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REVIEW ARTICLE

Nuclear phosphoinositides and their roles in cell biology and disease

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Abstract

Since the late 1980s, a growing body of evidence has documented that phosphoinositides and their metabolizing enzymes, which regulate a large variety of cellular functions both in the cytoplasm and at the plasma membrane, are present also within the nucleus, where they are involved in processes such as cell proliferation, differentiation, and survival. Remarkably, nuclear phosphoinositide metabolism operates independently from that present elsewhere in the cell. Although nuclear phosphoinositides generate second messengers such as diacylglycerol and inositol 1,4,5 trisphosphate, it is becoming increasingly clear that they may act by themselves to influence chromatin structure, gene expression, DNA repair, and mRNA export. The understanding of the biological roles played by phosphoinositides is supported by the recent acquisitions demonstrating the presence in the nuclear compartment of several proteins harboring phosphoinositide-binding domains. Some of these proteins have functional roles in RNA splicing/processing and chromatin assembly. Moreover, recent evidence shows that nuclear phospholipase Cβ1 (a key phosphoinositide metabolizing enzyme) could somehow be involved in the myelodysplastic syndrome, i.e. a hematopoietic disorder that frequently evolves into an acute leukemia. This review aims to highlight the most significant and updated findings about phosphoinositide metabolism in the nucleus under both physiological and pathological conditions.

Keywords: Signal transduction, proliferation, myogenic differentiation, phospholipase C, gene expression, myelodysplastic syndrome

Introduction

The inositol head group of phosphatidylinositol (PI) can be reversibly phosphorylated at the 3′, 4′, or 5′ position in all the possible combinations, thus generating seven different biologically relevant phosphoinositides that form the basis of an ubiquitous signaling system. Phosphoinositides comprise only a small fraction (less than 5%) of cell membrane phospholipids, yet they play roles of paramount importance in the control of an extremely wide range of cell functions. The subcellular profile of phosphoinositides is controlled by a wide array of kinases, phosphatases, and phospholipases (Sasaki *et al.*, 2009; Liu and Bankaitis *et al.*, 2010; Fukami *et al.*, 2010).

Phosphoinositides act as both direct messengers and precursors to messengers that are involved in regulating protein enzymatic activity. Moreover, it is now established that phosphoinositide head groups bind a variety of protein modules. Through these interactions, phosphoinositides play a major role in recruiting target proteins at the membrane interface. By doing so, phosphoinositides deeply impact on a wide array of cellular processes, which include

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cell proliferation, differentiation, survival, polarity, migration, vesicle transport, actin and microtubule dynamics, autophagy, ion channel function, and gene transcription (McCrea and De Camilli, 2009; Balla *et al.*, 2009). Moreover, deregulation of phosphoinositide signaling is being implicated in a growing number of human disorders, including cancer, type 2 diabetes, myopathies, Charcot-Marie-Tooth disease, and amyotrophic lateral sclerosis (McCrea and De Camilli, 2009; Majerus and York, 2009).

Phosphoinositides are tightly bound to the cytosolic-facing leaflet of biomembranes, thus phosphoinositide-driven signaling occurs on different membranes which include the plasma membrane, the endoplasmic reticulum, the Golgi apparatus, and on membrane vesicles moving between these compartments. The spatially- and temporally-restricted, subcellular distribution of specific phosphoinositide signaling pathways is mainly achieved through protein-protein interactions unique to each lipid kinase, which allow for the generation of lipid messengers at specific cell domains (Heck *et al.*, 2007).

The PI cycle was discovered in the 1950s by Lowell and Mabel Hokin (Hokin and Hokin, 1953). In the canonical plasma membrane phosphoinositide cycle, extracellular stimuli (growth factors, hormones, cytokines, etc.) trigger the generation of phosphoinositide-dependent signals via membrane receptors. In the early 1980s, it became clear that both phosphoinositides and their metabolizing enzymes were also present in the nucleus of mammalian cells. However, it was initially thought that nuclear phosphoinositide metabolism occurred at the nuclear envelope level (Smith and Wells, 1983). In 1987, Cocco and co-workers (Cocco *et al.*, 1987) documented that mouse erythroleukemia (MEL) cell nuclei, completely stripped of their nuclear membrane by detergent, were still able to synthesize *in vitro* phosphoinositides, as demonstrated by incorporation of ^{32}P into PI 4 phosphate (PI4P) and PI 4,5 bisphosphate ($PI4,5P_2$). Moreover, it was documented that when MEL cells were induced to differentiate along the erythroid lineage, the levels of nuclear $PI4,5P_2$ increased, while the total cellular amount of $PI4,5P_2$ remained unchanged. These findings implied that signaling by phosphoinositides occurred within the nucleus and that nuclear phosphoinositide metabolism and its regulation were independent from their cytoplasmic/plasma membrane counterparts. Subsequent studies, carried out both *in vitro* and *in vivo*, reinforced the idea that an independent nuclear phosphoinositide metabolism did indeed exist (Cocco *et al.*, 1988; Cocco *et al.*, 1989; Divecha *et al.*, 1991). Since then, nuclear phosphoinositide-based signaling pathways have been shown to play key roles in a wide range of events that include cell proliferation and differentiation, DNA repair, transcription, chromatin structure, and mRNA metabolism (Martelli *et al.*, 2004; Visnjic and Banfic, 2007; Ye and Ahn, 2008; Mellman and Anderson, 2009; Divecha, 2010).

In this review, we shall mostly summarize the most recent and significant findings regarding nuclear phosphoinositides and their metabolizing enzymes. In particular, we will focus on the emerging theme of nuclear phosphoinositide-binding proteins. Then, we will highlight novel functions played by phosphoinositides and their kinases within the nucleus. Finally, we will discuss the possible involvement of nuclear phosphoinositide metabolism in the evolution of the myelodysplastic syndrome (MDS) to acute leukemia. An overview, which highlights nuclear phosphoinositide-binding proteins and their interacting domains, the known functions of nuclear phosphoinositides, their intranuclear localization sites, and the enzymes involved in their synthesis and degradation within the nucleus, is presented in Table 1.

For detailed descriptions of phosphoinositide-metabolizing enzymes (kinases, phospholipases, phosphatases), phosphoinositide-binding protein domains, and functions of the protein interacting with phosphoinositides, we refer the readers to the many comprehensive reviews available on broader topics related to phosphoinositide signaling.

Nuclear structure

For a better understanding of this review, it is useful to briefly recapitulate the basic structure of the nucleus. The nucleus is separated from the cytoplasm by the nuclear envelope (NE) that comprises the outer and the inner membrane. Both of these membranes are phospholipid bilayers. The NE is pierced at intervals by nuclear pore complexes, highly structured focal continuities between the two membranes (Doucet and Hetzer, 2010). The outer nuclear membrane is an extension of the endoplasmic reticulum containing ribosomes, while the inner nuclear membrane contains specific proteins (nesprin, emerin, lamin B receptor, etc.), which bind the nuclear lamina and chromatin (Marmiroli *et al.*, 2009). The nuclear lamina is an intermediate filament protein meshwork, which is anchored to the inner nuclear membrane and provides structural support to the nucleus and interacts directly with chromatin (Kind and van Steensel, 2010). Regarding the nuclear interior, evidence has accumulated that the nucleus has a compartmentalized structure consisting of chromosome territories (CTs) and an interchromatin compartment (IC). CTs are built up from a hierarchy of chromatin domains starting with DNA loop domains with an average DNA content ranging from about 30–200 kilobases (Kb), referred to as 100 Kb chromatin domains. This model further predicts that a series of loop domains, forms larger chromatin domains with DNA contents of several hundred Kb to several megabases (Mb), termed 1 Mb chromatin domains (Cremer *et al.*, 2004).

The IC is envisaged as a three-dimensional network of lacunas and channels, which starts at nuclear pores and then expands both between neighboring CTs and into the interior of individual CTs, and is lined by the surface of smaller and larger chromatin domains. It has been hypothesized that the IC and the border zone between chromatin domains and the IC, termed the perichromatin region, have a defined topology essential

Table 1. Overview of nuclear phosphoinositides, their functions, and their metabolizing enzymes.

Nuclear lipid	Binding domain(s) recognizing the lipid at the nuclear level	Nuclear proteins binding the lipid	Known nuclear function(s)	Localization within the nucleus	Enzyme responsible for synthesis of the lipid within the nucleus	Enzyme responsible for degradation of the lipid within then nucleus
PI 4P	Unknown	Unknown	Precursor to PI $4,5P_2$	Unknown (Nuclear speckles?)	PIKIIIα/β	Unknown
PI 5P	PHD	ING2	Pro-apoptotic; Regulation of a nuclear ubiquitin ligase complex; Precursor to PI $4,5P_2$	Chromatin	Type I PI $4,5P_2$ 4-phosphatase	Unknown
PI $4,5P_2$	PDZ; PDZ-2; K/R motifs	Syntenin-2; ZO-2; U2 snRNP A'; U4/U6 snRNP Prp4; SF3A1; SPF27; Topoisomerase IIα; Aly	Precursor to DG and Ins $1,4,5P_3$; Regulation of chromatin structure;Regulation of pre-mRNA processing; Regulation of STAR-PAP complex; Regulation of DNA topology (?)	Nuclear speckles	Type I and II PIPKs	PLCβ1; Type I PI4,5P 4-phosphatase
PI $3,4,5P_3$	PH; GR-rich	PI $3,4,5P_3$BP; Centaurin-α1; Nucleolin/C23; Nucleophosmin/B23; PIKE-L; Aly	Chemoattractant for PKCζ translocation; Anti-apoptotic; DNA repair	Diffuse	Class I and II PI3Ks	PTEN; SHIP2

for transcription, RNA splicing, DNA replication, and presumably also for DNA repair (Fakan and van Driel, 2007).

It should be considered that the nucleus is unique amongst cellular organelles in that it contains a myriad of discrete suborganelles referred to as nuclear bodies or nuclear domains. These nuclear domains are morphologically and molecularly distinct dynamic entities, which further compartmentalize the IC (Zhao *et al.*, 2009). By doing so, they create microenvironments within the nucleus, which host specific nuclear processes. In sharp contrast to cytoplasmic organelles, nuclear domains are not surrounded by lipid membranes, and their structural integrity is entirely mediated by protein-protein and possibly protein-RNA interactions (Dundr and Misteli, 2010).

The nucleus can be stripped of the envelope by detergents, and then treated with DNase, RNase, and high salt buffers to remove chromatin, RNA, and soluble proteins. What is left is a residual protein network referred to as the nuclear matrix, which could act as the nuclear equivalent of the cytoskeleton. However, the existence of a nuclear matrix *in vivo* is highly controversial (Martelli *et al.*, 2002).

The chemical nature of nuclear phosphoinositides

Phosphoinositide molecules consist of two long hydrophobic fatty acyl tails linked to a glycerol group that is anchored through a phosphodiester bond to the phosphorylated inositol head group. This chemical structure is perfectly suited to form an interface between the hydrophobic plasma membrane and the cytosol, through the insertion of the fatty acyl tails. Indeed, such an interaction leaves the inositol head group exposed and accessible for phosphorylation/dephosphorylation by specific kinases/phosphatases.

