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Localization and function of phosphoinositides in the cell nucleus
Lokalizace a funkce fosfoinositidů v buněčném jádře

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ABSTRACT (ENGLISH)

Phosphoinositides (PIs) are negatively charged glycerol-based phospholipids. Their inositol head can be phosphorylated at three positions generating seven differently phosphorylated species. Cytoplasmic phosphoinositides regulate membrane and cytoskeletal dynamics, vesicular trafficking, ion channels and transporters and generate second messengers. In the nucleus, PIs are implicated in pre-mRNA processing, DNA transcription and chromatin remodelling. However, their nuclear functions are still poorly understood. Here we focus on nuclear phosphatidylinositol 4-phosphate (PI(4)P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2). We describe their localization and interaction with proteins involved in regulation of DNA transcription.

PI(4)P localizes to the nuclear membrane, nuclear speckles and nucleoplasm. The majority of nuclear PI(4)P is associated with chromatin and colocalizes with H3K4me2. PI(4,5)P2 localizes to nucleoli and nuclear speckles. Besides, 30 % of nuclear PI(4,5)P2 forms small nucleoplasmic PI(4,5)P2 islets. They have carbon rich core, which is probably formed by lipids, and are surrounded by proteins and nucleic acids. The active form of RNA polymerase II associates with PI(4,5)P2 islets and DNA is actively transcribed in the vicinity of PI(4,5)P2 islets. Moreover, nuclear myosin 1 (NM1) binds PI(4,5)P2 in the nucleus. This interaction targets NM1 to PI(4,5)P2 islets and is essential for NM1 interaction with transcription machinery and active DNA transcription. Therefore, we suggest that PI(4,5)P2 islets facilitate a spatial-temporal arrangement of transcription complexes assembly.

Moreover, we demonstrate that lysine-specific histone demethylase 1 (LSD1), enzyme that demethylates H3K4me2, interacts with both PI(4)P and PI(4,5)P2. While the interaction with PI(4)P leads to an inhibition of LSD1, the interaction with PI(4,5)P2 stimulates LSD1 H3K4me2 demethylase activity *in vitro*. Thus, PI(4)P and PI(4,5)P2 could regulate transcription at the epigenetic level also *in vivo*.

Another PI(4,5)P2 binding protein, actin, exists in the cytoplasm in monomeric form that can polymerize to filaments. However, in which form is actin present in the nucleus is still not sufficiently understood. After actin overexpression, we observed formation of actin filaments in the nucleus. These filaments resemble cytoplasmic F-actin and recruit cofilin and Arp3 actin binding proteins. The formation of actin filaments in the nucleus results in an increased transcription in S-phase, decreased cell proliferation and aberrant mitosis.

ABSTRACT (CZECH)

Fosfoinositidy jsou negativně nabité fosfolipidy. Jejich inositolová hlavička může být fosforylována na třech pozicích, a tak mohou tvořit sedm různě fosforylovaných forem. Cytoplasmatické fosfoinositidy regulují dynamiku buněčných membrán a cytoskeletu, transport membránových váčků, funkci iontových kanálů a transportérů a produkci druhých poslů. Jaderné fosfoinositidy ovlivňují postranskripční úpravy pre-mRNA, DNA transkripci a remodelování chromatinu. Jejich jaderné funkce jsou nicméně nedostatečně prozkoumány. Tato práce se zaměřuje na jaderný fosfatidylinositol 4-fosfát (PI(4)P) a fosfatidylinositol 4,5-bisfosfát (PI(4,5)P2), jejich lokalizaci a interakce s proteiny regulujícími transkripci.

PI(4)P je součástí jaderné membrány, nachází se v jaderných speckles a v nukleoplasmě. Většina jaderného PI(4)P je navázána na chromatin a kolokalizuje s H3K4me2 histonovou značkou. PI(4,5)P2 lokalizuje do jadérka a jaderných speckles. Přibližně 30 % jaderného PI(4,5)P2 tvoří malé ostrůvky v nukleoplasmě. Nitro těchto ostrůvků je bohaté na uhlík a je pravděpodobně tvořeno lipidy. Ostrůvky jsou obklopeny proteiny a nukleovými kyselinami. Aktivní forma RNA polymerázy II lokalizuje k PI(4,5)P2 ostrůvkům a v blízkosti ostrůvků dochází k aktivní transkripci. Jaderný myosin 1 (NM1) váže PI(4,5)P2. Tato interakce je zásadní pro vazbu NM1 na transkripční komplex a probíhající transkripci. Předpokládáme proto, že PI(4,5)P2 ostrůvky zprostředkovávají časově-prostorovou koordinaci tvorby aktivních transckripčních komplexů.

Lyzín-specifická histon demetyláza 1 (LSD1) interaguje s PI(4)P i PI(4,5)P2. Interakce s PI(4)P inhibuje, zatímco interakce s PI(4,5)P2 stimuluje LSD1 demetylační aktivitu a ovlivňuje tak hladinu H3K4me2 *in vitro*. Tímto způsobem by PI(4)P a PI(4,5)P2 mohly regulovat transkripční aktivitu na epigenetické úrovni i *in vivo*.

