. Examples are the proteins pREX2a and P-REX1 which function as GEFs for Rac and integrate PtdIns-3,4,5-P₃ and heterotrimeric G protein signaling in cancer (Fine et al. 2009; Sosa et al. 2010).

4.5 Challenges Ahead

Due to the enormous advances in PI 3-K signaling and biology during the past two decades, we are presently at the stage where this pathway has entered the clinical arena and there is the realistic expectation that current drugs, or their derivatives, that target enzymes in the PI 3-K pathway will ultimately prove to have therapeutic value.

This should not be taken as meaning that we now have learned the major mechanisms by which the PI 3-K pathway controls human biology and disease. There are numerous gaps in our understanding as to how PI 3-K and its lipid products mediate signal relay, and how deregulation of these mechanisms results in pathology, and yet new discoveries are constantly being made. Even though we knew back in the 1990s that PtdIns-3,4-P₂ has a signaling role *per se*, for example in the binding and regulation of Akt, it wasn't until the polyphosphates 4 phosphatase *INPP4B* was shown to function as a tumor suppressor that the exclusive role of this phosphoinositide was directly demonstrated (Gewinner et al. 2009). The implication is that in addition to Akt there likely exist other direct PtdIns-3,4-P₂ effectors. Another major unexplored area of signaling in the field is the specific roles played by p110 isoforms. Much information has been garnered from studies of individual p110 knockout mice, and also cell-based studies using RNAi. What is lacking is a more complete understanding how individual p110 isoforms, perhaps in specific cellular compartments, elicit pools of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ at discrete locations, and in turn how this affects downstream signaling. Compartmentalized PI 3-K signaling is likely to have a profound impact on certain cellular responses, for example remodeling of the actin cytoskeleton which requires specific temporal as well as kinetic regulation during directed cell migration. Genetically-encoded FRET-based sensors to monitor both PI 3-K effectors, as well as PtdIns-3,4,5-P₃/PtdIns-3,4-P₂ themselves will likely aid in this pursuit. This issue also applies to the nuclear phosphoinositide cycle, first recognized and pioneered by Irvine, Divecha and Cocco (Cocco et al. 1987; Divecha et al. 1991). Even before the discovery of PI 3-K we knew there exists a nuclear phosphoinositide cycle, and nuclear phosphoinositides had been detected (Cocco et al. 1987). Since then, both PI 3-K and its lipid products have been detected inside the nucleus, consistent with the model that certain targets of Akt, for example FOXO3a, are actually phosphorylated in the nucleus, and subsequently exported to the cytoplasm thus terminating transcriptional activity (Brunet et al. 2002). Yet there remain many gaps in our knowledge of the regulation and function of nuclear PI 3-K signaling. Many of the effectors of PI 3-K also exist as multi-gene families, a good example is Akt (Akt1, Akt2 and Akt3 in mammals). While it was originally assumed that they would function redundantly, we now know that there are specific functions and even specific substrates of Akt isoforms. For example, in the context of cancer progression, Akt2 promotes tumor invasion and metastasis, whereas Akt1 either does not or even attenuates this phenotype (Yoeli-Lerner et al. 2005; Irie et al. 2005; Maroulakou et al. 2007). These findings of opposing or non-redundant function for PI 3-K effectors have profound implications for drugs that are being developed for therapeutic intervention in the PI 3-K/Akt pathway. Whether similar opposing phenotypes exist for other PI 3-K effectors remains to be determined, but is likely to be the case. It is also very likely that additional feedback loops exist in the pathway, analogous to the mTOR/S6K1 negative feedback loop. If this is the case, this will make the use of PI 3-K and Akt inhibitors somewhat of a challenge. Moreover, although powerful genetic models exist, such as p110 isoform knockout as well as transgenes, the next generation models will likely be considerably more complex, and recapitulate the multiple genetic lesions in the PI 3-K pathway that we

now know exist in human cancer patients. These can then be used in combination with the latest PI 3-K inhibitors to more accurately model human cancer therapy in preclinical models.

In closing, this chapter has highlighted the major discoveries in the PI 3-K field in some two and a half decades. From the identification of the first novel phosphoinositide PtdIns-3-P in the mid 1980s, to the discovery of oncogenic *PIK3CA* mutations in the mid 2000s, to the present day with clinical trials for p110 inhibitors, the field has matured at a dizzying pace. Yet we have clearly much more to learn as we await the results from the PI 3-K inhibitor clinical trials. We are now at a point where biochemical studies, genome-wide screening approaches and clinical trials are all informing the basic and clinical arena, and the field is poised to capture this information and much more efficiently translate it into therapeutic benefit.

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