The exact chemical nature of phosphoinositides residing in the nucleus is far from being understood. In the late 1990s, it was reported that interphase nuclei of many mammalian cells contained deep, dynamic, branching, tubular membrane-bound invaginations of the NE (Fricker *et al.*, 1997; Lui *et al.*, 1998). The existence of this nucleoplasmic reticulum (Malhas *et al.*, 2011), lined by both the outer and the inner nuclear membranes, where intranuclear phosphoinositides might reside, has been demonstrated by electron microscopy (Fricker *et al.*, 1997), immunofluorescence microscopy (Lui *et al.*, 1998; Echevarria *et al.*, 2003), and more recently, also by three-dimensional structured illumination microscopy (Schermelleh *et al.*, 2008). However, there are some cell types, such as primary neurons, where the nucleoplasmic reticulum could not be observed (Bezin *et al.*, 2008).

Alternatively, nuclear phosphoinositides might form some micelle-like structures. However, one would imagine that, if indeed nuclear phosphoinositides were either components of a nucleoplasmic reticulum or formed micelles, they could be efficiently solubilized by detergents, whereas there is a nuclear phosphoinositide pool, which is highly resistant to detergent extraction (Vann *et al.*, 1997).

Therefore, two fundamental questions that need to be addressed are: Where are phosphoinositides localized and how are they maintained within the nucleus? Over the years, several techniques have been exploited to address these outstanding issues. An elegant electron microscopy analysis using a glutathione S-transferase-tagged phospholipase C (PLC) Δ1 pleckstrin homology (PH) domain which interacts specifically with $PI4,5P_2$, documented that $PI4,5P_2$ was clustered in electron dense nuclear structures referred to as interchromatin granules (Watt *et al.*, 2002). The interchromatin granules comprise regions highly enriched in factors involved in mRNA splicing and correspond to nuclear speckles, a type of nuclear domain, which could be identified by immunofluorescence microscopy using an antibody to protein SC-35 (Handwerger and Gall, 2006).

The presence of $PI4,5P_2$ in nuclear speckles has also been documented using a monoclonal antibody to the lipid and both immunogold (Mazzotti *et al.*, 1995) and immunofluorescence analysis (Boronenkov *et al.*, 1998; Mortier *et al.*, 2005; Mellman *et al.*, 2008; Meerschaert *et al.*, 2009). In contrast, PI3,4,5 trisphosphate ($PI3,4,5P_3$) displayed a more diffused distribution throughout the nucleus when its localization was analyzed by immunofluorescence microscopy using a monoclonal antibody raised against the lipid (Kwon *et al.*, 2010). Intriguingly, kinases involved in $PI4,5P_2$ generation were also found localized to speckles (Boronenkov *et al.*, 1998; Szivak *et al.*, 2006; Bultsma *et al.*, 2010). Other phosphoinositide metabolism-related enzymes that are resident in the speckles include PLCβ1 and diacylglycerol kinase (DGK) θ and ζ (Tabellini *et al.*, 2003; Evangelisti *et al.*, 2006). DGK converts diacylglycerol (DG) (which could be derived from PLC-mediated $PI4,5P_2$ hydrolysis, see later on) to phosphatidic acid (PA) (Topham and Epand, 2009).

Nuclear phosphoinositide binding proteins

Given their chemical structure, nuclear phosphoinositides most likely interact with proteins that hide the lipid hydrophobic tails, but are able to present the inositol head group for further phosphorylation/dephosphorylation by kinases/phosphatases or cleavage by PLC (Keune *et al.*, 2011).

Therefore, an answer to the outstanding issue regarding how phosphoinositides are maintained within the nucleus could come from studies aimed to identifying nuclear phosphoinositide-binding proteins.

In a seminal work, Gozani and co-workers (Gozani *et al.*, 2003) showed for the first time that the chromatin-associated protein inhibitor of growth 2 (ING2) binds nuclear PI 5 phosphate (PI5P) via its plant homeodomain (PHD) finger, and suggested that PHD fingers, a type of domain present in a large number of chromatin regulatory factors, could function as nuclear PI5P receptors (Gozani *et al.*, 2003). Nevertheless, also the 18-residue polybasic region C-terminal to the PHD domain of ING2 is necessary for binding PI5P (Huang *et al.*, 2007).

The ING family of proteins is involved in regulation of a wide variety of processes, which include gene transcription, DNA repair, tumorigenesis, apoptosis, cellular senescence, and cell cycle arrest (Aguissa-Toure *et al.*, 2011). A subsequent paper documented that the ING2 PHD finger also interacted with trymethylated lysine 4 of histone H3, implying that PHD fingers may have the capacity of translating the histone code into chromatin structure and gene expression changes (Shi *et al.*, 2006; Pena *et al.*, 2006).

Nuclear $PI4,5P_2$-binding proteins

Since $PI4,5P_2$ is by far the most abundant of phosphoinositides, it is not surprising that quite a few nuclear $PI4,5P_2$-interacting proteins have been identified.

Syntenin-2, a protein containing a PDZ (Post synaptic density protein, Drosophila disc large tumor suppressor, Zonula occludens-1 protein) (Zimmermann, 2006) has been shown to interact with $PI4,5P_2$ at the speckles (Kouci *et al.*, 2011). Consistently, syntenin-2 loss-of-function in cultured cells induced the dispersal of $PI4,5P_2$ from nuclear speckles (Mortier *et al.*, 2005). Therefore, it has been hypothesized that syntenin-2 could function as a scaffold, which maintains $PI4,5P_2$ in proximity of components of the phosphoinositide cycle machinery or other speckle components (Zimmermann, 2006).

Another speckle-resident protein is zonula occludens-2 (ZO-2), a protein displaying a PDZ-2 domain. ZO-2 concentrates in the nucleus either in response to chemical stress or mechanical injury, or when cells are cultured at sparse density (Islas *et al.*, 2002; Traweger *et al.*, 2003). When expression of ZO-2 was reduced by siRNA, a dispersed nuclear $PI4,5P_2$ staining pattern was observed. These findings suggested that ZO-2 could also function as a scaffold in the organization of $PI4,5P_2$ within the speckles (Meerschaert *et al.*, 2009).

The most comprehensive investigation on nuclear $PI4,5P_2$-binding proteins published so far is the one by Divecha and co-workers (Lewis *et al.*, 2011). Since neomycin is known to bind phosphoinositides with high affinity (Gabev *et al.*, 1989) and could compete with $PI4,5P_2$-specific antibodies for nuclear $PI4,5P_2$ binding sites in intact nuclei (Osborne *et al.*, 2001), Divecha and co-workers reasoned that extraction of purified nuclei with neomycin could yield samples with reduced complexity and enriched for a pool of potential phosphoinositide-binding proteins devoid of phosphoinositides (Lewis *et al.*, 2011). Indeed, neomycin binds to phosphoinositides with high affinity through electrostatic interactions between the basic amino groups of the antibiotic and the negatively charged groups of phosphoinositides. It is well known that phosphoinositide-binding proteins interact in a similar manner via basic residues present in their lipid-binding domains (Lewis *et al.*, 2011). Interestingly, the neomycin-extracted nuclear proteins were very similar even if nuclear preparations had been done in the presence of detergents, implying that these proteins were highly insoluble and could be part of a nuclear matrix. Proteomic analysis allowed the identification of 168

proteins harboring phosphoinositide-binding domains. While some of the identified proteins [dynamin-2 and BTK (Bruton agammaglobulinemia Tyrosine Kinase), for example] were already known as phosphoinositide-binding proteins and contained PH or PHD domains, others had been previously uncharacterized from this point of view. Forty-eight percent of the proteins displaced by neomycin possessed at least one K/R-($X_{n=3-7}$)-K-X-K/R-K/R motif, known to be present in well-characterized cytoplasmic PI4,5P_2-binding protein, which include cytoskeletal components such as gelsolin, cofilin, and villin (Lewis *et al.*, 2011). At present, we not do know how exactly cytoskeletal proteins interact with phosphoinositides. We ignore if the interactions take place either via a component of a cytoplasmic membrane structure or through a PI4,5P_2 pool distinct from any membranous structures. As stated above, the *in vivo* existence of a nucleoskeleton is highly controversial nevertheless, it might be that similar mechanisms of interaction exist for both cytoskeletal and nucleoskeletal phosphoinositide-binding proteins via the K/R motifs in a membrane-free microenviroment.

The analysis was further refined by performing quantitative lipid pull-down experiments to identify specific PI4,5P_2-binding proteins from neomycin supernatants. Twenty-eight proteins, known for residing in the nucleus, were specifically pulled-down by PI4,5P_2 beads. Clustering analysis of the newly-identified phosphoinositide-binding proteins revealed functions related to chromatin assembly/disassembly, RNA splicing, nucleosome positioning/assembly and DNA packaging, and DNA topological changes. Some of the identified proteins, associate with nuclear speckles including U2 snRNP A', U4/U6 snRNP Prp4, SF3A1, and SPF27 (Lewis *et al.*, 2011).

Topoisomerase IIα, which displays seven K/R motifs located in its C-terminal regulatory domain, was also identified in the extracts, and this was in agreement with a previous report hinting at a possible link between regulation of topoisomerase IIα activity and phosphoinositides (Yu *et al.*, 1998). Accordingly, *in vitro* enzymatic assays documented that PI4,5P_2 and other phosphoinositides decreased topoisomerase IIα activity (Lewis *et al.*, 2011).

Nuclear PI3,4,5P3-binding proteins

Over the years, quite a few PI3,4,5 trisphosphate (PI3,4,5P_3)-interacting proteins residing in the nucleus have also been identified. Tanaka and co-workers, by overexpressing a hybrid fused with green fluorescent protein, documented in both COS-7 and PC12 cells the intranuclear presence of PI3,4,5P_3BP, a PI3,4,5P_3-binding protein. PI3,4,5P_3BP was originally purified from rat brain as a protein with a molecular mass of about 43-kDa, containing one zinc finger motif and two PH domains (Tanaka *et al.*, 1997).

Centaurins are a family of proteins containing GTP-ase-activating protein domains. Centaurins, that reside also in the nucleus, display a PH domain, which binds PI3,4,5P_3 (Soundararajan *et al.*, 2007; Haase *et al.*, 2008). Centaurins activate PI3K, however, their function in the nucleus is not understood. Centaurin-α1 (also referred to as p42IP4), besides binding PI3,4,5P_3 and inositol 1,3,4,5 tetrakisphosphate (Ins 1,3,4,5P_4), interacts with nucleolin (Reiser and Bernstein, 2004). Nucleolin/C23 is an abundant, ubiquitously expressed protein that is found in various cell compartments, especially in the nucleolus, of which it is a major component. Nucleolin/C23 is a multifunctional protein which impinges on many pathways, from interactions with viruses at the cellular membrane to processing of the ribosomal RNA in the nucleolus, to histone chaperoning, to chromatin co-remodeling (Mongelard and Bouvet, 2007).