Dalším PI(4,5)P2 vazebným proteinem, který jsme studovali, je aktin. Aktin existuje v cytoplasmě ve formě monomerů, které mohou polymerizovat a vytvářet vlákna. Zatím není dostatečně objasněno, v jaké formě se aktin vyskytuje v buněčném jádře. Po exogenní overexpresi aktinu jsme pozorovali tvorbu jaderných aktinových vláken. Tato vlákna připomínala svými vlastnostmi cytoplasmatická aktinová vlákna. Aktin vazebné proteiny cofilin a Arp3 lokalizovaly k těmto jaderným vláknům. Následkem tvorby jaderných aktinových vláken byla zvýšená transkripce během S fáze buněčného cyklu, snížená buněčná proliferace a aberantní mitóza.

1. INTRODUCTION

Phosphatidylinositol (PI) is a negatively charged glycerol-based phospholipid. As an amphipathic molecule, PI is formed by hydrophobic acyl tail and hydrophilic inositol head. The inositol head can be phosphorylated at three different positions generating 7 phosphorylated species, phosphoinositides (PIs) – phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3; Fig. 1). Their metabolism is very dynamic, regulated by numerous PIs kinases, phosphatases and phospholipases. Although PIs represent only about 2 % of cellular phospholipids, they are very important signalling molecules (reviewed in Viaud et al. 2015; Tan et al. 2015).

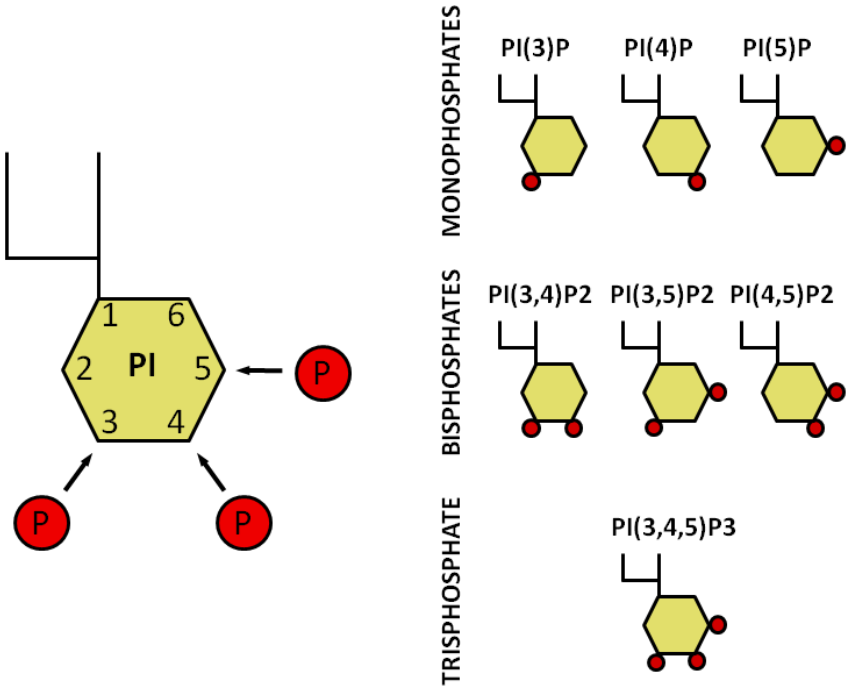

Fig. 1 Schematic representation of PI and seven differently phosphorylated PIs. PI can be phosphorylated at 3', 4' and 5' positions yielding three phosphatidylinositol monophosphates (PI(3)P, PI(4)P, PI(5)P), three phosphatidylinositol bisphosphates (PI(3,4)P2, PI(3,5)P2, PI(4,5)P2) and one phosphatidylinositol trisphosphate (PI(3,4,5)P3). Red circles depict sites of inositol ring phosphorylation.

1.1 Phosphoinositides in the cytoplasm

As phospholipids, cytoplasmic PIs are components of cellular membranes. Their localization can be visualized by specific PIs-binding protein domains coupled to GFP and can be concluded also from localization of their metabolizing enzymes. There are many PIs

metabolizing enzymes, which differ in their specificity, activity and localization. Therefore, several pools of particular PIs are produced in different membranes and organelles. PIs then recruit their binding partners to these sites, which results in unique functions of individual PIs within the cell. Cytoplasmic PIs regulate vesicular trafficking, membrane and cytoskeletal dynamics, ion channels and transporters, and generation of second messengers (reviewed in Balla 2013; Tan et al. 2015).

