# CHARLES UNIVERSITY

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## Developmental and Cell Biology

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## The role of Nuclear Phosphatidylinositol 4,5-bisphosphate in RNA Polymerase II Transcription

Doctoral Thesis

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## DECLARATION

I hereby declare that I wrote this work independently and that I did my best to acknowledge all people and literature. I did not use this work or a substantial part of it to obtain another academic degree or equivalent.

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## ACKNOWLEDGEMENT

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# List of Abbreviations

| | |
|---|---|
| 5-FU | 5-Fluorouridine |
| 5HT | 5-hydroxytryptamine |
| AKT | Protein Kinase B |
| ALK1 | Activin A receptor like type 1 |
| ARP2/3 | Actin Related Protein 2/3 complex |
| ArPIKfyve | Associated Regulator of PIKfyve |
| B23 | Nucleophosmin |
| BAF | BRG1/BRM-associated factor |
| BASP1 | Brain acid soluble protein 1 |
| BRD4 | Bromodomain-containing protein 4 |
| Btk | Bruton tyrosine kinase |
| CC | Coiled-coil |
| CDK9 | Cyclin-Dependent Kinase 9 |
| Chip-Seq | Chromatin immunoprecipitation Sequencing |
| CHMP3 | Charged Multivesicular Body Protein 3 |
| CTD | Carboxy terminal domain |
| Cul3-SPOP | Cullin3 - Speckle-type POZ protein |
| DAG | Diacylglycerol |
| DDR1 | Discoidin domain receptor 1 |
| DSIF | DRB-sensitivity inducing factor |
| EBP1 | ErbB3-binding protein 1 |
| EGF | Epidermal growth factor |
| EHD | Eps15 Homology Domain |
| ENTH/ANTH | Epsin n-terminal homology/ AP180 N-terminal homology |
| ER | Endoplasmic reticulum |
| f1, 2 | Fragment 1, 2 |
| FABPs | Fatty Acid-Binding Proteins |
| F-actin | Filamentous actin |
| FAK | Focal Adhesion Kinase |
| FERM | 4.1 protein, Ezrin, Radixin and Moesin |
| FRAP | Fluorescence recovery after photobleaching |
| FYVE | Fab1p, YOTB, Vac1p, and EEA1 |
| GFP | Green Fluorescent Protein |

| | |
|---|---|
| GOCC | Gene ontology cellular component |
| GPL | Glycerophospholipids |
| GTFs | General Transcription Factors |
| H3K9meX | X-methylated lysine 9 at histone H3 |
| HDAC1 | Histone deacetylase 1 |
| hnRNPs | Heterogeneous nuclear ribonucleoproteins |
| IDR | Intrinsically disordered regions |
| ING2 | Inhibitor of Growth Family Member 2 |
| INPP4B | Inositol polyphosphate 4-phosphatase type II |
| INPP5 | Inositol polyphosphate-5-phosphatase E |
| IPMK | Inositol polyphosphate multikinase |
| JNK1 | Mitogen-activated protein kinase 8 |
| LIMK | LIN-11, Isl-1 and MEC-3 Kinase |
| LINK-A | Long intergenic non-coding RNA for kinase activation |
| LiP-MS | Limited Proteolysis-Coupled Mass Spectrometry |
| LLPS | Liquid-liquid phase separation |
| LRH-1 | Liver Receptor Homolog-1 |
| MAL | Myelin and lymphocyte protein |
| MARCKS | Myristoylated Alanine Rich C-Kinase Substrate |
| mDia | Diaphanous-related formin |
| Med1 | Mediator of RNA polymerase II transcription subunit 1 |
| MLCP | Myosin Light Chain Phosphatase |
| MPRIP | Myosin Phosphatase Rho-Interacting Protein |
| MRTF-A | Myocardin-related transcription factor A |
| mTORC1,2 | mammalian target of rapamycin complex 1,2 |
| MVI | Myosin VI |
| MYPT1 | Myosin Phosphatase Target Subunit 1 |
| NDP52 | Nuclear domain 10 protein 52 |
| NELF | Negative elongation factor |
| NES | Nuclear Export Signal |
| NGF | Nerve growth factor |
| nLDs | Nuclear lipid droplets |
| NLIs | Nuclear Lipid Islets |
| NLS | Nuclear Localization Signal |