Another nuclear PI3,4,5P_3-binding protein is nucleophosmin/B23 protein (Ahn *et al.*, 2005). Nucleophosmin/B23 protein is mainly localized to the nucleolus and is thought to have a relevant role in diverse cellular functions, including ribosome biogenesis, centrosome duplication, DNA repair, and response to stress (Okuwaki, 2008). Moreover, it has been implicated in the pathogenesis of several human malignancies, including acute myelogenous leukemia (AML). Intriguingly, it has been described both as an activating oncogene and a tumor suppressor, depending on cell type and protein levels (Colombo *et al.*, 2011). Nucleophosmin/B23 protein does not possess any phosphoinositide-binding module, yet it interacts with PI3,4,5P_3 via several lysine residues within its C-terminus (Ahn *et al.*, 2005). By binding nucleophosmin/B23 protein, nuclear PI3,4,5P_3 regulated the interaction between nucleophosmin/B23 protein and Akt and controlled the concentration and the subcellular dynamics of these two proteins (Kwon *et al.*, 2010).

Another nuclear PI3,4,5P_3-interacting protein is PI 3-kinase (PI3K) enhancer (PIKE) -L, which exclusively resides in the nucleus. PIKE family proteins are GTP-ases that upregulate PI3K activity (Ye, 2006). The PI3,4,5P_3-binding activity of PIKE-L is due to its PH domain. A PH mutant (K679,687N) of PIKE-L, unable to bind PI3,4,5P_3, translocated to the cytoplasm and substantially compromised the stimulatory effects on PI3K by PIKE-L (Hu *et al.*, 2005).

Aly is a speckle-located protein, which is a downstream substrate of nuclear PI3K signaling (Okada *et al.*, 2008). Aly interacted with both PI4,5P_2 and PI3,4,5P_3 and this interaction was essential for Aly localization to nuclear speckles. The PI3,4,5P_3-interacting site of Aly was mapped to its N-terminus, which contains a GR-rich domain. Nuclear Akt phosphorylates Aly on Thr 219 and this phosphorylation was necessary for Aly binding to PI3,4,5P_3. Depletion of Aly by siRNA resulted in reduced cell proliferation and mRNA export, and these two processes required Aly phosphorylation by Akt and Aly interaction with PI3,4,5P_3 (Okada *et al.*, 2008).

Nuclear phosphoinositide metabolizing enzymes

Cytoplasmic and nuclear phosphoinositide metabolism shares common enzymes and there is an extensive

literature dealing with phosphoinositide kinases, phosphatases, and phospholipases localized to the nucleus.

Kinases

As to kinases, much of the information available in the literature regards nuclear PIK and PIPK, i.e. the enzymes that synthesize PI4P and PI4,5P_2, respectively, as well as PI3K, i.e. the enzymes involved in the generation of 3′ phosphorylated phosphoinositides.

Nuclear PIK and PIPK

Four distinct PIKs have been identified so far and are classified as follows: the type II isozymes, PIKIIα and PIKIIβ, and the type III isozymes, PIKIIIα and PIKIIIβ (Sasaki *et al.*, 2009). They all synthesize PI4P from PI.

There exist three classes of PIPK, referred to as type I, II and III. Both type I and type II PIPK comprise the α, β, and γ isozymes. Moreover, at least five splice variants of PIPKIγ have been identified in mammals (Schill and Anderson, 2009). In contrast, the type III PIPK is a large protein product of a single-copy gene (Sasaki *et al.*, 2009). Type I and type II PIPK generate PI4,5P_2, although by utilizing different substrates, PI4P and PI5P, respectively (van den Bout and Divecha, 2009; Kwiatkowska, 2010). *In vitro* and *in vivo* studies support the concept that type III PIPK phosphorylates the 5′ position of the inositol ring, thus generating PI3,5P_2 (Sasaki *et al.*, 2009). There also is some evidence that type III PIPK could generate PI5P (Sbrissa *et al.*, 2002). However, it has been documented that most of cell PI5P arises from the action of a phosphatase rather than a kinase (Roberts *et al.*, 2005).

PIKIIIα has been detected in the nucleolus and in the nucleus of various mammalian cells (Kakuk *et al.*, 2006). Accordingly, PIKIIIα contains a bipartite nuclear localization sequence (NLS), which is absent in PIKIIIβ (Sasaki *et al.*, 2009). Nevertheless, the presence of nuclear PIKIIIβ has been reported (de Graaf *et al.*, 2002) and was found to be dependent on its phosphorylation on Ser 496 or Thr 504 residues (Szivak *et al.*, 2006). As several other phosphoinositide-metabolizing enzymes, also PIKIIIβ was detected at the nuclear speckle level. When antibodies to Ser 496 p-PIKIIIβ were microinjected into the nucleus of HS68 cells, it was possible to see by immunofluorescence staining, a much lower amount of nuclear PI4,5P_2 than the non-injected control cells, suggesting that the antibody blocked the production of PI4,5P_2 due to inhibition of PI4P synthesis. Interestingly, the nuclear decrease was not reflected in the amount of PI4,5P_2 in cell membranes, as the staining level of PI4,5P_2 in cytoplasmic membranes, including the Golgi apparatus, did not show any changes (Szivak *et al.*, 2006). PIKIIIβ must also have a nuclear export sequence (NES), as treatment with leptomycin B, a selective inhibitor of NES-dependent nuclear export, increased the intranuclear amount of the enzyme (de Graaf *et al.*, 2002). However, the NES of PIKIIIβ has not been identified so far.

PI4,5P_2 is by far the most abundant of cell phosphoinositides (Meerschaert *et al.*, 2009). Since the cellular levels of PI4P are much greater than PI5P, it is likely that the majority of cell PI4,5P_2, including nuclear PI4,5P_2, is synthesized through type I PIPK. However, there is clear evidence for a minor route of nuclear PI4,5P_2 synthesis through the phosphorylation of PI5P by type II PIPK (Clarke *et al.*, 2001). In MEL cells, the synthesis of nuclear PI4,5P_2 dramatically increased during progression from G_1 to S phase of the cell cycle. However, the overall mass of PI4,5P_2 did not change in a significant manner during the cell cycle, whereas a transient, but marked, increase in PI5P mass at the G_1 phase was detected (Clarke *et al.*, 2001).

Four members of the PIPK family, PIPKIα, PIPKIγ_i4, PIPKIIα, and PIPKIIβ are localized inside the nucleus at the speckle level (Boronenkov *et al.*, 1998; Ciruela *et al.*, 2000; Richardson *et al.*, 2007; Schill and Anderson, 2009; Bultsma *et al.*, 2010). PIPKIIβ displays no obvious NLS. However, a detailed analysis of the localization of chimaeras and mutants of PIPKIIα and β revealed that the nuclear localization required the presence of a 17-amino-acid length of α-helix (α-helix 7) that is specific to the β isoform, and that this helix must be present in its entirety, with a precise orientation (Ciruela *et al.*, 2000). This resembles the nuclear targeting of the HIV protein Vpr, and PIPKIIβ was therefore the first example of an eukaryotic protein that uses the same mechanism (Chen *et al.*, 1999).

The regulation of nuclear PIPK activity has been only partially clarified. Nevertheless, it has been documented that the retinoblastoma protein (pRB) interacts with all isoforms (α, β, and γ) of PIPKI, and stimulated their activity (Divecha *et al.*, 2002). The protein pRB is a master regulator of cell differentiation, survival, and progression through the cell cycle (Rizzolio *et al.*, 2010). Moreover, the pRB pathway is somehow deregulated in nearly all human tumors (Chinnam and Goodrich, 2011).

PI3K

The nucleus also contains PI3Ks, i.e. the kinases that catalyze the phosphorylation of the 3′ position of phosphoinositides. PI3Ks have been categorized into three classes according to sequence homology, substrate preference, and mechanisms of regulation (Vanhaesebroeck *et al.*, 2010).

Class IA enzymes consist of a 110 kDa catalytic subunit (α, β, ω) and an adaptor protein (p85α, p85β, p55Δ, p55α, and p50α) which links the enzyme to tyrosine kinases, whereas the class IB enzymes are composed of a p110γ catalytic subunit and a subunit (p101, p87, or p84) regulated by G proteins. Class I PI3Ks phosphorylates both PI4P and PI4,5P_2 to yield *in vivo* PI3,4P_2 and PI3,4,5P_3, respectively. PI3,4,5P_3 is a crucial activator of phosphoinositide-dependent kinase 1 (PDK1) and thus the serine/threonine protein kinase Akt.

Class II PI3Ks, which comprise the PI3K-C2α, -C2β, and -C2γ isoforms, preferentially phosphorylate PI to yield PI3P, however, they can also yield PI3,4P_2 (Vanhaesebroeck *et al.*, 2005).

Vacuolar protein sorting 34 (vps34) is the only class III PI3K and exists as a heterodimer bound to the vps15 regulatory subunit (formerly called p150 in mammals) (Hirsch *et al.*, 2009). Vps34 only phosphorylates PI to generate PI3P and is important both for vesicular trafficking in the endosomal/lysosomal system and for autophagy (Sasaki *et al.*, 2009).

Both class I and class II PI3Ks have been reported to be localized in the nucleus (Didichenko and Thelen, 2001; Sindic *et al.*, 2001; Visnjic *et al.*, 2002; Visnjic *et al.*, 2003; Ahn *et al.*, 2004).

Regarding class IA PI3Ks, p110α localized mainly in the cytoplasm of several cells lines, while p1110β was mainly nuclear (Kumar *et al.*, 2011). While p110α displayed a diffuse distribution throughout the nucleus (Neri *et al.*, 1999), p110β showed a more discrete localization (Marques *et al.*, 2009). As far as the p85 regulatory subunits are concerned, the majority of p85α localized in the cytoplasm, but p85β was more abundantly expressed in the nuclear compartment (Kumar *et al.*, 2011). The heterodimeric p110γ translocated to the nucleus in response to serum stimulation of HepG2 cells (Metjian *et al.*, 1999). The mechanisms regulating nuclear shuttling of class I PI3K have long escaped clarification. However, it has been recently documented that the C2 domain of p110β possesses an NLS (residues 310 to 318: KVKTKKSTK), which mediates its nuclear entry, while p85β displays an NES (residues 25–32: LLPGDLLV) (Kumar *et al.*, 2011). Deletion or mutation of this region rendered p85β predominantly nuclear and insensitive to leptomycin B treatment. Interestingly, the authors demonstrated that p85β regulates both the nuclear entry and exit of p110β. They speculated that in the p85β/p110β complex, p85β contributes by supplying an NES, whereas p110β supplies an NLS. However, the p110β NLS sequence is not functional, as overexpressed p110β stayed in the cytoplasm, while concomitant p85β overexpression increased the amount of nuclear p110β. This finding could be explained by the fact that the predicted structure of the p85β/p110β complex reveals that the NLS sequence in the C2 domain of p110β stays in close proximity to p85β. Therefore, it might be that association of p85β with p110β alters the structure of the latter to yield a functional NLS (Kumar *et al.*, 2011). There is no detailed information available regarding the mechanisms that regulate nuclear shuttling of p110γ; however, truncation of the N-terminal 82 residues resulted in a p110γ, which was constitutively localized to the nucleus and did not associate with p101 (Metjian *et al.*, 1999). This implies that p101 could somehow be involved in controlling nuclear import/export of p110γ.