1.2 Phosphoinositides in the nucleus

Beside their cytoplasmic functions, PIs play an important role also in the cell nucleus (reviewed in Shah et al. 2013). It is still unclear whether or how PIs are transported from the sites of their synthesis to the nucleus. However, two isoforms of PI transfer proteins (PIPTs) are able to translocate to the nucleus (De Vries et al. 1996). As many PIs metabolizing enzymes also localize to the nucleus (Keune et al. 2011; Martelli et al. 2011), it is plausible that PI is transported to the nucleus, where it is further metabolized. Indeed, several studies have confirmed an active intranuclear PIs metabolism (Smith and Wells 1983; Cocco et al. 1987; Déléris et al. 2003; Yildirim et al. 2013). These results point towards existence of an intranuclear PIs cycle, which is independent on cytoplasmic PIs metabolism (Fig. 3).

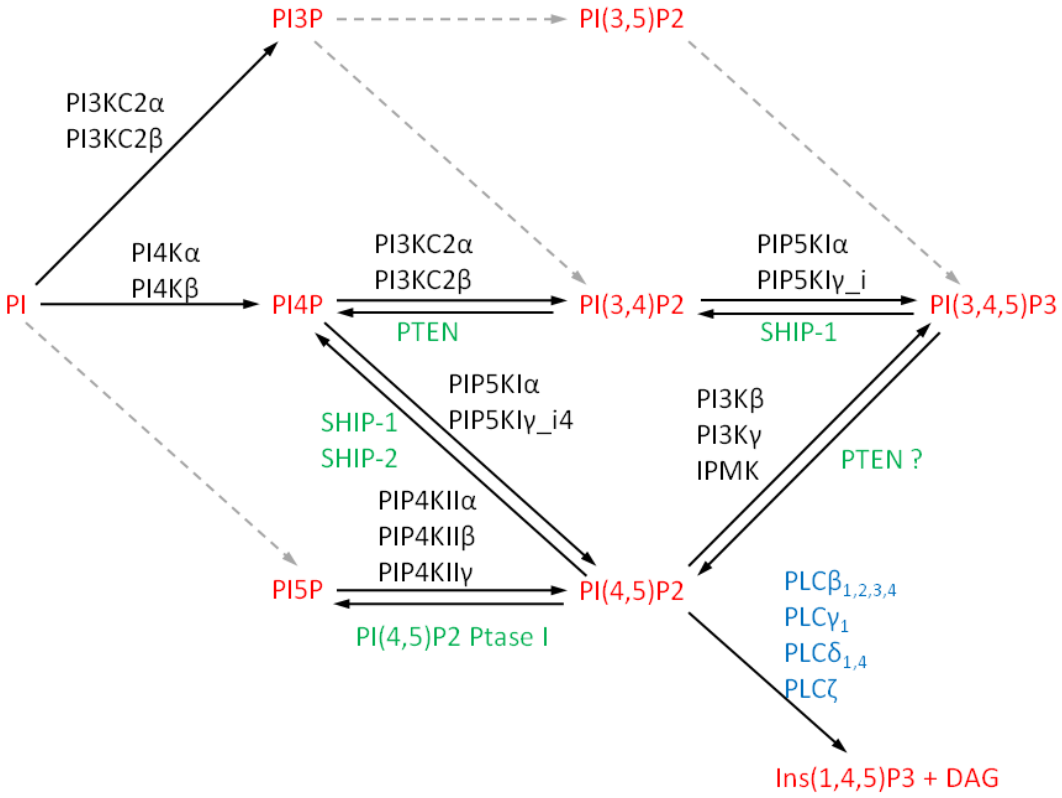

Fig. 2 PIs metabolism in the nucleus. Enzymes involved in PIs metabolism that localize to the nucleus are depicted. PIs kinases (black), phosphatases (green), and phospholipases (blue) form a metabolic network that most likely facilitates formation of different PIs species (red) in the nucleus. Pathways catalyzed by enzymes confirmed to be

present in the nucleus (solid black arrows) and also possible pathways without known nuclear enzyme (dashed grey arrows) are illustrated. A specific case is phosphatase and tensin homolog (PTEN), which localizes to the nucleus but has been shown to be unable to dephosphorylate nuclear PI(3,4,5)P3 (Lindsay 2006).

1.3 Functions of nuclear PIs

Approximately 15 % of cellular PIs reside in the nucleus (York and Majerus 1994). Recent high-throughput studies identified more than 120 nuclear PIs-interacting proteins involved in different nuclear functions including DNA repair, chromatin remodelling and rRNA or mRNA processing (Lewis et al. 2011; Jungmichel et al. 2014). In the following section, data about nuclear PIs and their roles in these processes will be summarized.

1.3.1 Pre-rRNA and pre-mRNA processing

We have recently shown that PI(4,5)P2 binds to nucleolar protein fibrillarin (Yildirim et al. 2013). Fibrillarin functions in pre-rRNA processing, modification, and ribosomal assembly (Tollervey et al. 1993). PI(4,5)P2 binds to fibrillarin in the transcriptionally active regions of the nucleolus, alters its conformation and modulates the binding of fibrillarin to pre-rRNA (Yildirim et al. 2013).