| | |
|---|---|
| NM1 | Nuclear myosin 1 |
| NOR | Nucleolus organizer region |
| NTPs | Nucleoside triphosphates |
| OSH1 | Oxysterol-binding protein homolog 1 |
| p53 | Protein 53 |
| Paf1C | Polymerase-associated factor 1 complex |
| PC | Phosphatidylcholine |
| PCG | Procyanidin C-13,3',3"-tri-O-gallate |
| PDGF | Platelet-derived growth factor |
| PDK1 | Phosphoinositide-dependent kinase-1 |
| PH | Pleckstrin Homology |
| PHD | Plant homeodomain |
| PHF8 | PHD Finger Protein 8 |
| PI(3)P | Phosphatidylinositol 3-phosphate |
| PI(3,4)P2 | Phosphatidylinositol 3,4-bisphosphate |
| PI(3,4,5)P3 | Phosphatidylinositol 3,4,5-trisphosphate |
| PI(3,5)P2 | Phosphatidylinositol 3,5-bisphosphate |
| PI(4)P | Phosphatidylinositol 4-phosphate |
| PI(4,5)P2 | Phosphatidylinositol 4,5-bisphosphate |
| PI(5)P | Phosphatidylinositol 5-phosphate |
| PI3KC2α | Phosphatidylinositol 3-kinase type IIα |
| PI3K | Phosphoinositide 3-kinase |
| PI3KC2β | Phosphatidylinositol 3-kinase type IIβ |
| PI4Kα | Phosphatidylinositol 4-kinase α |
| PI4Kβ | Phosphatidylinositol 4-kinase β |
| PI | Phosphatidylinositol |
| PIC | Pre-initiation complex |
| PIKfyve | 1-phosphatidylinositol 3-phosphate 5-kinase |
| PIP4KIIα | Phosphatidylinositol 5-phosphate 4-kinase type IIα |
| PIP4KIIβ | phosphatidylinositol 5-phosphate 4-kinase type IIβ |
| PIP4KIIγ | Phosphatidylinositol 5-phosphate 4-kinase type IIγ |
| PIP5KIα | Phosphatidylinositol 4-phosphate 5-kinase type Iα |
| PIP5KIγ_i4 | Phosphatidylinositol 4-phosphate 5-kinase type |
| PI(5)P4Kγ | Phosphatidylinositol-5-phosphate 4-kinase, type II γ |

| | |
|---|---|
| PIPs | Phosphoinositide phosphates |
| PIS | Phosphatidylinositol synthase |
| PITPs | Phosphatidylinositol transfer proteins |
| PLC | Phospholipase C |
| PML | Promyelocytic leukemia protein |
| PM | Plasma membrane |
| PP1 | Protein Phosphatase 1 |
| PPARs | Peroxisome proliferator-activated receptors, PPARs |
| PROPPIN | β-propellers that bind polyphosphoinositides |
| PTB | Phosphotyrosine binding |
| P-TEFb | Positive transcription elongation factor b |
| PTEN | Phosphatase and tensin homolog |
| PX | Phox homology |
| RhoA | Ras homolog family member A |
| RNAPII | RNA polymerase II |
| RPB1 | DNA-directed RNA polymerase II subunit rpb1 |
| Sac1 | Phosphatidylinositol-3-phosphatase, *Saccharomyces cerevisiae* |
| SAP30L | Co-repressor sin3A-associated protein 30-like |
| SC-35 | Serine/arginine-rich splicing factor 2 |
| Ser2P-CTD | Serine 2 phosphorylated CTD of RNAPII |
| Ser5P-CTD | Serine 5 phosphorylated CTD of RNAPII |
| SHIP1-2 | Src homology 2 domain-containing inositol phosphate phosphatase |
| SNX1, 2 | Sorting nexins 1,2 |
| SRF | Serum response factor |
| SRRM2 | Serine/arginine repetitive matrix protein 2 |
| Star-PAP | Speckle targeted PIPKI regulated-poly(A) polymerase |
| STED | Stimulated emission depletion |
| STF-1 | Steroidogenic factor 1 |
| SWI/SNF | Switching and sucrose non-fermenting complex |
| TAF3 | TFIID subunit 3 |
| TFIID | Transcription Factor II D |
| TGN | Trans-Golgi Network |
| TNF | Tumor necrosis factor |
| TRAF | Tumor necrosis factor receptor (TNFR) associated factors |

| | |
|---|---|
| Tyr1P-CTD | Tyrosine 1 phosphorylated CTD of RNAPII |
| UBF | Upstream binding factor |
| UHRF1 | Ubiquitin-like, containing PHD and RING finger domains 1 |
| WASP | Wiskott–Aldrich Syndrome protein |
| WIPI1,2 | WD repeat domain phosphoinositide-interacting protein 1,2 |
| Xrn2 5'–3' exonuclease | 5'-3' Exoribonuclease 2 |

