Chapter 3

NUCLEAR PHOSPHOLIPIDS AS EPIGENETIC REGULATORS

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PREFACE

Nuclear phospholipids are important for regulating many essential processes such as DNA replication, RNA processing, cell cycle progression and gene expression. Recent advances in the field of epigenetics have indicated involvement of nuclear phospholipids in chromatin remodelling and gene transcription in yeast and animals. However, the molecular function of the nuclear phospholipids has remained elusive. In this review, the potential function of nuclear phospholipids like inositol polyphosphates, sphingolipds and phosphoinositides in remodelling of chromatin and epigenetic regulation of gene expression are discussed within the context of recent literature. Future research will help shed light on the functional significance of nuclear phospholipids as epigenetic therapeutic targets.

INTRODUCTION

Phospholipids are a large family of signaling lipids that play important roles in the control of a very wide range of cellular functions. Most of our knowledge on phospholipid signaling is derived from events occurring at the cytoplasm and plasma membrane; yet phosphoinositide signaling has been established in the nucleus as well. Although the existence of nuclear phospholipids has been known for a while, their functions remain largely unknown. This is partly because this research field has long been hampered by technical difficulties demonstrating nuclear phospholipid synthesis and explaining their endonuclear state. Once their presence was demonstrated in the nucleus and several phospholipids, such as phosphoinositides, phosphatiylcholine, sphingolipids but also inositol polyphosphates, were found associated with several components of the nuclear matrix, the question arose as to what their nuclear function might be. Recent findings have suggested that the interaction of phospholipids with transcription factors or chromatin-modifying enzymes may serve a role in regulation of gene expression. This review will focus on the most significant and recent findings about phospholipid signaling in the nucleus and their emerging role as important modulators of chromatin and gene transcription.

NUCLEAR PHOSPHOLIPIDS

In the cytosol, phospholipids are located in the (plasma) membrane bilayers, whereas in the nucleus a subset of these lipids resides outside membrane structures (Rees, 1963) (Barlow *et al.*, 2010). Several reports (that assessed the nuclear localization of these phospholipids) indicated their presence in nucleoli and were found to colocalize with RNA in interchromatin granules, which are known sites of transcription (La cour Lf *et al.*, 1958; Rose *et al.*, 1965). The cellular phospholipid levels were also found to change in a cell- cycle dependent manner, which suggested a role in transcription (Fakan, 1986; Fraschini, 1992). Thereupon, the idea evolved that phospholipids might be involved in mediating nuclear events like chromatin remodeling and transcription (Fraschini *et al.*, 1999; Manzoli, 1976). This idea was reinforced when evidence accumulated that various phospholipid-metabolizing enzymes (like phospholipases and PIPkinases), largely known for their signaling functions in the cytoplasm and at the plasma membrane, were also present in the nuclear compartment (Capitani, 1989; Payrastre, 1992; Smith, 1983; Vann, 1997).

NUCLEAR PHOSPHOINOSITIDES

Phosphoinositides were among the first families of phospholipids identified in the nucleus. Phosphoinositides represent a small (less than 5%) proportion of the total cell phospholipids, yet they play crucial roles in the regulation of cell metabolism through their involvement in intracellular signaling mechanisms (Hammond, 2004). Phosphoinositides contain two long hydrophobic fatty acyl tails linked to a glycerol group, which is coupled via a phosphodiester linker to an inositol head group. Combination of phosphorylation at the 3, 4 or 5 position of the inositol head group generates different biologically relevant variants that form the basis

of a ubiquitous signaling system. In the "canonical" phosphoinositide cycle, extracellular stimuli trigger the metabolism of phosphoinositides by the action of kinases, phosphatases and phospholipases (Irvine, 2005). Phosphoinositides are sensed by specific phosphoinositide-binding domains present in a diverse array of proteins (Lemmon, 2008). Much of our knowledge about phosphoinositide signaling is derived from cytosolic phosphoinositide pathways. However, early experimental data from Cocco and colleagues demonstrate that isolated intact nuclei from MEL cells were still able to synthesize phosphoinositides *in vitro* (Cocco, 1987). Moreover, they also showed that upon differentiation there was a change in the phospholyration of phosphoinositides present in the isolated intact nuclei. Subsequent biochemical and immunehistochemical studies identified phosphoinositide-metabolizing enzymes in the nucleus, responsible for the change in phosphoinositide levels (Divecha, 1993; Martelli, 1992; Vann, 1997). Collectively, *in vitro* and *in vivo* studies reinforced the existence of an endonuclear phosphoinositide pathway, and that its regulation and metabolizing enzymes are independent of the cytosolic pathway (Gonzales, 2006; Irvine, 1992).