Also PI3K-C2α localized to nuclear speckles (Didichenko and Thelen, 2001), whereas PI3K- C2β was detected in the nuclear periphery, at the nuclear lamina level (Banfic *et al.*, 2009). Sequence alignment of all three class II PI3K enzymes reveals a conserved KRKTKxxxK motif located at the C-terminal of the C2 domain of the kinase. This motif serves as an NLS in both PI3K-C2α and β (Didichenko and Thelen, 2001; Banfic *et al.*, 2009). Indeed, C-terminal deletion and point mutations of this motif impaired epidermal growth factor (EGF)-driven PI3K-C2β translocation to the nucleus in HEK-293 cells (Banfic *et al.*, 2009). Such an NLS is homologous to that found in p110β, suggesting a potential conservation of structural elements for nuclear import also between PI3K classes.

Phosphatases

Type I PI4,5P_2 4-phosphatase is one of the two enzymes that convert PI4,5P_2 to PI5P, the other being type II PI4,5P_2 4-phosphatase (Ungewickell *et al.*, 2005). Type I PI4,5P 4-phosphatase (but not type II PI4,5P_2 4-phosphatase) translocated to the nucleus of cells treated with DNA damaging agents such as doxorubicin and etoposide (Zou *et al.*, 2007). This in turn mediated p53-dependent apoptosis through interaction with ING-2 in response to genotoxic stress (see also later on).

PTEN is a dual specificity lipid and protein phosphatase that preferentially removes the 3′ phosphate mainly from PI3,4,5P_3, but is also active on PI3,4,P_2 (Stiles, 2009). Many studies, using either primary tumors or cell lines, have established PTEN to be the most deleted phosphatase and second most deleted gene next to p53 in human cancer. Given its ability to downregulate signaling downstream of oncogenic PI3K, PTEN is considered as a powerful oncosuppressor gene (Zhang and Yu, 2010). PTEN has been detected in the nucleus (Deleris *et al.*, 2003) and could have an impact on the levels of PI3,4,5P_3 (Kwon *et al.*, 2010). Indeed, the lipid phosphatase activity of nuclear PTEN was found to be important for the CDX2-mediated intestinal differentiation of gastric carcinoma cells, implying that PI3,4,5P_3 plays an important role in this process (Semba *et al.*, 2009). Nevertheless, a growing body of evidence indicates that nuclear PTEN has other functions that are unrelated to its lipid phosphatase activity (Shen *et al.*, 2007; Song *et al.*, 2011). Furthermore, recent findings have documented that the transcription factor cAMP response element binding protein (CREB) is a protein target of PTEN in the nucleus, implying that the protein phosphatase activity of PTEN can modulate CREB-mediated gene transcription (Gu *et al.*, 2011). Therefore, the physiological relevance of the lipid phosphatase activity of nuclear PTEN has yet to be determined, also in consideration of the fact that re-introduction of PTEN in PTEN-null U87MG cells, did not affect the quantity of PI3,4,5P_3 present in the nucleus (Lindsay *et al.*, 2006). Nuclear PTEN resides in PML (promyelocytic leukemia) bodies (Song *et al.*, 2008), another class of nuclear bodies. PML bodies are matrix-associated domains that recruit an astonishing variety of seemingly unrelated proteins, and in many ways they still constitute an enigma in cell biology (Lallemand-Breitenbach and de The, 2010). A considerable number of PML body components are critically involved in apoptosis, senescence, tumor suppression, gene expression, DNA damage repair, and stress response (Bernardi and Pandolfi,

2007; Borden, 2008; Krieghoff-Henning and Hofmann, 2008). Moreover, PML bodies have been implicated to play an important role during viral infections (Tavalai and Stamminger, 2008).

SHIP1 and SHIP2 (for Src homology domain-containing inositol phosphatase) are two other phosphatases that remove the 5′ phosphate from $PI3,4,5P_3$ to yield $PI3,4,P_2$ (Hamilton *et al.*, 2011).

SHIP2 localizes to nuclear speckles (Deleris *et al.*, 2003). However, no information is at present available regarding the exact role(s) played by nuclear SHIP2.

PLC

PLC is a key enzyme of phosphoinositide metabolism, as it hydrolyzes $PI4,5P_2$ into the two second messengers, inositol 1,4,5 trisphosphate ($Ins1,4,5P_3$) and DG. $Ins1,4,5P_3$ triggers the release of Ca^{2+} from intracellular stores, and DG mediates the activation of DG-dependent protein kinase C (PKC) isoforms. By doing so, PLC acts as a fundamental modulator of phosphoinositide balance. Thirteen PLC isozymes have been identified and categorized into six classes, the β (1-4), γ (1, 2), δ (1, 3, 4), ε, ζ, and η (1, 2) types, on the basis of domain structure and regulatory activation mechanisms (Fukami *et al.*, 2010).

PLCβ1 was the first PLC isoform identified in the nucleus (Martelli *et al.*, 1992; Divecha *et al.*, 1993). Subsequently, other PLC isoforms have been localized to the nucleus, including β2 (Bertagnolo *et al.*, 1997), β3 (Faenza *et al.*, 2004), γ1 (Bertagnolo *et al.*, 1998), Δ1 (Yamaga *et al.*, 1999), Δ4 (Liu *et al.*, 1996), and ζ (Sone *et al.*, 2005; Cooney *et al.*, 2010).

PLCβ1 exists as two splicing variants, **a** and **b**. Of these, the PLCβ1a splicing subtype displays both nuclear and cytoplasmatic localization, while PLCβ1b splicing subtype is localized only in the nucleus (Cocco *et al.*, 2006). PLCβ1 localizes to nuclear speckles (Tabellini *et al.*, 2003), whereas the γ1 isoform was identified in the PML bodies (Ferguson *et al.*, 2007). Also PLCΔ1 displays a discrete subnuclear distribution; however, it is not known where exactly it localizes in the nucleus (Stallings *et al.*, 2005).

Some of the mechanisms that govern nuclear import/export of PLC isozymes have been identified.

As to PLCβ1, the nuclear localization of this enzyme is determined by a cluster of lysine residues (between positions 1055 and 1072) which is common to both isoforms (Kim *et al.*, 1996). However, it not clear why the **b** splicing variant is completely nuclear, whereas the **a** variant is located both in the cytoplasm and in the nucleus. This might depend on interactions with different partner proteins at the nuclear level, or on differences in nuclear export. The last 32 amino acids of PLCβ1b located at the C-terminus are different from those of PLCβ1a. These amino acids form an α-helix/proline/basic residue motif which might act as an additional NLS. Moreover, PLCβ1a has in its unique C-terminus a typical NES (LxLxxLxxV), which may result in this splicing variant being less concentrated in the nucleus. In keeping with this, recent evidence has highlighted that phosphorylation of Ser 887 by PKC somehow influenced nuclear localization of PLCβ1, as overexpression of a PLCβ1 mutant mimicking the unphosphorylated state (S887A) in both HEK and PC12 cells, resulting in a much more abundant nuclear localization, than the enforced expression of a mutant mimicking the phosphorylated state (S887D) (Aisiku *et al.*, 2011). Translin-associated factor X (TRAX) is a recently identified binding partner of PLCβ1 (Aisiku *et al.*, 2010). TRAX binds the C-terminal region of PLCβ1 (undefined splicing variant). TRAX is a cytosolic protein that can migrate to the nucleus as it possesses an NLS (Cho *et al.*, 2004). In C6 glioma cells, endogenous PLCβ1 and TRAX colocalized in the cytosol and in the nucleus, but not at the plasma membrane. Moreover, Förster resonance energy transfer (FRET) analysis of Neur2a cells overexpressing fluorescent-tagged PLCβ1 and TRAX, revealed that the two proteins interacted mostly in the cytosol, and to a lower level, also in the nucleus (Aisiku *et al.*, 2010). Additional studies on TRAX/PLCβ1 interactions could help clarifying the mechanisms, which control nuclear import of this PLC isoform.

Both the export and import signals that regulate PLCΔ1 trafficking from/to the nucleus are known. Export from the nucleus required a typical NES, which was mapped at amino acid residues 164–177 of the EF-hand sequence (Yamaga *et al.*, 1999). This leucine-rich functional NES is absent from PLCΔ4. As expected, nuclear export of PLCΔ1 was sensitive to leptomycin B. PLCδ1 displays a basic amino acid-rich region covering the C-terminus X domain and the XY-linker is necessary for the nuclear import of PLCδ1 (Okada *et al.*, 2002). Two lysine residues (K432 and K434) in the region are important for nuclear import, since a deletion mutant lacking the region or a site-directed mutant of the lysine residues did not accumulate in the nucleus, even in the presence of leptomycin B. Ca^{2+}-binding to the catalytic domain is essential for the nuclear import of PLCδ1, suggesting that Ca^{2+} causes a structural change in PLCδ1 exposing the positively charged cluster recognized by importin β1, which then carries the cargo molecule to the nuclear pore complex (Yagisawa *et al.*, 2006). In primary rat hippocampal neurons, ionomycin or thapsigargin caused the nuclear localization of PLCδ1. Moreover, overexpression of wild type PLCδ1 facilitated ionomycin-induced nuclear shrinkage in embryonic fibroblasts derived from PLCδ1 gene-knockout mice. In contrast, an E341A mutant of PLCδ1 that cannot be imported into the nucleus by ionomycin and also lacks enzymatic activity, did not cause nuclear shrinkage in fibroblasts from the same animal model. Therefore, nuclear translocation and the enzymatic activity of PLCδ1 may regulate the nuclear shape during stress-induced cell death caused by high levels of Ca^{2+} (Okada *et al.*, 2010). However, the mechanisms through which PLCδ1 could control nuclear shape during apoptosis are not understood.

An NLS has also been identified at the amino acids 374–381 in the XY-linker region of PLCζ (Kuroda *et al.*, 2006). PLCζ translocated to the nucleoplasm of the

newly formed pronucleus in mouse fertilized eggs and remained nuclear during the first prophase. As the zygote then entered first mitosis, the pronuclear envelope breakdown took place, and PLCζ was released back to the cytoplasm (Larman *et al.*, 2004). It is interesting that nuclear translocation of PLCζ represents a mechanism for sequestering it and is not associated with Ins1,4,5P_3 generation. Indeed, preventing pronuclear localization of PLCζ by mutation of the NLS, prolonged Ca^{2+} oscillations in the cytosol.

Although PLCγ1 localizes to the nucleus of highly proliferating and transformed cell lines (but not in primary embryo skin or lung fibroblasts), its NLS is not known (Ye, 2006).

DGK

Although DGKs are not, strictly speaking, phosphoinositide-metabolizing enzymes, they are nevertheless included in this review, as they metabolize DG, one of the end-products of PLC-mediated PI4,5P_2 hydrolysis. DGKs are enzymes that convert DG to PA. This conversion terminates DG signaling and, at the same time, initiates additional signaling events downstream of PA, which also acts as a lipid-signaling molecule (Topham and Epand, 2009; Cai *et al.*, 2009). For example, PA acts as a key regulator of several members of the Ras superfamily of GTP-ases (Zhang and Du, 2009). Moreover, PA derived from DGKζ activity could stimulate PIPKIα activity and PI4,5P_2 synthesis (Luo *et al.*, 2004). However, it is not known if this actually happens also in the nucleus (Cai *et al.*, 2009). DGKs represent one of the two routes for cell PA synthesis, the other one being phospholipase D (PLD)– mediated phosphatidylcholine hydrolysis (Merida *et al.*, 2008; Raghu *et al.*, 2009).