# Abstract (in English)

The phosphatidylinositols are a subclass of glycerophospholipids with their inositol head group linked to the diacylglycerol backbone. The differential phosphorylation of the inositol head group yields seven different phosphoinositide phosphates (PIPs) which can be mono-, bis,- or tris-phosphorylated. The roles of the cytoplasmic PIPs have been extensively studied in vesicular trafficking, ion channels, generating second messengers and, membrane and cytoskeletal dynamics. While their cytoplasmic functions are very well described, the molecular mechanism of their nuclear functions are still poorly understood.

From the nuclear PIPs, the Phosphatidylinositol 4,5-bisphosphate (PIP2) is the most abundant phosphoinositide in the cell nucleus and it participates to the nuclear architecture by regulating processes such as chromatin remodeling, DNA-damage response and gene expression. In the cell nucleus, it localizes mostly to nuclear speckles where it interacts with the splicing machinery. In nucleolus, PIP2 is involved in the RNA Polymerase I machinery to regulate rDNA transcription. Recently, we have defined a nucleoplasmic pool of PIP2 which is observed in 40 to 100nm foci. The nascent transcripts of RNA Polymerase II (RNAPII) were visualized at their periphery and RNA was shown to be essential for their integrity. In this study, we sought to examine the role of PIP2 in the RNAPII transcription machinery.

The RNAPII transcription cycle is generally divided into four steps; Initiation, promoter-proximal pausing, elongation, and termination. The specific post-translational modifications of the C-terminal domain (CTD) of RNAPII correlates with the stages of the RNAPII transcription and coins the term "CTD code". The phosphorylation of the Serine5 residues of this domain associates with the initiation condensates which are formed through liquid–liquid phase separation (LLPS). The Tyrosine1 phosphorylation of the CTD occurs during the promoter-proximal pausing and marks the release of the RNAPII from the initiation condensate. This process is essential to trigger the release of RNAPII, as the subsequent Serine2 phosphorylation of the CTD abolishes the affinity of RNAPII for the initiation condensate.

With this study, we have identified the nuclear interactome of PIP2 and defined the processes that are associated with PIP2-effectors. The Myosin Phosphatase Rho-Interacting Protein (MPRIP) was revealed as a promising effector to mediate PIP2-associated RNAPII transcription. Accordingly, we have identified MPRIP in the same complex with RNAPII and NMI. By using super-resolution microscopy, we have localized MPRIP at nuclear PIP2-containing structures. In the overexpression experiments, MPRIP shows LLPS characteristics in nucleus and

exhibits a novel phase behavior that points to a regulatory function regarding nuclear actin polymerization. Furthermore, we show that the depletion of MPRIP promotes the formation of the initiation condensates and increases the association of Ser5P-CTD (Serine 5 phosphorylated CTD) with PIP2. It is then revealed that MPRIP mediates the association between Tyr1P-CTD and PIP2. Finally, we show that MPRIP determines the localization of the Tyr1P-CTD to nuclear speckles and NLIs. Overall, our data shed light on the role of PIP2 in RNAPII transcription through identifying a novel transcription regulator that defines the association between Tyr1P-CTD and PIP2.

# Abstrakt (Česky)

Fosfatidylinositoly jsou podtřídou glycerofosfolipidů s jejich inositolovou hlavní skupinou spojenou s diacylglycerolovou kostrou. Diferenciální fosforylace inositolové hlavní skupiny poskytuje sedm různých fosfoinositidfosfátů (PIP), které mohou být mono-, bi- nebo tri-fosforylované. Role cytoplazmatických PIP byly rozsáhle studovány ve vezikulárním transportu, iontových kanálech, transportérech, generujících druhé posly a membránové a cytoskeletální dynamice. Zatímco jejich cytoplazmatické funkce jsou velmi dobře popsány, molekulární mechanismus jejich jaderných funkcí je stále málo pochopen.

Z jaderných PIP je fosfatidylinositol 4,5-bisfosfát (PIP2) nejhojnějším fosfoinozitidem v buněčném jádru a podílí se na vytváření jaderné architektury regulací procesů, jako je remodelace chromatinu, reakce na poškození DNA a genová exprese. V buněčném jádře se lokalizuje většinou do jaderných skvrn, kde interaguje se sestřihovým apárátemmRNA. V jadérku je PIP2 zapojen do mechanismu regulace transkripce rDAN RNA polymerázou I. Nedávno jsme definovali nukleoplazmatický pool PIP2, který je pozorován v ostrůvcích o průměru 40 - 100 nm (NLI). Vznikající transkripty RNA polymerázy II (RNAPII) byly vizualizovány na jejich periferii a ukázalo se, že RNA je nezbytná pro jejich integritu. V této studii jsme se snažili prozkoumat roli PIP2 v transkripčním aparátu RNAPII.