NUCLEAR PI(4,5)P2 METABOLISM AND CHROMATIN STRUCTURE

The identification of an autonomous nuclear phosphoinositide cycle suggested that these phospholipids in the nucleus might function to regulate nuclear processes. In 1965, Rose and colleagues first identified phospholipids that were associated with the chromatin fraction. *In vitro* studies have indicated that nuclear phospholipids could be involved in DNA associated process(Rose *et al.*, 1965). For instance, adding phospholipids to purified nuclei influences DNA replication and transcription (Capitani, 1986; Kuvichkin, 2002). Another study showed that *in vitro* addition of positively charged lipids lead to chromosome condensation whereas negatively charged lipids caused decondensation, however questions regarding the mode of action remained (Kuvichkin, 2002). A clue came from the study of the phosphoinositide species phosphatidylinositol 4,5-bisphosphate PI(4,5)P_2 . This phosphoinositide was shown to bind to the C-terminal tail of histone H1. Depletion of histone H1 unfolds chromatin fibers and increases RNA polymerase II transcriptional activity, and therefore histone H1 is a potent inhibitor of RNA pol II. PI(4,5)P_2 binding to H1 releases H1 from DNA and therefore reverses this inhibition of RNA polymerase II (Yu, 1998). This provided an example regarding how chromatin structure could be regulated by phosphoinositides.

PI(4,5)P2 can also influence chromatin structure by facilitating the interaction between the nuclear matrix and the SWI/SNF-like chromatin remodeling complex called BAF (Zhao, 1998). The mammalian BAF complex is a very large ATP-dependent complex containing BAF53, actin and a central ATPase subunit called BRG-1. Within the BAF complex, BAF53 and actin interact with BRG-1 through two actin-binding domains, one of which contains a lysine-rich patch. Interestingly, this lysine-rich patch of BRG-1 is essential for its function *in vivo* and PI(4,5)P2 inhibits actin binding by this domain *in vitro* (Bourachot, 1999; Rando, 2002). This suggests that PI(4,5)P2 disrupts the interaction of BRG-1 with actin/BAF53 and consequently expose uncapped actin/BAF53 allowing actin to interact with other components of the in