Anti-PI(4,5)P2 antibody co-immunoprecipitates both small nuclear RNAs and the active form of RNA Pol II, and inhibits pre-mRNA splicing *in vitro* (Osborne et al. 2001). These results indicate that PI(4,5)P2 forms RNA-protein-lipid complexes which are involved in pre-mRNA splicing. However, the exact molecular mechanism remains unknown.

PI(4,5)P2 acts also as a regulatory molecule in mRNA polyadenylation. PI(4,5)P2 synthesized by PIP5KIα stimulates processivity of a non-canonical Speckles targeted PIP5KIα regulated poly(A) polymerase (Star-PAP) resulting in a stabilization of target mRNA coding proteins involved in detoxification and oxidative stress response (Mellman et al. 2008).

Once mRNA is maturated, it is exported to the cytoplasm upon association with RNA-interacting protein complexes (Nojima et al. 2007; Fuke and Ohno 2008). PI(4,5)P2 and PI(3,4,5)P3 directly interact with the N-terminal part of export factor Aly/REF. (Okada et al. 2008). PI3K activity of IPMK and nuclear PI(3,4,5)P3 formation is essential for Aly binding to target mRNA. The downregulation of IPMK activity inhibits export of mRNA coding proteins implicated in DNA repair and homologous recombination (Wickramasinghe et al. 2013).

1.3.2 DNA damage response and apoptosis

The activity of class I PI3Ks, kinases that generate PI(3,4,5)P3, increases after growth factor stimulation and during cell differentiation (Bertagnolo et al. 1999; Tanaka et al. 1999; Ahn et al. 2004). After nerve growth factor (NGF) stimulation, PI(3,4,5)P3 binds directly to nuclephosmin (B23). The PI(3,4,5)P3-B23 complex then mediates anti-apoptotic signalization (Ahn et al. 2005). The action of both SHIP-2 and phosphatase and tensin homolog (PTEN) phosphatases (Ahn et al. 2004; Ahn et al. 2005) as well as overexpression of B23 PI(3,4,5)P3-binding mutant (Ahn et al. 2005) can inhibit NGF induced anti-apoptotic actions.

Besides its role in stabilization of particular mRNAs, Star-PAP-PIP5KIα complex takes part in DNA damage response and apoptosis. Via PIP5KIα, Star-PAP interacts with PKCδ (Li et al. 2012), which is a key regulator of apoptosis (reviewed in Brodie and Blumberg 2003). Following DNA damage, PI(4,5)P2 generated by PIP5KIα stimulates PKCδ activity. PKCδ then phosphorylates Star-PAP, leading to Star-PAP activation and stabilization of a pro-apoptotic protein Bcl2-interacting killer (BIK) mRNA (Li et al. 2012).

Moreover, PI(5)P is an important player in DNA damage response through its interaction with inhibitor of growth protein 2 (ING2; Gozani et al. 2003). Following DNA damage, ING2 stimulates acetylation of cellular tumour antigen p53 leading to G1-phase cycle arrest, and apoptosis (Nagashima et al. 2001). PI(5)P binds to ING2 (Gozani et al. 2003) and stabilizes it at the promoters of target genes (Bua et al. 2013). PIP4KIIβ kinase, which phosphorylates PI(5)P to PI(4,5)P2, is inhibited upon cellular stress (Jones et al. 2006). Similarly, PI(4,5)P2 4-Ptase I, which dephosphorylates PI(4,5)P2 to PI(5)P, translocates into the nucleus upon DNA damage (Zou et al. 2007). Elevated PI(5)P levels result in increased ING2 association with chromatin (Jones et al. 2006) and induction of apoptosis through ING2-p53 pathway (Zou et al. 2007).

1.3.3 Chromatin remodeling and modifications

The accessibility of DNA is crucial for gene expression regulation. There are data demonstrating that PI(4,5)P2 plays an important role in DNA topological change and chromatin remodelling, since it interacts with proteins involved in these processes (Yu et al. 1998; Zhao et al. 1998; Rando et al. 2002; Lewis et al. 2011; Toska et al. 2012).

Nuclear PI(4,5)P2 interacts with transcriptional co-repressor brain acid soluble protein 1 (BASP1) and facilitates BASP1 interaction with histone deacetylase 1 (HDAC1). BASP1-PI(4,5)P2-HDAC1 complex is then recruited to the promoter of target genes, where HDAC1 deacetylates histones and reduces promoter accessibility for RNA Pol II transcription machinery (Toska et al. 2012). Another mechanism of PI(4,5)P2 action in chromatin remodelling is through its direct interaction with SWI/SNF-like BAF chromatin remodelling complex (Rando et al. 2002). PI(4,5)P2 binds BAF complex, targets it to chromatin, and facilitates changes in chromatin structure during T-lymphocyte activation (Zhao et al. 1998).