Transkripční cyklus RNAPII je obecně rozdělen do čtyř kroků; Iniciace, proximální pauza promotoru, elongace a ukončení. Specifické posttranslační modifikace C-terminální domény (CTD) RNAPII koreluje se stádii transkripce RNAPII a vytváří termín „CTD kód". Fosforylace zbytků Serinu5 této domény je spojena s iniciačními kondenzáty, které se tvoří separací kapalina-kapalina (LLPS). Fosforylace tyrosinu1 CTD nastává během proximální pauzy promotoru a označuje uvolnění RNAPII z iniciačního kondenzátu. Tento proces je nezbytný pro spuštění uvolňování RNAPII, protože následná fosforylace Serine2 CTD ruší afinitu RNAPII k iniciačnímu kondenzátu.

Pomocí této studie jsme identifikovali jaderný interaktom PIP2 a definovali procesy, které jsou spojeny s některými PIP2-efektory. Myosin Phosphatase Rho-Interacting Protein (MPRIP) byl odhalen jako slibný efektor pro zprostředkování PIP2-asociované RNAPII transkripce. V souladu s tím jsme identifikovali MPRIP ve stejném komplexu s RNAPII a NMI. Pomocí mikroskopie s vysokým rozlišením jsme lokalizovali MPRIP na jaderné struktury obsahující PIP2. V experimentech s vysokou expresí vykazuje MPRIP charakteristiky LLPS v jádře a vykazuje nové fázové chování, které ukazuje na regulační funkci týkající se polymerace jaderného aktinu. Dále

jsme ukázali, že vyčerpání MPRIP podporuje tvorbu iniciačních kondenzátů RNA polymerázy II a zvyšuje asociaci Ser5P-CTD (serin 5 fosforylovaný CTD) s PIP2. Poté se ukázalo, že MPRIP zprostředkovává spojení mezi Tyr1P-CTD a PIP2. Nakonec jsme ukázali, že MPRIP určuje lokalizaci Tyr1P-CTD na jaderné skvrny a NLI. V úhrnu naše data poskytují nový pohled na roli PIP2 v transkripci RNAPII prostřednictvím nového regulátoru transkripce, který definuje spojení mezi Tyr1P-CTD a PIP2.

# 1. INTRODUCTION

## 1.1. Phospholipids

The lipids in mammalian cells can be classified into three main groups: Triglycerides, Phospholipids, and Sterols. Triglycerides are non-polar lipids that travel in the bloodstream by binding to the lipoproteins. They are the energy sources taken from food and reserved in the adipose cells (Alberts *et al.,* 2002). The phospholipids and sterols, on the other hand, have their own metabolisms in mammalian cells and are structurally important molecules constituting cell and organelle membranes (Vance, 2015; Maxfield and Mondal, 2006). In each membrane, the lipid composition is different, also from the inner to the outer layer, favoring the functions of the surrounded compartment (Alberts *et al.,* 2002).