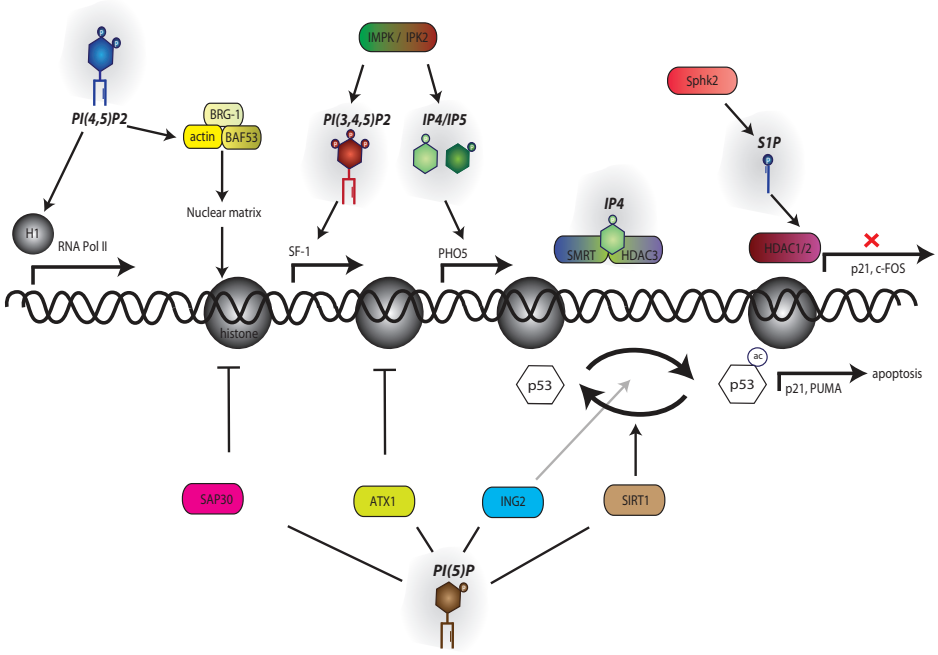

Figure 1. *Examples of regulation of chromatin remodeling and gene expression by phospholipids. Different phospholipids indicated in italic. PI(4,5)P_2 binding to Histone 1 (H1) releases H1 from DNA allowing RNA polymerase II binding. PI(4,5)P_2 binding stabilizes BAF complex by increasing its interaction with nuclear matrix and chromatin. Also PI(4,5)P_2 can mediate SF-1 mediated transcription by interaction with IMPK. Inositol polyphosphates can regulate gene transcription at PHO5 promotor trough the generation of IP_4/IP_5 by Ipk2 in yeast. IP_4 can also act as a modulatory bridging factor between HDAC3 deacetylase and co-repressor component SMRT. S1P formed by SphK2 inhibits histone deacetylases. Complexes containing SphK2 and histone deacetylases are enriched in the promotor regions of p21 and c-fos genes. Binding of PI(5)P to ATX1 inhibits association with chromatin and can also displace SAP30 from chromatin. Furthermore, PI(5)P can influence both acetylation and deacetylation of p53 trough binding of respectively ING2 and SIRT1 deacetylase and induction of p53 target genes and apoptosis.*

nuclear matrix (Janmey *et al.*, 1999; Shen, 2003). PI(4,5)P_2 binding to BRG1 may facilitate recruitment to chromatin and stabilize the chromatin remodeling complex by an increased interaction with nuclear matrix. These findings provide an example of phospholipid-induced chromatin remodeling. A question remains: how is phospholipids synthesis, in this example PI(4,5)P2, regulated at sites where it can affect chromatin (BAF complex) function? An attractive hypothesis suggests that pRB, a tumor suppressor gene that functions trough binding to BAF complex, interacts with and activates PIPKIα (Divecha, 2002). PIPKIα is the enzyme responsible for almost all PI(4,5)P2 synthesis. Therefore, it is plausible that pRB recruits PIPKIα to the BAF complex to control localized PI(4,5)P2 production.

INOSITOL POLYPHOSPHATES REGULATING GENE TRANSCRIPTION

Next to PIPKIα, nuclear PI(4,5)P2 levels can also be regulated via the activity of other metabolizing enzymes such as nuclear phospholipase C isoforms (PLCs). These PLCs catalyze the hydrolysis of PI(4,5)P2, thereby generating the two second messengers DAG and IP_3 (van den Bout *et al.*, 2009). Phosphorylation of IP_3 by inositol polyphosphate kinases (Ipks) produces a set of inositol polyphosphate second messengers. Intriguingly, studies in yeast have implicated these inositol polyphosphates in nuclear transcription (Odom, 2000). In yeast IP3, generated by PI(4,5)P2 hydrolysis, is utilized by Ipk2 to produce IP4 and IP5, which can be further phosphorylated by Ipk1 to IP6 (York, 1999). Strikingly, in yeast this kinase Ipk2 is also known as a transcription factor named ARG82. Steger *et al*. showed that in an *Ipk2/arg82* deficient yeast strain, chromatin remodeling at PHO5, a phosphate responsive promoter was impaired (Steger, 2003). PHO5 transcription depends on either IP4 or IP5, because mutations blocking the pathways leading to IP6 have no effect on transcription. Their data showed that chromatin remodeling at the PHO5 nucleosome required for PHO5 transcription depended on Ipk2/ARG82 inositol kinase activity in yeast. IPMK, the human homologue of ARG82/ Ipk2 has also been reported to phosphorylate IP3 to IP4 and IP5. Blind *et al*. demonstrated a role for IPMK in gene transcription in mammalian cells. IPMK can not only function as an inositol kinase, but also as phosphoinositide 3-kinase (PI3K) that can phosphorylate PI(4,5)P2 to phosphatidylinositol 3,4,5-trisphosphate PI(3,4,5)P3 (Blind, 2012). IPMK was also found to interact with nuclear receptor steroidogenic factor 1 (SF-1) and to phosphorylate its bound ligand PI(4,5)P2 to PI(3,4,5)P3, which subsequently affects SF-1-mediated transcription. A structural study has shown that phosphoinositides, including PI(4,5)P2 and PI(3,4,5)P3, are ligands for nuclear receptor (Krylova *et al*., 2005). The ability of Ipk2 and IPMK to directly remodel and alter the activity of a transcription factor-lipid complex exemplifies how phospholipids can regulate gene expression (Chakraborty, 2011).