Ten mammalian isoforms of DGKs have been cloned, characterized, and classified in 5 classes based on their primary structure (Topham and Epand, 2009). Class I comprises the a, ß, and γ isozymes; class II the Δ, η, and κ; class III the ε isoform; class IV the ζ and ι; class V the θ. It has been demonstrated that several DGK isoforms localize to the nucleus, including α, γ, δ, θ, ζ (Evangelisti *et al.*, 2007a; Raben and Tu-Sekine, 2008).

The most thoroughly characterized nuclear DGK isoform is DGKζ. DGKζ resides in nuclear speckles, where it physically interacts with PLCβ1, as documented by co-immunoprecipitation experiments (Evangelisti *et al.*, 2006).

This DGK isozyme, by controlling the levels of nuclear DG, is involved in the progression from G_1 to S phase of the cell cycle. Indeed, overexpression of DGKζ within the nucleus inhibited cell cycle progression (Topham *et al.*, 1998). In particular, cell cycle arrest of cells overexpressing in the nucleus wild type DGKζ was accompanied by decreased levels of pRB phosphorylated on Ser 807/811 residues (Evangelisti *et al.*, 2007b). The protein pRB is a critical regulator of the cell cycle transition from G_1 to S phase by interacting with and attenuating the activity of the E2F transcription factor family. As cells progress through late G_1 to S phase, pRB becomes increasingly phosphorylated (Giacinti and Giordano, 2006). Interestingly, two reports have documented that the Ser 807/811 residues are key determinants of pRB activity. In fact, it has been shown that a pRB mutant with alanine substitutions at Ser 807/811 had enhanced growth suppressing activity (Driscoll *et al.*, 1999), and phosphorylation of Ser 807/811 led to an inactivation of pRB tumor suppressor activity in uveal melanoma (Brantley and Harbour, 2000).

Moreover, nuclear DGKζ has been demonstrated to play an important role during myogenic differentiation of C2C12 myoblasts, which is characterized by a progressive decrease in cell proliferation (Evangelisti *et al.*, 2006). Indeed, nuclear DGKζ was upregulated when C2C12 cells were challenged with insulin and started to differentiate along the myogenic pathway. If upregulation of DGKζ was prevented by siRNA, myogenic differentiation was impaired (Evangelisti *et al.*, 2006). We have subsequently demonstrated that nuclear DGKζ controls the expression of TPA-Inducible Sequences 21/B-cell Translocation Gene 2/PC3 (BTG2), a negative transcriptional regulator of cyclin D1. BTG2, whose levels increase during myogenic differentiation, displays a strong anti-proliferative action, which could be related to cyclin D1 dowregulation and decreased pRB phosphorylation on Ser 807/811 residues (Evangelisti *et al.*, 2009) (Figure 1). If cells overexpressing DGKζ were exposed to phorbol esters (PMA), which could substitute for DG, the increased expression of BTG2 could not be detected anymore. However, if cells with forced expression of DGKζ were treated with PMA + Gö6976 (a pharmacological inhibitor of PKC conventional isoforms, including PKCα), increased expression of BTG2 gene was again detectable (Evangelisti *et al.*, 2009). It might be that the increase in nuclear DGKζ which occurs during myogenic differentiation of C2C12 myoblasts is related to the upregulation of nuclear PLCβ1 (and hence of DG levels, see later on) which also takes place in this model (Faenza *et al.*, 2004).

The NLS of DGKζ is a bipartite type sequence that overlaps with a sequence similar to the myristoylated alanine-rich C kinase substrate (MARCKS). Also class IV DGKι displays an NLS; however, it does not localize to the nucleus (Ito *et al.*, 2004). This observation suggests that nuclear localization of DGKζ could depend on other features of its structure, as well as interactions with specific binding partners. Indeed, a truncated form of DGKζ, which lacks the C-terminus domain, led the protein to localize in the cytoplasm, although the primary structure still contained the NLS (Evangelisti *et al.*, 2010). Therefore, the NLS could be a cryptic site whose exposure is regulated by the C-terminal region (Hozumi *et al.*, 2003).

We have recently shown that DGKζ possesses a canonical leucine-rich, leptomycin B-sensitive NES (residues 362–370: LSTLDQLRL), located next to the second zinc finger-like sequence in the regulatory domain (Evangelisti *et al.*, 2010). The identification of this NES

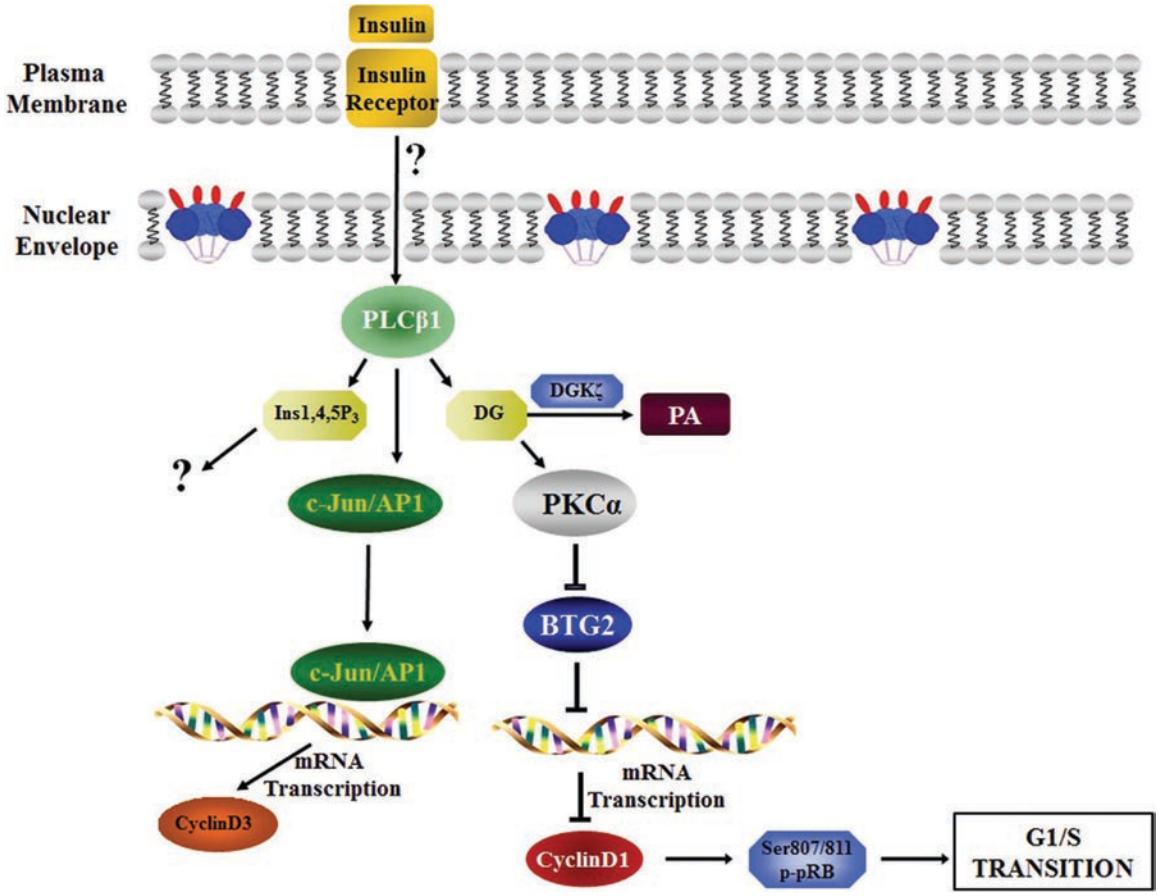

Figure 1. Schematic representation of nuclear PLCβ1 and DGKζ signaling in C2C12 rat myoblasts treated with insulin. Insulin activates nuclear PLCβ1 which then hydrolyses $PI4,5P_2$ to DG and $Ins1,4,5P_3$. The PLCβ1 catalytic activity is required for upregulating c-Jun/AP1 function which ensues in enhanced transcription of the cyclin D3 gene. The role of $Ins1,4,5P_3$ in this context is unknown. DGKζ metabolizes DG to PA within the nucleus. If nuclear DGKζ is overexpressed (as it happens during myogenic differentiation), the levels of nuclear DG are strongly reduced. Since DG is an activator of PKCα, low levels of DG result in a lower activity of this PKC isoform. PKCα is a negative regulator of BTG2 which in turn targets cyclin D1. Hence, high levels of expression of DGKζ could result in increased expression of BTG2 and decreased expression of cyclin D1 with a subsequent G1/S phase transition block through downregulation of Ser 807/811 p-pRB. Arrows indicate activating events, whereas perpendicular lines highlight inhibitory events. PLC: phospholipase C; DGK: diacylglycerol kinase; $PI4,5P_2$: phosphatidylinositol 4,5 bisphosphate; DG: diacylglycerol; PA: phosphatidic acid; PKC: protein kinase C; BTG2: TPA-Inducible Sequences 21/B-cell Translocation Gene 2/PC3; pRB: retinoblastoma protein; $Ins1,4,5P_3$: inositol 1,4,5 trisphosphate.

seems particularly intriguing as in neurons, there are some conditions where DGKζ migrates outside from the nucleus *in vivo* and never relocates to the nucleus. These conditions, which result in cell death, include transient ischemia-reperfusion of the forebrain and kainate-induced seizures of hippocampal neurons (Ali *et al.*, 2004; Nakano *et al.*, 2006; Saino-Saito *et al.*, 2011). It has been therefore hypothesized that nuclear export of DGKζ could somehow facilitate neuronal apoptosis.

It has been reported that nuclear DGKζ activity is controlled by members of the pRB family, including the p107 and p130 members (Los *et al.*, 2006). The protein pRB binds *in vitro* and *in vivo* to the MARCKS phosphorylation-site domain of DGKζ that can be phosphorylated by PKC. Activation of PKC by phorbol esters inhibited DGKζ binding to pRB. Mimicking of PKC phosphorylation of serine residues (by S/D but not S/N mutations) within the DGKζ-MARCKS phosphorylation-site domain also prevented DGKζ binding to pRB, implying that phosphorylation of these residues negatively regulated the interactions between DGKζ and pRB. Interestingly, overexpression of DGKζ in pRB-null fibroblasts reconstituted a cell cycle arrest induced by γ-irradiation, suggesting that DGKζ may act *in vivo* as a downstream effector of pRB (Los *et al.*, 2006). In a subsequent study, the same group identified PKCα as being particularly important for inhibiting DGKζ binding to pRB (Los *et al.*, 2007). This PKC-mediated, pRB-dependent control of DGKζ activity may have important implications for the regulation of DG and PA levels during the cell cycle.

However, for the sake of completeness, it should be pointed out that PLD also localizes to the nucleus and could be involved in PA and DG generation (Gayral *et al.*, 2006). A very recent study has led to the identification of the NLS of PLD1 and has highlighted how this enzyme could be responsible for the activation of both PKCα and extracellular-regulated kinase (ERK) signaling in the nucleus of HEK293 cells. However, how PLD1

could regulate these two pathways within the nucleus remains to be elucidated (Jang and Min, 2011).