In addition, PI(5)P binds directly to ubiquitin-like PHD and really interesting new gene (RING) finger domain-containing protein 1 (UHRF1) and allosterically regulates its function. When not bound to PI(5)P, UHRF1 recognizes unmodified histone H3 tail, while PI(5)P-UHRF1 complex binds histone H3K9me3 (Gelato et al. 2014). Therefore, PI(5)P levels could regulate UHFR1 association with chromatin and heterochromatic state of the genome.

1.3.4 DNA transcription

Recent findings show that PI(4,5)P2 and PI(3,4,5)P3 can interact with steroidogenic factor 1 (SF-1) trough their acyl chains (Blind et al. 2012; Blind et al. 2014) and stabilize the tertiary structure of SF-1. SF-1 in a complex with PI(3,4,5)P3 displays significantly higher affinity for a coactivator peptide than in a complex with PI(4,5)P2 (Blind et al. 2014).

Therefore, the action of IMPK kinase or PTEN phosphatase can regulate SF-1 activity and SF-1 target genes expression (Blind et al. 2012).

Furthermore, PI(4,5)P2 interacts with RNA Pol II and RNA Pol I transcription machinery (Osborne et al. 2001; Toska et al. 2012; Sobol et al. 2013; Yildirim et al. 2013). PI(4,5)P2 forms a complex with the active form of RNA Pol II (Osborne et al. 2001; Toska et al. 2012). Yet, there is no evidence of a direct interaction between PI(4,5)P2 and RNA Pol II.

In comparison to RNA Pol II, the mode of PI(4,5)P2 action in RNA Pol I transcription is more understood. The upstream binding factor (UBF), which is recruited to the rDNA promoter and facilitates the initiation of RNA Pol I transcription (Bell et al. 1988), interacts with PI(4,5)P2. The interaction with PI(4,5)P2 enhances the binding of UBF to the rDNA promoter. Moreover, the depletion of PI(4,5)P2 from HeLa nuclear extract decreases the level of RNA Pol I transcription *in vitro* (Yildirim et al. 2013). These data suggest that PI(4,5)P2-UBF interaction might be required for association of the transcription initiation complex with rDNA and activation of RNA Pol I transcription.

2. AIMS

PIs and PIs-interacting proteins are regulators of essential nuclear processes. Yet, PIs nuclear functions are still poorly understood. Therefore we addressed these questions:

1) **Which PIs localize to the cell nucleus?**
2) **In which subnuclear domains are they localized?**
3) **What are the binding partners of PIs in the nucleus?**

Actin is a well-known PI(4,5)P2 interacting protein. The state of actin in the nucleus (monomeric, filamentous, alternative polymeric) is unclear. We addressed these questions:

4) **Can actin form filaments in the nucleus? Does the filament formation affect nuclear functions?**
5) **Is the localization of actin in the nucleus regulated by PI(4,5)P2?**

3. OVERVIEW OF USED METHODS

Tissue cultures, transfections
Cloning and standard molecular biology techniques
Cell fractionation
Fluorescence microscopy, Super-resolution microscopy
Electron microscopy and electron energy loss spectroscopy
Protein expression and purification
Co-immunoprecipitations and pull-downs
In vitro demethylation assay
SDS-PAGE and immunoblotting
RNA isolation and RT-qPCR

4. RESULTS

4.1 Nuclear PIs can be visualized by specific antibodies and PIs-binding domains.

Overexpressed EEA1-FYVE domain detects PI(3)P in nucleoli.

Overexpressed PIs-binding domains conjugated with GFP are widely used for detection of PIs in cellular membranes. We tested these domains for detection of PIs in the nucleus. Most of PIs-binding domains we tested display the same nuclear pattern as their mutant forms, which are not able to bind PIs. However, EEA1-FYVE domain specifically detected PI(3)P foci in nucleoli of U2OS cells.

Specific antibodies and purified PIs-interacting domains detect PI(4)P and PI(4,5)P2 in the nucleus.

As most of the overexpressed PIs-binding domains are not suitable for PIs detection in the nucleus, we prepared purified PLCδ1-PH, Tubby and OSH1-PH domains fused with eGFP, which recognize PI(4,5)P2 and PI(4)P, respectively. Using OSH1-PH and anti-PI(4)P antibody, we demonstrated for the first time that nuclear PI(4)P can be visualized in the cell nucleus. Using PLCδ1-PH and Tubby domains and anti-PI(4,5)P2 antibody we detected PI(4,5)P2 in nucleoli, nuclear speckles and in the nucleoplasm.

PI(4)P localizes to nuclear membrane, nuclear speckles and nucleoplasm and is associated with active chromatin.