LINKING PHOSPHOLIPIDS TO ACETYLATION

The role of the other inositol phosphates and their kinases in mammalian chromatin remodeling complexes, or how they might exert these roles, has not been demonstrated yet. However, a recent study by Watson *et al*., has provided a mechanistic insight into how inositol phosphate IP4 can regulate chromatin structure through the study of histone deacetylases in repressive complexes (Kutateladze, 2012).

Acetylation is a key posttranslational modification on lysine residues in many histone and non-histone proteins. Acetylation of histones by histone acetyltransferases (HAT's) directly influences chromatin remodeling, presumably by reducing the positive charge on lysines hence the affinity of histones for negatively charged DNA. The chromatin is then allowed to become more accessible for binding to the basal transcription machinery (Choudhary *et al*., 2009). Conversely, histone deacetylases can remove acetyl groups from histones and other nuclear proteins, thereby inducing chromatin condensation and transcriptional repression. Watson *et al*. resolved the structure of a complex between HDAC3 deacetylase and the deacetylase activation domain (DAD) of co-repressor SMRT, and revealed IP4 to function as a

modulatory bridging factor between the two proteins, which contributes to the stabilization and activation of HDAC3 (Watson, 2012). IP4 binding was shown to be essential for both the incorporation of the histone deacetylase HDAC3 into a repressive complex and its deacetylase activity. The authors argued that a conformational change of HDAC3, which occurs upon its interaction with IP4 and DAD-SMRT, facilitates the access of substrates to the HDAC3 active site(Kutateladze, 2013). This study provides a possible explanation of why inositol lipids and their kinases are found in the chromatin fraction, and reveals an interesting link between inositol phosphates signaling and epigenetic regulation.

Similarly, another family of phospholipids, nuclear sphingolipids and their kinases can also affect HDAC activity. New evidence revealed that HDAC1 and HDAC2 activity can be inhibited by the sphingolipid metabolite sphingosine-1-phosphate (S1P). Both nuclear S1P and sphingosine kinase 2 (SphK2), the enzyme that synthesizes S1P, were found to be part of a corepressor complex containing HDAC1 and HDAC2 (Hait *et al.*, 2009). SphK2 binding to p21 and c-fos promotors enhanced acetylation of histone H3 and transcription. Furthermore, S1P was shown to inhibit deacetylase activity of HDAC1 and HDAC2. These studies exemplify nuclear lipids that are associated with an epigenetic modification and shows how nuclear phospholipid signaling can regulate chromatin-remodeling enzymes that influence histone acetylation and gene expression (Riccio, 2010). However, it remains to be determined how these nuclear phospholipid signalling pathways can influence HDAC dependent gene repression and how specificity towards acetylated targets is regulated.

CHROMATIN REMODELING AND PI(5)P; DOES IT TAKE A PHD?

Like nuclear S1P and inositol phosphates, nuclear phosphoinositides are implicated in gene transcription and chromatin remodeling by several studies. Many of these studies have been centered on the role of nuclear PI(4,5)P2 in gene expression, as discussed above (Bunce, 2006). There are however, other examples of phosphoinositide species that interact with chromatin. Phosphatidylinositol mono-phosphates (PI3P, PI4P and PI5P) have long been considered to be just intermediate metabolites for the synthesis of PI(4,5)P2 and other phosphoinositides via their (de)phosphorylation by PIP kinases and phosphatases (Doughman *et al.*, 2003). However, it is now clear that phosphatidylinositol monophosphates levels are dynamic and serve important signaling functions by themselves (Pendaries, 2005). For instance, they change abundantly when cells progress through the cell cycle. Nuclear PI(3)P accumulates during G2/M phase in HL-60 cells by activation of the phosphatidylinositol 3 kinase , PI3K-C2β. In MEL cell nuclei, the abundance of PI(4)P and PI(5)P (but not PI(3)P) increases as cells progress through the G1-S phase of the cell cycle, which suggests a potential role for these phosphoinositides in cell cycle progression (Clarke *et al.*, 2001a; Visnjic, 2003). The nuclear levels of phosphoinositide mono-phosphates can be increased by various cellular responses and stresses as well. Notably PI(3)P levels respond to RA treatment and PI (4)P, PI(5)P upon UV etoposide treatment (Jones *et al.*, 2006; Visnjic, 2002).