Conventional functions of nuclear phosphoinositides

In the canonical phosphoinositide cycle occurring at the plasma membrane, $PI4,5P_2$ is hydrolyzed by a PLC yielding the two second messengers, DG and $Ins1,4,5P_3$. However, $PI4,5P_2$ can also be phosphorylated to $PI3,4,5P_3$ by PI3K. A wide array of agonists (hormones, cytokines, growth factors, etc.) can activate PLC and/or PI3K. Also, in the nucleus $PI4,5P_2$ is hydrolyzed by PLC to generate DG and $Ins1,4,5P_3$ or phosphorylated to $PI3,4,5P_3$ by PI3K.

Signaling by nuclear PLCβ1

It has long been known that the mitogen insulin-like growth factor-1 (IGF-1), but not bombesin, activated nuclear PLCβ1 in Swiss 3T3 cells and this resulted in a decrease in $PI4,5P_2$ and an increase in DG mass (Cocco *et al.*, 1988; Divecha *et al.*, 1991; Martelli *et al.*, 1992). Activation of nuclear PLCβ1 in response to IGF-1 was dependent on a ERK 1/2-mediated phosphorylation of Ser 982, as forced expression of a PLCβ1 mutant, which could not be phosphorylated at Ser 982 attenuated both the increase in nuclear PLCβ1 activity and the mitogenic effect of IGF-1 on Swiss 3T3 fibroblasts (Xu *et al.*, 2001a). Although ERK 1/2-dependent phosphorylation at Ser 982 was essential for PLCβ1 activation (as demonstrated in mutants carrying Ser 982 Gly), it was not sufficient alone. However, other possible components of the mechanism of activation remain to be identified.

There exists quite an extensive literature regarding the role played by nuclear PLCβ1 during myogenic differentiation of C2C12 rat myoblasts (Faenza *et al.*, 2004; Faenza *et al.*, 2007; Ramazzotti *et al.*, 2008). Overexpression of PLCβ1 mimicked insulin action on C2C12 cells, as far as myogenic differentiation was concerned, and the cyclin D3 promoter was identified as a target of nuclear PLCβ1 signaling elicited by insulin in these cells (Faenza *et al.*, 2007). Cyclin D3 expression was much lower in C2C12 cells overexpressing a catalytically inactive form of nuclear PLCβ1 than in control cells, suggesting that DG and/or $Ins1,4,5P_3$ generation within the nucleus were required during myogenic differentiation. PLCβ1 signaled through a c-jun/AP1 module, which impacted on cyclin D3 gene promoter (Ramazzotti *et al.*, 2008). It is worth recalling here several studies have established that cyclin D3 is essential for myogenesis (De Santa *et al.*, 2007; Salisbury *et al.*, 2008). However, we do not know how PLCβ1 signaling upregulated the transcriptional activity of c-jun/AP1 (Figure 1).

Nuclear DG

Nuclear DG levels fluctuate during the cell cycle and in some cell types they peak at the G1/S phase transition of the cell cycle (Banfic *et al.*, 1993; Topham *et al.*, 1998; Evangelisti *et al.*, 2007b). However, an increase in nuclear DG mass has been reported to occur also at the early G_1 and G_2/M phases of the cell cycle (Sun *et al.*, 1997; Lukinovic-Skudar *et al.*, 2005; Lukinovic-Skudar *et al.*, 2007).

DG is a powerful chemoattractor/activator of some PKC isoforms (Rosse *et al.*, 2010) and it has been hypothesized that nuclear DG also could somehow be involved in PKC signaling (Divecha *et al.*, 1991). Accordingly, in response to IGF-1 stimulation of Swiss 3T3 fibroblasts, DG-sensitive PKCα migrated to the nucleus (Neri *et al.*, 1994), where it provided a negative feedback regulation for terminating the IGF-1-induced activation of nuclear PLCβ1 (Xu *et al.*, 2001b). Indeed, PKCα phosphorylated PLCβ1 on Ser 887 and this resulted in decreased activity of the phospholipase. A time course study revealed an inverse relationship between nuclear PKC activity and the activity of nuclear PLCβ1 in IGF-1-treated cells. A time-dependent association between PKCα and PLCβ1 in the nucleus was also observed following IGF-1 treatment. Interestingly, PLCβ1 phosphorylation on Ser 887 is somehow related to its nuclear localization (see above), and thus we could suppose that after being phosphorylated on Ser 887, PLCβ1 exits the nucleus, which would explain the decrease in PLC activity (Aisiku *et al.*, 2011). Nevertheless, at least in IGF-1-stimulated Swiss 3T3 cells, no shuttling of PLCβ1 outside and inside the nucleus has been reported (Xu *et al.*, 2001b).

Recently, it has been demonstrated that in MEL cells, DG generated by PLCβ1 activated PKCα, which in turn phosphorylated lamin B1. In cells with downregulated PLCβ1, or PKCα, or lamin B1, an accumulation of cells in the G_2/M phase of the cell cycle was observed (Fiume *et al.*, 2009).

Therefore, it was hypothesized that PLCβ1/PKCα signaling, by controlling lamin B1 phosphorylation levels, facilitates lamin B1 depolymerization, which leads to NE breakdown and mitosis. These findings are in agreement with a previous report, which highlighted an involvement of PLCβ1 in NE breakdown in primary mouse oocytes (Avazeri *et al.*, 2000) and with a much earlier paper dealing with the role of an undefined nuclear PLC activity in DG generation, PKCβ2 activation, and lamin B1 phosphorylation in HL60 human leukemia cells (Sun *et al.*, 1997). It is interesting that in MEL cells, nuclear PLCβ1 activation was demonstrated to be downstream of jun NH2-terminal kinase (JNK) which migrated to the nucleus in response to a mitogenic stimulus (serum stimulation of starved cells) (Fiume *et al.*, 2009). Nevertheless, we still do not know if JNK directly phosphorylates PLCβ1, as ERK 1/2 does, or if there are other signaling intermediates between JNK and PLCβ1 (Figure 2). Moreover, we ignore which growth factor(s) could activate this signaling pathway, as MEL cells were challenged with serum. Also, the findings by Fiume *et al.* (Fiume *et al.*, 2009) documented that lamin B1 and PLCβ1 colocalized at the nuclear periphery, implying that PLCβ1 could migrate from nuclear speckles to other nuclear districts, where its substrate $PI4,5P_2$ would be present.

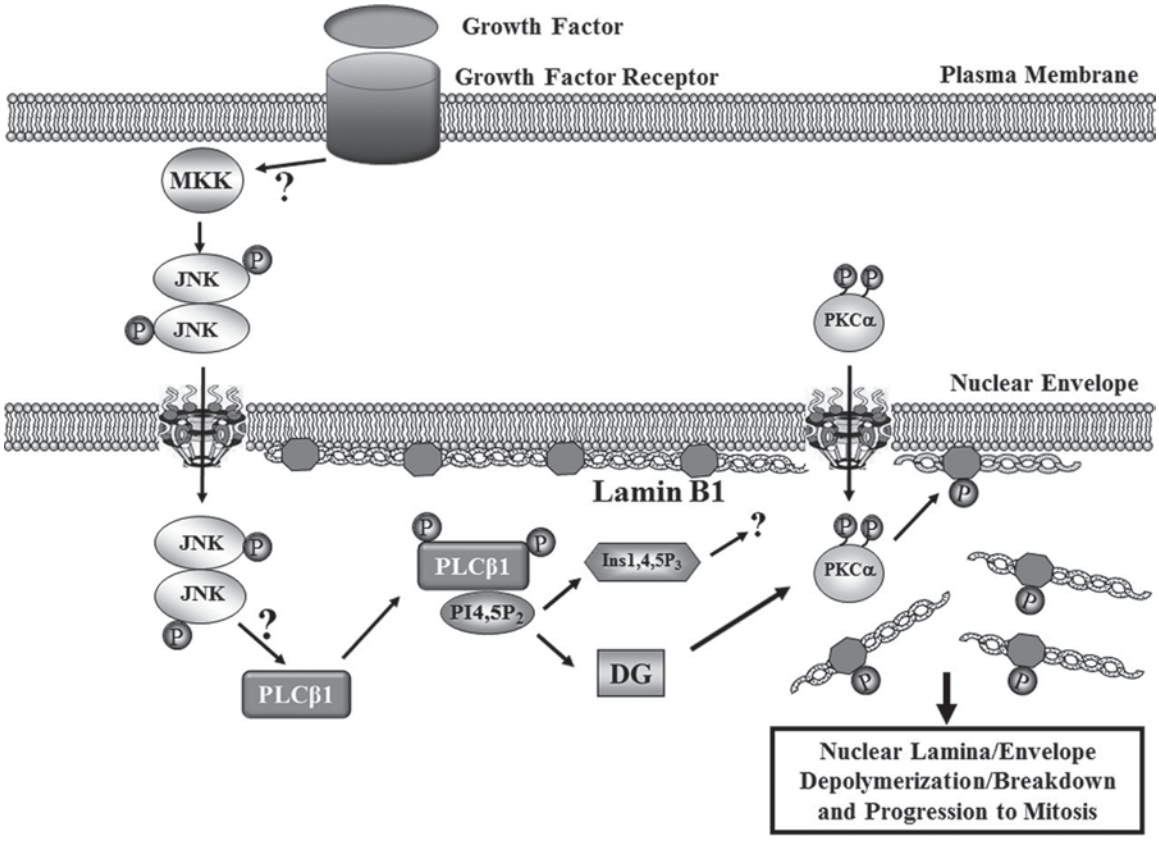

Figure 2. Schematic representation of a possible nuclear phosphoinositide-dependent signaling pathway activated in starved mouse erythroleukemia cells by serum. Undefined growth factors present in the serum cause MKK-dependent JNK activation and its translocation to the nucleus. JNK then activates PLCβ1, however it is still unclear whether it does so directly or through signaling intermediates. Once activated, PLCβ1 generates DG from $PI4,5P_2$. DG chemoattracts PKCα to the nucleus, which in turn phosphorylates lamin B1. Lamin B1 phosphorylation causes its depolymerization and subsequent nuclear envelope breakdown and mitosis. The role of $Ins1,4,5P_3$ in this setting is not understood. Arrows indicate activating events. MKK: MAP kinase kinase; JNK: jun NH2-terminal kinase; PLC: phospholipase C; DG: diacylglycerol; $Ins1,4,5P_3$: inositol 1,4,5 trisphosphate; PKC: protein kinase C.

Nuclear DG, generated by PLCβ1, could also be responsible for nuclear translocation of PCKβ1 in C2C12 cells treated with insulin. PCKβ1 then phosphorylates the eukaryotic elongation factor 1α (eEFIα) (Piazzi *et al.*, 2010).

Nuclear $Ins1,4,5P_3$

The presence of the $Ins1,4,5P_3$ receptor on the inner nuclear membrane has long been known (Malviya *et al.*, 1990). Moreover, the receptor could also cluster in the nuclear interior in apoptotic PC12 cells (Ondrias *et al.*, 2011). This could, however, depend on the fact that the $Ins1,4,5P_3$ receptor localizes to the nucleoplasmic reticulum (Marius *et al.*, 2006).

Although, the existence of independent Ca^{2+} fluxes within the nucleus is still a debated issue (Bootman *et al.*, 2009; Alonso and Garcia-Sancho, 2011), evidence exists that localized Ca^{2+} release in the nucleus could elicit responses (gene expression changes) that are unique and distinct from those elicited by cytosolic Ca^{2+} release (contraction) (Garcia *et al.*, 2004).