We show that PI(4)P is present in nuclear membrane, it localizes to nuclear speckles and forms small nucleoplasmic foci. The pattern of PI(4)P in nuclear speckles is not homogenous, it forms small foci inside and at the edges of nuclear speckles, where the active transcription takes place (reviewed in Spector and Lamond 2011). The majority of nuclear PI(4)P localizes outside of nuclear speckles and most of nuclear PI(4)P is associated with chromatin. Moreover, small nucleoplasmic foci of PI(4)P partially colocalize with H3K4me2, the mark of active chromatin (Wang et al. 2014).

Nucleoplasmic PI(4,5)P2 forms small PI(4,5)P2 islets.

Nearly 30% of total nuclear PI(4,5)P2 detected by anti-PI(4,5)P2 antibody is located in foci outside of nuclear speckles and nucleoli. This nucleoplasmic PI(4,5)P2 forms 40-100 nm roundish structures, which we termed PI(4,5)P2 islets. They have carbon-rich interior and phosphorus- and nitrogen-rich exterior.

PI(4,5)P2 islets are associated with active transcription.

The majority of PI(4,5)P2 islets colocalizes with RNA and can be disturbed by RNase treatment. The active form of RNA Pol II and transcription machinery proteins as well as nascent RNA are associated with PI(4,5)P2 islets. Moreover, PI(4,5)P2 hydrolysis results in a significant decrease of transcription level.

4.2 PI(4,5)P2 binds NM1 in the nucleus and mediates association of NM1 with transcription machinery.

NM1 interacts with PI(4,5)P2 in the nucleus.

We demonstrate that NM1 interacts with PI(4,5)P2 in the nucleus through its C-terminal PH domain. This interaction anchors NM1 to bigger protein-lipid complexes. Moreover, we show that NM1 localizes to PI(4,5)P2 islets.

PI(4,5)P2 is required for the association of NM1 with transcription machinery.

It has been show before that the C-terminal part of NM1 is required for NM1-mediated chromatin remodelling and activation of transcription (Hofmann et al. 2006; Almuzzaini et al. 2015). We show that mutation of PI(4,5)P2 binding site results in loss of interaction with RNA Pol II and downregulation of transcription.

4.3 PI(4)P and PI(4,5)P2 interact with LSD1 and regulate its activity.

LSD1 is a general PIs interactor with the highest affinity for PI(4)P.

LSD1 demethylates H3K4me2 and H3K4me1 histone methylation marks and therefore represses transcription of its target genes (Shi et al. 2004). By direct binding assay, we show that LSD1 interacts with PI, PI(4)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3 but displays the highest affinity towards PI(4)P.

PI(4)P and PI(4,5)P2 regulate LSD1 activity.

The PIs binding site of LSD1 is located in the C-terminal catalytically active domain of LSD1. We demonstrate that PI(4)P binding inhibits LSD1 H3K4me2 demethylase activity *in vitro* whereas PI(4,5)P2 binding has a stimulatory effect on LSD1 activity.

4.4 Actin is able to form filaments in the cell nucleus.

NLS-actin forms nuclear actin bundles, which recruit cofilin and Arp3.

After over-expression of EYEP-NLS-actin (EN-actin), 1 to 5 % of cells formed long thick nuclear filaments, which in their length and thickness resembled cytoplasmic F-actin. The rest of the cells displayed diffused nuclear actin signal. Cofilin, actin-depolymerizing factor, and Arp3, a component of actin nucleation complex Arp2/3 colocalizes with nuclear actin filaments.

Actin filaments increase transcription in S-phase and block mitosis.

EN-filaments do not preferentially localize to either active or inactive chromatin. Although EN-actin does not colocalize with RNA polymerases, the level of transcription increases in S-phase in presence of EN-actin filaments, by unknown mechanism. Moreover, we observed that cells with EN-actin filaments do not undergo mitosis. These cells are often binucleic or generate micronuclei.

4. DISCUSSION

Nuclear PIs are important regulators of various nuclear processes (reviewed in Shah et al. 2013). To better understand their role in the nucleus, tools for nuclear PIs visualization both *in vivo* and *in vitro* are needed.

In order to detect nuclear PIs, we tested several antibodies and PIs-binding protein domains. Overexpressed EEA1-FYVE specifically detected PI(3)P foci in nucleoli of U2OS cells. In support of our data, PI(3)P has been previously detected in nucleoli of human fibroblasts and baby hamster kidney cells (Gillooly et al. 2000). However, the function of nucleolar PI(3)P is completely unknown. Moreover, we prepared purified PLCδ1-PH, Tubby and OSH1-PH domains fused with eGFP, which recognize PI(4,5)P2 and PI(4)P, respectively. We compared nuclear pattern of these domains with patterns of anti-PI(4,5)P2 and anti-PI(4)P antibodies.

Using OSH1-PH and anti-PI(4)P, we demonstrate that nuclear PI(4)P is present in nuclear membrane, it localizes to nuclear speckles and forms small nucleplasmic foci. The majority of nuclear PI(4)P is associated with chromatin. Moreover, small nucleoplasmic foci of PI(4)P partially colocalize with H3K4me2, the mark of active chromatin (Wang et al. 2014).