Recently, it was shown that PI(5)P can act as a redox-regulated second messenger, in that its levels regulate oxidative stress responses (Keune *et al.*, 2012). To date, there is no evidence for a role of nuclear PI3P or PI4P in chromatin remodeling. However, the identification of PI(5)P binding proteins and their nuclear functions are now beginning to be elucidated (Keune *et al.*,

2011). A breakthrough came from the discovery that ING2, which regulates p53 acetylation and function, binds to PI(5)P through its PHD domain (plant homeo domain) (Gozani *et al*., 2003). ING2 promotes the acetylation of p53 and induces p53-mediated apoptosis in response to UV and etoposide. In contrast to wild-type ING2, point mutated ING2 that resulted in loss of PI(5)P binding was compromised in the ability to induce apoptosis and p53 acetylation. In addition, overexpression of PIP4Kβ, which depletes nuclear PI5P by converting it to PI(4,5)P2, significantly inhibited ING2-mediated apoptosis. This study was the first to identify the PHD motif as a PI(5)P-binding module and ING2 as the first PI(5)P binding protein. Expanding on these studies, Jones *et al*. showed that PIP4Kβ becomes phosphorylated by p38 MAPK in response to genotoxic stress (Jones *et al*., 2006). This phosphorylation inhibits PIP4Kβ resulting in accumulations nuclear PI(5)P. This stress induces increase in nuclear PI(5)P causes translocation of ING2 to the chromatin-enriched fraction, where it binds and activates ING2 thereby modulating p53 acetylation and function. The ING2 experimental data exemplifies that phosphoinositides, notably PI(5)P, can modulate the association of remodelling enzymes with chromatin and can be regulated by phosphoinositide metabolizing enzymes (Jones *et al*., 2004). Precisely how PI(5)P promotes binding or detachment of protein-chromatin interactions is currently not known. One could imagine that, similar to PKB activation, interaction of PI(5)P with ING2 could lead to its allosteric activation or recruitment of other binding partners that change ING2 association with chromatin(Franke *et al*., 1997), or that PI(5)P can act as a modulatory bridging factor, similar to the IP4-HDAC association described above (Kutateladze, 2012).

In addition to PI(5)P binding, the PHD motif of ING2 also binds histone H3 trimethylated at lysine 4 (H3K4me3). Upon DNA damage, H3K4me3 binds to ING2, which is essential for the activity of ING2 (Shi, 2006). Together with HDAC1 and Sin3A, ING2 forms a complex that transcriptionally represses active promoters. Interestingly, PHD fingers are mostly found on nuclear proteins involved in regulating gene transcription through the modulation of chromatin structure (Bienz, 2006). An attractive hypothesis is that upon DNA damage, elevated PI(5)P levels recruit ING2 to specific (target) promotors, which would facilitate H3K4me3 binding to ING2 and stabilize ING2 to regulate transcriptional activity at these promotors. However, it is currently not clear how PI(5)P binding can modulate H3K4me3, or vice versa.

There is however some data in plants suggesting that PI(5)P might regulate the levels of H3K4me3 under certain conditions (Alvarez-Venegas *et al*., 2006). ATX1, the Arabidopsis homolog of trithorax, is a trimethyltransferase that trimethylates histone 3 lysine 4 (H3K4me3) at the nucleosome of promotor regions. Others show not only that ATX1 binds PI(5)P through the ATX-PHD domain, but also that PI(5)P binding could negatively affect ATX1 trimethyltransferase-activity and causes ATX1 to detach from promoters and translocate from the nucleus to the cytosol (Ndamukong *et al*., 2010). This is opposite to the effect (described above) where PI(5)P positively affected ING2 activity by translocation into to the nucleus and facilitating recruitment of ING2 to chromatin. This suggests that the effect of the interaction of PI(5)P with PHD domains can have different consequences on the activity of (and therefore association with) PHD-containing proteins with chromatin. In addition, PI(5)P dependent chromatin remodelling can also occur on proteins that do not contain PHD fingers (Viiri, 2009). Components of the Sin3A corepressor complex SAP30 and SAP30L were found to bind

immobilized phosphoinositides, particularly PI(5)P through a zinc motif that overlaps with its DNA binding site. The authors proposed that PI(5)P binding displaces DNA from the Sin3A corepressor complex by modulating SAP30 repressor activity and translocate them from the nucleus to the cytoplasm (Viiri, 2012). Possibly, the displacement of these proteins from the DNA by PI(5)P might be similar to the Histone H1 displacement from RNA by PI(4,5)P2 binding describe above.