Moreover, recent findings have highlighted that nuclear $Ins1,4,5P_3$ might specifically regulate increases in nuclear Ca^{2+} induced by insulin in hepatocytes. Insulin is a powerful mitogen for this kind of cells (Rodrigues *et al.*, 2007; Rodrigues *et al.*, 2008). Nuclear Ca^{2+} could impact on a number of transcription factors involved in cell proliferation, such as CREB and its coactivator, CREB-binding protein (Chawla *et al.*, 1998), as well as Elk-1, a downstream mediator of EGF signaling (Pusl *et al.*, 2002).

Moreover, $Ins1,4,5P_3$ can be further phosphorylated in the nucleus to yield several inositol phosphates, a family of water soluble second messengers, that have been implicated in several nuclear functions including mRNA export, chromatin remodeling, and DNA repair (Monserrate and York, 2010). Accordingly, inositol phosphate multikinase, which is involved in regulating the levels of $Ins1,4,5,6P_4$ (Chang and Majerus, 2006; Resnick and Saiardi, 2008), is localized in the nucleus (Nalaskowski *et al.*, 2002; Resnick and Saiardi, 2008). Interestingly, nuclear inositol phosphate multikinase is also endowed with a PI3K activity and is involved in transcriptional regulation (Resnick *et al.*, 2005).

Signaling by nuclear PI3K

Nerve growth factor (NGF) is an example of a growth factor that stimulates nuclear translocation of class IA PI3K

and generation of PI3,4,5P_3 within the nucleus of PC12 cells (Neri *et al.*, 1999; Ye *et al.*, 2000). PI3,4,5P_3 has been shown to be involved in attracting to the nucleus PKCζ in NGF-treated PC12 cells (Neri *et al.*, 1999). However, nuclear PI3,4,5P_3 has other functions, including the control of PI3K activity through PIKE-L (Hu *et al.*, 2005). It is interesting that PLCγ1, which also translocates to the nucleus of PC12 cells challenged with NGF, is an upstream regulator of PIKE-L (Ye *et al.*, 2002). Moreover, PI3,4,5P_3 generated by PI3K binds nucleophosmin/B23 protein, which mediates the antiapoptotic effects of NGF in the nucleus of PC12 cells by inhibiting the DNA fragmentation activity of caspase-activated DNase (CAD) (Ahn *et al.*, 2005). Another agonist that increases nuclear PI3,4,5P_3 levels is platelet-derived growth factor (Lindsay *et al.*, 2006).

There also is quite an extensive literature demonstrating the involvement of class IA PI3K during myeloid differentiation of human leukemia cells, in particular after exposure to stimuli such as all-*trans*-retinoic acid (ATRA) or vitamin D3 (Bertagnolo *et al.*, 1998; Neri *et al.*, 1999). Although, the exact roles played by nuclear class I PI3K during myeloid differentiation have so far escaped clarification, these findings are intriguing, as ATRA is now being successfully employed in combination with other drugs for treating patients with acute promyelocitic leukemia (Ablain and de The, 2011).

Also, PI3K-C2β activity and protein increase during ATRA-induced differentiation of HL60 cells (Visnjic *et al.*, 2002), as well as during the G_2/M phase of the cell cycle in nocodazole-treated HL60 cells (Visnjic *et al.*, 2003).

Novel functions of nuclear phosphoinositides and their metabolizing enzymes

Phosphoinositides and their metabolizing enzymes can also act as direct regulators of diverse cellular functions. These novel roles of polyphosphoinositide are mostly based on interactions between the lipids and their specific binding proteins. An ever growing body of evidence indicates that, depending on the subcellular localization, a single phosphoinositide species can fulfill strikingly different roles by interacting with different protein partners.

Regulation of Nuclear Actin

Actin is a fundamental component of the cytoskeleton and PI4,5P_2 acts as a key regulator of actin dynamics in the cytoplasm by modulating the activity of several proteins that control actin polymerization and association with other proteins (D'Angelo *et al.*, 2008; Myers and Casanova, 2008).

Actin is present in the nucleus (Zhong *et al.*, 2010) and has been implicated in several functions that include transcription, mRNA processing, chromatin remodeling, and long-range chromatin organization (Visa and Percipalle, 2010; Castano *et al.*, 2010).

There exist several nuclear actin-binding proteins known for being regulated by PI4,5P_2. These include profilin I, which localizes to nuclear speckles and Cajal bodies and has been implicated in pre-mRNA splicing (Skare et al., 2003; Birbach *et al.*, 2006); the Arp2/3 complex, which interacts directly with RNA polymerase II and participates in transcription (Yoo *et al.*, 2007); myosin I, which regulates RNA polymerase I- and II-dependent transcription (Ye *et al.*, 2008; Obridlik *et al.*, 2010; Philimonenko *et al.*, 2010) and the p53 cofactor, junction mediating and regulatory protein or JMY (Zuchero *et al.*, 2009).

PI4,5P_2 can influence chromatin structure by facilitating the interactions between a nuclear matrix and the chromatin remodeling complex referred to as Brahma-related gene Associated Factors [BAF; (Zhao *et al.*, 1998)]. We now know a few details about the molecular mechanisms that regulate this interaction. It has been shown that PI4,5P_2 upregulates actin binding by the BAF complex (Rando *et al.*, 2002). Because there are reports indicating that actin is a nuclear matrix protein (Zhong *et al.*, 2010), PI4,5P_2 is an attractive candidate for a matrix localization signal for the BAF complex. The BAF complex comprises several polypeptides (at least 13) including actin, BAF53, and Brahma-Related Gene 1 (Brg1). A full BAF complex was required for PI4,5P_2 binding and stabilization of actin filaments. Moreover, it was found that Brg1 interacted with actin using at least two separate domains and PI4,5P_2 could selectively displace actin from one of these sites, thus relieving capping of BAF53 and actin by the Brg1 C-terminus (Rando *et al.*, 2002).

INO80 is another evolutionarily conserved, ATP-dependent chromatin-remodeling complex that contains actin (Shen *et al.*, 2003a). Moreover, recent studies have revealed that the INO80 complex has crucial functions in many other essential nuclear processes, including DNA repair, checkpoint regulation, DNA replication, telomere maintenance, and chromosome segregation (Morrison and Shen, 2009). Actin plays an important role in the regulation of DNA binding, ATP-ase activity, and nucleosome mobilization capability of the INO80 complex (Farrants, 2008). Although we do not know if PI4,5P_2 somehow directly regulates INO80 activity, it is interesting that Ins1,2,3,4,5,6P_6 (which could derive from Ins1,4,5P_3 generated through PI4,5P_2 hydrolysis) inhibits nucleosome mobilization by the INO80 complex (Shen *et al.*, 2003b).

Nuclear phosphoinositide metabolism, stress response, and apoptosis

Upon exposure of mammalian cells to oxidative and UV damage, there are changes in the amount of phosphoinositides depending on both the cell type and the stressing stimulus, which indicates the activation of specific pathways (Roberts *et al.*, 2005; Halstead *et al.*, 2006; Zou *et al.*, 2007; Chen *et al.*, 2009). PIPKIIβ and PI5P have been related to nuclear stress response pathways (Jones *et al.*, 2006). It has been hypothesized that PIPKIIβ signaling within the nucleus links PI5P and type I PI4,5P_2 4-phosphatase to p38 mitogen-activated protein kinase

(MAPK)-mediated stress response signaling (Jones *et al.*, 2006). Indeed, PIPKIIβ was phosphorylated by p38 MAPK at Ser 326 in response to oxidative or UV stress. This phosphorylation inhibited PIPKIIβ activity, resulting in the accumulation of PI5P within the nucleus. How then could PIPKIIβ, which has very little intrinsic 4-kinase activity (Wang *et al.*, 2010), regulate the levels of nuclear PIP5? PIPKIIβ is 95% nuclear, where about 60% of PIPKIIα is cytoplasmic and 40% resides in the nucleus (Wang *et al.*, 2010). Unexpectedly, it was found that PIPKIIβ associated *in vitro* and *in vivo* with PIPKIIα. Actually, it was demonstrated that *in vivo* the majority of 4-kinase activity in a PIPKIIβ immunoprecipitate was derived from its association with PIPKIIα (Bultsma *et al.*, 2010), and that PIPKIIβ was able to target PIPKIIα to the nucleus. What is even more interesting is that when HEK-293 cells were stably suppressed for PIPKIIβ expression, the majority of endogenous PIPKIIα was cytoplasmic, but the total amount of PIPKIIα localized to the nucleus did not change in a significant manner. Overexpressed PIPKIIα was predominantly cytoplasmic, while overexpressed PIPKIIβ was present both in the cytoplasm and in the nuclear speckles. However, when the two kinases were co-expressed, PIPKIIα and PIPKIIβ colocalized at the speckles. Overall, these findings could indicate that PIPKIIβ may specifically target PIPKIIα to nuclear speckles where it could act on its substrate, PI5P (Bultsma *et al.*, 2010). Intriguingly, essentially similar results were reported by another group (Wang *et al.*, 2010). It is still unclear how phosphorylation by p38 MAPK could impact on the levels of PI5P; however, it might be that it regulates the association between PIPKIIα and PIPKIIβ, hence the amount of PIPKIIα which localizes to the speckles (Keune *et al.*, 2011).

Nevertheless, upon cellular stress, the levels of nuclear PI5P could be upregulated by yet another mechanism. It has been shown that type I PI4,5P_2 4-phosphatase translocated to the nucleus in response to etoposide or doxorubicin treatment and yielded PI5P by dephosphorylating PI4,5P_2. This indicated that PIPKIIβ and type I PI4,5P_2 4-phosphatase could act in concert for increasing nuclear PI5P levels (Zou *et al.*, 2007).

How could these changes in nuclear phosphoinositide metabolism be related to stress-induced apoptosis? Increased PI5P levels caused translocation of the tumor suppressor ING2, a nuclear PI5P-binding protein, to a chromatin-enriched fraction (Gozani *et al.*, 2003). ING2 is a member of the inhibitor of growth family and acts as a cofactor on the histone acetyltransferase complex that functions in chromatin remodeling and p53 acetylation and activation. Indeed, ING2 associated with and modulated the activity of histone acetylases and deacetylases, and induced apoptosis through p53 acetylation on Lys 382 (Gozani *et al.*, 2003). Additionally, it was documented that ING2 regulation of p53 acetylation and apoptosis required both PI5P generation and an intact PI5P-binding domain. The accumulation of nuclear PI5P, which could be due to both a decrease in PIPKIIα activity and an increase in type I PI4,5P_2 4-phosphatase activity, facilitated the ING2-p53 apoptotic pathway by promoting ING2-dependent p53 acetylation (Gozani *et al.*, 2003; Zou *et al.*, 2007). Intriguingly, it has been reported that ING2 expression is lost in hepatocellular carcinoma, and it could be involved in the progression of the disease (Zhang *et al.*, 2008). Moreover, ING2 nuclear expression level is reduced in human melanomas when compared to dysplastic nevi. However, no correlation between ING2 nuclear expression and tumor stage was found, suggesting that reduced ING2 expression may be involved in the initiation rather than progression of melanoma (Lu *et al.*, 2006). It remains to be established whether these changes in ING2 expression are somehow related to an altered nuclear PI5P metabolism in cancer cells when compared to healthy cells. In any case, p53 is a master regulator of cell proliferation and is highly mutated and/or inactivated in human tumors (Goh *et al.*, 2011). Therefore, the above-discussed findings, link stress-activated modulation of nuclear PIP5 to the function of an important human tumor suppressor gene.