Since PI(4)P is present in nuclear speckles it could be implicated in splicing of newly transcribed pre-mRNA. The other possibility is that PI(4)P serves as a precursor for PI(4,5)P2. Enzymes, which convert PI(4,5)P2 to PI(4)P and *vice versa,* localize to nuclear speckles (Boronenkov et al. 1998; Déléris et al. 2003; Mellman et al. 2008; Schill and Anderson 2009; Elong Edimo et al. 2011) and therefore it is highly probable that an active metabolism of these PIs in nuclear speckles indeed occurs.

Using PLCδ1-PH and Tubby domains and anti-PI(4,5)P2 antibody we detected PI(4,5)P2 in nucleoli, nuclear speckles and nucleoplasm. We show that the nucleoplasmic pool forms nearly 30 % of detected nuclear PI(4,5)P2. This nucleoplasmic PI(4,5)P2 forms 40-100 nm large PI(4,5)P2 islets. They have carbon-rich interior and phosphorus- and nitrogen-rich exterior. Therefore, we suggest that the inner core of PI(4,5)P2 islets is composed of lipids while their outer surface is surrounded by proteins and nucleic acids.

The active form of RNA Pol II and transcription machinery proteins as well as nascent RNA are associated with PI(4,5)P2 islets. The majority of PI(4,5)P2 islets colocalizes with RNA and can be disturbed by RNase treatment. Therefore, we suggest that PI(4,5)P2 islets might serve as a scaffold for the arrangement of RNA Pol II transcription machinery. In agreement, PI(4,5)P2 hydrolysis results in a significant decrease of transcription level. Similarly, several studies demonstrated contribution of other lipid-based structures in transcription (Scassellati et al. 2010; Layerenza et al. 2013; Yoo et al. 2014).

Nuclear phosphoinositides associate with histones and other chromosomal proteins to regulate chromatin remodelling, DNA modifications and transcription (reviewed in Viiri et al. 2012). We describe two novel nuclear PIs interacting proteins - nuclear myosin 1 (NM1) and lysine-specific histone demethylase 1 (LSD1).

NM1 contains C-terminal PH domain that specifically binds PI(4,5)P2 and tethers NM1 to the plasma membrane (Hokanson and Ostap 2006; Hokanson et al. 2006). We show that NM1 interacts with PI(4,5)P2 also in the cell nucleus and this interaction anchors NM1 to bigger nuclear complexes. In the nucleus, NM1 is involved in chromatin remodelling and RNA Pol II transcription (Hofmann et al. 2006; Almuzzaini et al. 2015). Mutation of PI(4,5)P2 binding site results in loss of interaction with RNA Pol II and downregulation of transcription. As NM1 localizes to the surface of PI(4,5)P2 islets, we hypothesize that NM1 is sequestered to PI(4,5)P2 islets through the interactions with PI(4,5)P2. In such way, NM1 could recruit chromatin remodelling complexes to PI(4,5)P2 islets to create open chromatin structure and promote transcription.

We found that PI(4)P associates with chromatin and colocalizes with H3K4me2. Moreover, we show that PI(4)P interacts with LSD1, which demethylates H3K4me2 and H3K4me1 histone methylation marks and therefore represses transcription of its target genes (Shi et al. 2004). LSD1 interacts directly with PI, PI(4)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2

and PI(3,4,5)P3 but displays the highest affinity towards PI(4)P. As PI(4)P and PI(4,5)P2 are the most abundant PIs species (reviewed in Viaud et al. 2015), we studied how these two PIs regulate LSD1 activity. We demonstrate that PI(4)P binding inhibits LSD1 activity *in vitro* whereas PI(4,5)P2 binding has a stimulatory effect. Interestingly, LSD1 binds PI(4,5)P2 but not PI(4)P in nuclear lysates. Since LSD1 interacts with PI(4)P after overexpression, we hypothesize that PI(4)P binds to LSD1 only when downregulation of H3K4me2 demethylase activity is necessary. It has been shown that a single change in phosphorylation of inositol ring can regulate affinity of a PIs-interacting protein to its binding partners (Blind et al. 2012). Since the activity of LSD1 is highly dependent on its interacting partners (reviewed in Amente et al. 2013), a single phosphorylation of PI(4)P or dephosphorylation of PI(4,5)P2 could provide rapid and dynamic regulation of LSD1 function also *in vivo*.