Interestingly, PI(5)P can bind to another histone-protein deacetylase that does not contain a PHD domain. We found that nuclear PI(5)P can modulate the activity of another deacetylase namely human sirtuin 1 SIRT1, a NAD+dependent deacetylase that belongs to the class III deacetylases. SIRT1 is recruited to chromatin where it modifies and silenced transcription by histone deacetylation or influences acetylation of many transcriptional regulators including transcription factor p53 (Rajendran *et al.*, 2011; Vaziri *et al.*, 2001). We identified a previously unrecognized polybasic region (PBR) that binds to PI(5)P, and this interaction is essential for the SIRT1-mediated deacetylation of p53 both *in vitro* and *in vivo* (Elouarrat *et al.*, submitted). Subsequently, manipulation of nuclear PI(5)P (by overexpression/depletion of PIP4K) changed SIRT1 deacetylase activity and SIRT1 dependent p53 transcription and apoptosis. As nuclear PI(5)P can influence both acetylation (by binding to PHD domain of ING2) and deacetylation of p53 (by binding to PBR of SIRT1), we expect that the temporal and spatial regulation of PI(5)P likely determines the strength and duration of p53 activation and transcriptional activity. Together, these studies emphasize the role of nuclear phosphoinositide monophosphates in regulating chromatin-modifying enzymes and suggest an important general role for them as phospholipid signal transducing enzymes.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Studies over the last decades in plants, yeast and mammalian cells have revealed the existence of phospholipid signaling and metabolism in the nucleus. The multitude of roles they play in nuclear events like chromatin structure and gene expression are only now beginning to be uncovered. The recent advances in identifying the specific nuclear proteins that interact with and are regulated by nuclear phospholipids have been pivotal to reveal these roles. Despite significant advances in this field, many questions still remain regarding the mechanistic regulation of distinct nuclear phospholipids associating with target proteins. One could speculate that (local) changes of phospholipid levels can induce the recruitment of chromatin-remodeling proteins. This recruitment may induce a conformational change of the protein, which changes their interaction partners, localization or activity. It is therefore required to gain a better understanding of how phospholipid levels are regulated by their metabolizing enzymes, as was revealed in the studies on S1P/SphK2 regulating HDAC deacetylase activity or PI(4,5)P2/IMPK regulating SF-1 transcriptional activity (Blind, 2012; Krylova *et al.*, 2005; Watson, 2012). Further studies on these and other phospholipid metabolizing enzymes will provide new insights into how signaling phospholipids are loaded and gain access to their nuclear target protein.

One might wonder why nuclear processes like chromatin remodeling would be regulated by phospholipid signaling. Phospholipids are known to act as acutely regulated signaling

platforms that assemble and coordinately regulate components of cellular pathways (Irvine, 2005). Therefore, they would provide an ideal signal transducing mechanism that can acutely respond to external cues and by transducing to chromatin-associated proteins can rapidly alter gene transcription program. Indications for this come from for example, when chromatin-modifying enzymes associate with active or repressor complexes on promoters. One could speculate that upon the assembly of chromatin complexes on promotors, the production of phospholipid signalling molecules provides a means of rapidly modulating the transcription of their target genes upon differential stimuli and keeping the promotors in an (in) active state.

Recent advances in nuclear phospholipid signaling have opened a field of epigenetics that was previously unknown. Obviously, much work still lies ahead in order to better understand the mechanisms by which phosphoinositides participate in chromatin dynamics and gene transcription. Future efforts should focus on the identification of new (nuclear-specific) phosphoinositide-binding motifs, which will constitute a key step in our comprehension of nuclear phosphoinositides, their transducing mechanism and their functions. A recent unbiased proteomics study for nuclear PI(4,5)P2 interacting proteins has revealed novel nuclear phosphoinositide-binding proteins, many of which have known functional roles in chromatin assembly and no prior history of phosphoinositides interactions (Lewis *et al.*, 2011). Efforts like these and on other nuclear phospholipids will enhance our knowledge on their roles in chromatin remodeling. Additionally, the use of new emerging techniques for sensitive lipidomics profiling of the nucleus will provide a powerful tool for further insight in understanding phospholipid nuclear functions (Hunt, 2006). Elucidating the molecular and cellular consequences of phospholipids as lipid mediator of epigenetic modifications and establishing how widespread this is are going to be crucial. Ultimately, the nuclear roles of phospholipids might offer some new insights in to the development of novel small molecule targets for cancer therapy. Like their counterparts in the cytosol, nuclear phosphoinositides turn out to be essential regulators of diverse cellular processes and functions.

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