Nuclear phosphoinositide metabolism and regulation of the ubiquitin ligase complex

Recently, a novel mechanism has been identified by which PIPKIIβ and PI5P accumulation regulated a nuclear ubiquitin ligase complex (Bunce *et al.*, 2008). The authors, using yeast two-hybrid screen, identified speckle-type POZ domain protein (SPOP), an adaptor protein that recruits substrates to cullin 3 (Cul3) -based ubiquitin ligases (Li *et al.*, 2011), as a PIPKIIβ-binding protein. Ubiquitin ligases are enzymes that covalently attach ubiquitin to lysine residues on proteins to target for degradation or to modify activity. Ubiquitination is a post-translational modification pathway involved in myriad cellular regulation and disease pathways (Wenzel *et al.*, 2010). PIPKIIβ and SPOP interacted both *in vitro* and *in vivo*. The authors also demonstrated that type I PI4,5P_2 4-phosphatase generated PI5P from PI4,5P_2, leading to the stimulation of a MAP kinase kinase (MKK) 6/p38 MAPK pathway that activated the Cul3-SPOP ubiquitin ligase complex (Bunce *et al.*, 2008). The Cul3-SPOP ubiquitin ligase complex then ubiquitinylated PIPKIIβ, and PIPKIIβ downregulated this pathway by phosphorylating PI5P to PI4,5P_2. Overexpression of a PIPKIIβ kinase dead mutant stimulated the ubiquitinylation of itself and other Cul3-SPOP targets, including the Fas receptor binding protein Daxx and the pancreatic transcription factor Pdx1. PIPKIIβ and SPOP colocalized at the nuclear speckle level. These findings supported the idea that PI5P generation leads to activation of Cul3-SPOP activity. However, physiological or pathological conditions that activate or downregulate the PI5P/Cul3-SPOP pathway have yet to be identified.

Regulation of STAR-PAP

Using a yeast two-hybrid screen, Anderson and coworkers have recently identified STAR-PAP (speckle

targeted PIPKIα regulated-poly(A) polymerase) as a PIPKIα interactor (Mellman *et al.*, 2008). STAR-PAP is a poly(A) polymerase which regulates 3′-end cleavage and polyadenylation of a select set of mRNAs. Some of these mRNAs, such as heme oxygenase-1 (HO-1) mRNA, are involved in regulating the response to oxidative stress (Laishram and Anderson, 2010).

The interaction between STAR-PAP and PIPKIα dictates PIPKIα localization to the speckles, where $PI4,5P_2$ (presumably synthesized by PIPKIα itself) regulates STAR-PAP activity. If PIPKIα expression was suppressed by siRNA, a decrease in the mRNA subset regulated by STAR-PAP was observed (Mellman *et al.*, 2008). PIPKIα and STAR-PAP interacted directly *in vivo* and *in vitro*, and $PI4,5P_2$ stimulated both cell-purified and recombinant STAR-PAP activity by more than tenfold. Nevertheless, the PIPKIα-STAR-PAP complex also contains the $PI4,5P_2$-sensitive protein kinase CKIα, which directly phosphorylates STAR-PAP (Gonzales *et al.*, 2008). Both CKIα and PIPKIα were required for the expression of specific, STAR-PAP-regulated mRNAs. Therefore, it might be that $PI4,5P_2$ does not regulate STAR-PAP directly, but does so by affecting CKIα activity.

Scaffolding functions of nuclear PI3K

A relatively new theme regarding PI3K is that some members of this family (p110β and p110γ) might have a "double identity", i.e. PI3Ks have been found to act not only as classical kinases, but also as scaffolding proteins. This implies that generation of 3′ phosphorylated phosphoinositides is not necessarily linked with the presence of PI3K isoforms in a given cell domain (Costa and Hirsch, 2010). For example, both conditional $p110\beta^{-/-}$ mouse phenotype and that of inactive $p110\beta^{-/-}$ knock-in mice have highlighted that this PI3K isoform has kinase-independent function during embryonic development (Jia *et al.*, 2008; Ciraolo *et al.*, 2008).

It has been reported that nuclear PI3K p110β plays an important role in the control of DNA replication through both kinase-dependent and kinase-independent mechanisms (Marques *et al.*, 2009). The catalytic activity of p110β was required for regulating the nuclear activation of Akt during the S phase of the cell cycle and in turn the phosphorylation of the proliferating cell nuclear antigen (PCNA) negative regulator, $p21^{Cip}$, which is an Akt substrate. By doing so, p110β affected DNA replication by tuning PCNA binding to both chromatin and DNA polymerase Δ. However, p110β associated with PCNA and controlled PCNA binding to chromatin through a kinase-independent manner. In agreement with a possible scaffolding role, p110β was found to be associated with Akt and PCNA in a complex residing within the nucleus. In two subsequent studies, the same group demonstrated that nuclear p110β is involved in both double-strand DNA repair (Kumar *et al.*, 2010) and cell survival (Kumar *et al.*, 2011). Similarly to DNA replication, nuclear p110β played an important role also in DNA damage repair through both kinase-dependent and kinase-independent mechanisms. Indeed, endogenous p110β formed large nuclear foci after NIH 3T3 cell exposure to ionizing radiation, and simultaneous immunostaining of p110β and γ-H2AX documented partial colocalization of the two proteins at sites of double-strand DNA breaks. Reduction of p110β PI3K expression markedly diminished both the ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinase pathways, whereas inhibition of p110β only partially reduced the ATR route (Kumar *et al.*, 2010).

In particular, the kinase-independent ability of nuclear p110β to associate with Nbs1 (also referred to as nibrin, which is one of the members of the double-strand DNA break repair complex and is considered to be the earliest sensor of DNA damage (Stracker and Petrini, 2011) and to recruit it to damaged DNA, was critical for the DNA repairing activity. The kinase-dependent effects of nuclear p110β were related to $PI3,4,5P_3$ synthesis at the sites of double-strand DNA breaks. It was hypothesized that $PI3,4,5P_3$, through its negative charge, could help in maintaining DNA in an open conformation that would facilitate repair. Alternatively, $PI3,4,5P_3$ could interact with positively-charged histones, and this would also contribute to stabilization of chromatin in an open configuration at the sites of DNA damage (Kumar *et al.*, 2010). Regarding cell survival, it is not clear yet whether or not p110β catalytic activity was required (Kumar *et al.*, 2011).

PLCβ1 involvement in Mds

MDS are heterogeneous clonal disorders of hematopoiesis characterized by inefficient hematopoiesis, peripheral blood cytopenias, and risk of progression to AML. Although the pathogenesis of MDS is still unknown, deregulated signaling pathways are thought to play an important role in MDS pathophysiology (Bejar *et al.*, 2011). Identification of altered signal transduction mechanisms in MDS patients could be important for developing novel targeted therapies and for identifying molecular predictors of response to currently employed therapies. Moreover, such studies could lead to an improved classification and prognostic scoring schemes of the disorder.

It has been reported that the expression profile of both PLCβ1a and PLCβ1b mRNAs in bone marrow mononuclear cells is altered in MDS patients with a high risk of evolution to AML, as compared to healthy donors. In particular, it was documented that all of the patients with high risk MDS displayed a marked decrease in the amount of PLCβ1a mRNA, whereas most of the patients displayed low levels of PLCβ1b. This suggested that an imbalance between nuclear (PLCβ1b) and cytoplasmic (PLCβ1a) PLCβ signaling could somehow affect cell cycle progression and apoptosis resistance of MDS cells (Follo *et al.*, 2006). Lower levels of PLCβ1b expression correlated with enhanced activation of Akt in high risk MDS patients (Follo *et al.*, 2009). Activated Akt is a key determinant of cancer cell proliferation and survival and is a common feature displayed by AML patients (Martelli *et al.*, 2010).

However, it is still unclear how a lower amount of PLCβ1b expression could impact on Akt signaling upregulation. It might be that higher levels of PI4,5P_2 could result in a higher amount of PI3,4,5P_3, the upstream activator of PI3K/Akt.

Conclusion

It is almost 25 years since the concept of a distinct nuclear phosphoinositide signaling system emerged. Although considerable progress has been made in understanding some facets of it, yet there are still many key aspects that we do not understand at all. Some of these outstanding issues have been recently emphasized by Keune and co-workers (Keune *et al.*, 2011). For example, we do not know how phosphoinositides enter the nucleus or how they gain access to their interacting proteins. In case of PI4,5P_2-binding proteins, which phosphoinositide is first loaded onto the proteins, PI4P or PI4,5P_2? How could kinases, phosphatases, and PLC interact with nuclear phosphoinositides? What happens after PLC-mediated cleavage of PI4,5P_2? How is PI4,5P_2 resynthesized in the nucleus?

It is becoming increasingly clear that nuclear phosphoinositide signaling is as complex as its counterparts in other cell districts. If we aim to understand how nuclear phosphoinositide-based signaling networks operate, we first need to understand how phosphoinositide levels are regulated within the nucleus and how changes in their mass are transduced into output signals. A considerable insight into phosphoinositide-controlled cytoplasmic functions has been gained through the analysis of phosphoinositide-effector proteins. Therefore, identification of further specific nuclear phosphoinositide-interacting partners will be central for understanding how phosphoinositides regulate nuclear processes. Divecha and co-workers (Keune *et al.*, 2011) have proposed that nuclear phosphoinositides, concentrated in "hot spots", may regulate the functions of histone-interacting proteins. Two possible scenarios have been hypothesized. In the first one, the phospholipid "hot spots" would act as a hub where chromatin loops out and nucleosomes and phosphoinositides are brought together. Hubs of this type have been described where transcription occurs (Malyavantham *et al.*, 2008). In the second scenario, the "hot spots" would function rather like a drive through. Changes in phosphoinositide levels could induce the recruitment of a given protein to the "hot spot". Interactions with the phosphoinositides may then induce post-translational modification of the protein such as phosphorylation or acetylation, which may influence the location, interacting partners or the activity of the protein (Keune *et al.*, 2011). The data by Kumar and colleagues (Kumar et al., 2010), although related to DNA repair, seems to support the latter scenario, suggesting that PI3,4,5,P_3, synthesized by nuclear p110β PI3K, stabilizes or facilitates the recruitment of key proteins such as Nbs1, PCNA, and the ATM pathway effector 53BP1 to DNA double-strand breaks.

The identification of events regulated by nuclear phosphoinositides has lagged behind those in the cytoplasm and at the plasma membrane; however, nuclear phosphoinositides control functions of paramount importance which include gene expression, mRNA export, and chromatin structure. It is also clear that nuclear phosphoinositide metabolism requires specific regulatory factors that are only utilized within the nucleus. Moreover, nuclear phosphoinositides impinge on pRB, p53, and nucleophosmin/B23 protein pathways, and it is beginning to emerge that nuclear PLCβ1 could be involved in the evolution of MDS to acute leukemia.

These lines of evidence lead us to believe that dissecting the daunting complexity of nuclear phosphoinositide signaling networks will be highly rewarding, as it could undoubtedly offer valuable insights into the development of novel drug targets for several disorders, including cancer.

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