In the cytoplasm, actin is present in two different forms - as monomeric globular actin (G-actin), which can polymerize and form actin filaments (F-actin). In the nucleus, actin is present in monomeric form (reviewed in Jockusch et al. 2006). Initially, it was uncertain whether actin polymerization can occur in the nucleus. Eventually, several studies reported F-actin formation in the nucleus (Miyamoto et al. 2011; Belin et al. 2013; Baarlink et al. 2013; Kokai et al. 2014). We investigated whether nuclear actin filaments recruit actin-binding proteins and affect nuclear processes. After over-expression of EYEP-NLS-actin (EN-actin), 1 to 5 % of cells formed long thick nuclear filaments, which in their length and thickness resembled cytoplasmic F-actin. The rest of the cells displayed diffused nuclear actin signal. We show that cofilin, actin-depolymerizing factor, and Arp3, a component of actin nucleation complex Arp2/3 colocalizes with nuclear actin filaments. EN-filaments do not preferentially localize to either active or inactive chromatin. Although EN-actin does not colocalize with RNA polymerases, the level of transcription increases in S-phase in presence of EN-actin filaments. In support of this finding, earlier studies reported increased transcription in presence of polymeric actin in the nucleus (Ye et al. 2008; Baarlink et al. 2013; Kokai et al. 2014).

The formation of EN-actin filaments decreases cell proliferation rate and increases incidence of formation of additional micronuclei or retention of both daughter nuclei within one cell. Similar results were reported previously as a consequence of cytoplasmic F-actin assembly (Moulding et al. 2007). However, it could be also a result of aberrant chromosome segregation (reviewed in Fenech et al. 2011). Since EN-actin polymerization, its behaviour during the cell cycle, colocalization with actin binding proteins and transcriptional activity are in agreement with previous studies, we suggest that EN-actin could be used as a tool in future research regarding actin functions in the nucleus.

5. SUMMARY AND CONCLUSIONS

5.1 PI(4,5)P2 islets are associated with active transcription

PI(4,5)P2 forms 40-100 nm large structures in the nucleoplasm, which we call PI(4,5)P2 islets. Their interior is composed of carbon rich structures, probably lipids. They are

surrounded by proteins and nucleic acids. PI(4,5)P2 islets surface is associated with active form of RNA polymerase II, transcription machinery proteins and nascent RNA.

5.2 NM1 interacts with PI(4,5)P2 in the nucleus

NM1 interacts with PI(4,5)P2 in the nucleus through its PH domain. PI(4,5)P2 anchors NM1 to PI(4,5)P2 islets and is essential for NM1 interaction with transcription machinery. Mutation of PI(4,5)P2 binding site results in loss of interaction with RNA Pol II and decreased transcription.

5.3 PI(4)P localizes to nucleus and is associated with chromatin

In the nucleus, PI(4)P is present in the nuclear membrane, nuclear speckles and small nucleoplasmic foci. The majority of nuclear PI(4)P is associated with chromatin and colocalizes with H3K4me2, a mark of active chromatin.

5.4 LSD1 is regulated by nuclear phosphoinositides

LSD1 binds directly to both PI(4)P and PI(4,5)P2. While the interaction with PI(4)P leads to inhibition of LSD1, the interaction with PI(4,5)P2 stimulates LSD1 H3K4me2 demethylase activity.

5.5 Nuclear actin filaments alter cellular behaviour

After overexpression of nuclear targeted EN-actin, 1-5 % of cells display formation of nuclear actin filaments. These filaments resemble cytoplasmic F-actin and recruit cofilin and Arp3 actin binding proteins. Formation of actin filaments in the nucleus results in increased transcription in S-phase, decreased cell proliferation and aberrant mitosis.

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10. RESEARCH PAPERS PRESENTED WITHIN THE THESIS

Tools for visualization of phosphoinositides in the cell nucleus
Kalasova I, Fáberová V, Kalendová A, Yildirim S, Uličná L, Venit T and Hozák P
Histochem Cell Biol. 2016 Feb 4 [Epub ahead of print]. doi: 10.1007/s00418-016-1409-8.
IF: 3.054 (2014)
I. K. designed and performed experiments (DNA cloning, DNA mutagenesis, fluorescence microscopy, protein expression and purification) and wrote the manuscript.

Nuclear phosphatidylinositol 4,5-bisphosphate islets contribute to efficient DNA transcription
Sobol M, Kalendová A, Yildirim S, Philimonenko V, Marášek P, Kalasová I, Pastorek, Hozák P
Manuscript.
I.K. performed experiments (pull-down assay and western blotting).

Chromatin associated PI(4)P regulates lysine-specific histone demethylase 1
Kalasova I, Kalendová A, Fáberová V, Marášek P, Uličná L, Vacík T and Hozák P
Manuscript.
I.K. designed and performed most of the experiments (DNA cloning, DNA mutagenesis, protein expression and purification, cellular fractionations, pull-down assays, fluorescence microscopy, western blotting, demethylation assays, qPCR) and wrote the manuscript.

Nuclear actin filaments recruit cofilin and Arp3 and their formation is connected with a mitotic block
Kalendová A, Kalasová I, Yamazaki S, Uličná L, Harata M and Hozák P
Histochem Cell Biol. 2014 Aug;142(2):139-52. doi: 10.1007/s00418-014-1243-9. Epub 2014 Jul 8.
IF: 3.054 (2014)
I. K. performed experiments (fluorescence microscopy)

11. CV

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