The phospholipids are divided into two subclasses: Glycerophospholipids, and Sphingolipids. In general, lipids are classified not by their function but by their structure. This structural classification is based on the backbone of the molecule where each class is diversified by various head groups, chain modifications, and bonding types (ester, ether, vinyl-ether) (Figure 1, Harayama and Riezman, 2018). The glycerophospholipids and sphingolipids have similar structures with hydrophobic fatty acid chains and polar head groups. Similarly, the cholesterols (as most common sterols in mammals) carry a polar hydroxyl head group but it is fused to a much more rigid backbone composed of four steroid rings and a hydrocarbon tail. These essential lipids with amphipathic characteristics regulate cellular processes such as signaling, vesicle trafficking, and membrane fluidity (reviewed in Harayama and Riezman, 2018).
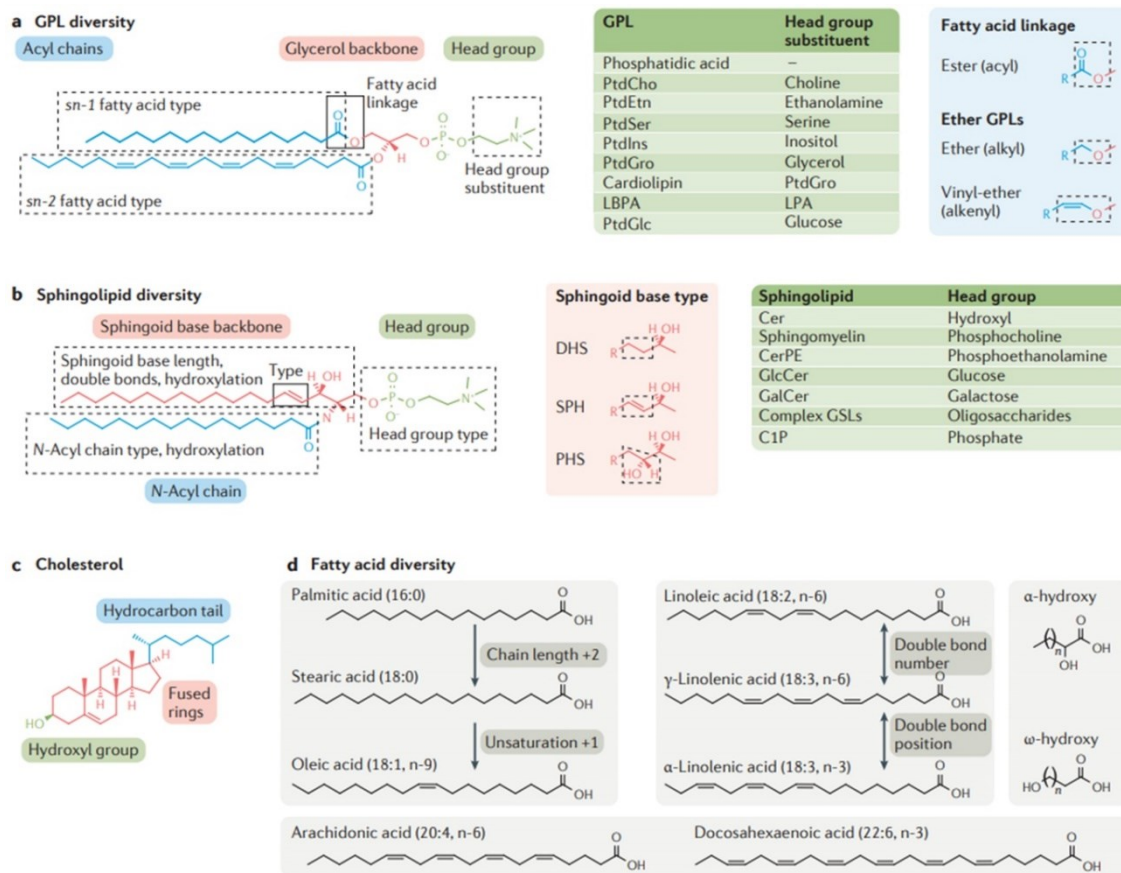
**Figure 1:** Chemical diversity of membrane lipids in mammals. **a**- Glycerophospholipids (GPLs) have a glycerol backbone with fatty acids at the sn-1 and sn-2 positions. The head group consists of a phosphate and an alcohol, which defines the GPL name. Ether-GPLs are GPLs with ether or vinyl-ether linkage at the sn-1 position. Boxed parts of the GPL structure represent building blocks that confer diversity (same for **b**). Cardiolipin and lysobisphosphatidic acid have acyl chains in their glycerol head group substituent; thus, they cannot be accommodated by the illustrated structure. B- Sphingolipids consist of a sphingoid base (which is simultaneously the backbone and a hydrophobic tail), an N-acyl chain and a head group. Hydroxylation and unsaturation define the sphingoid base type, whereas the head group defines the sphingolipid name. **c**- The major mammalian sterol, cholesterol. D- Fatty acids differ in chain length, level of unsaturation and the position of double bonds, illustrated as (XX:Y, n-Z), where XX, Y and Z are carbon number, double bond number and the position of the first double bond from the *omega end*, respectively. Fatty acids can be hydroxylated (Harayama and Riezman 2018).

## 1.2. Phosphoinositide Phosphates

The phosphatidylinositols are a subclass of glycerophospholipids with the inositol head group linked to the diacylglycerol (DAG) backbone, having a fatty acid composition of 18:0, 20:4

(Figure 1A, D). Inositol is a cyclohexane sugar alcohol where all six carbon atoms carry one hydroxyl group (Figure 2). The D-myo-inositol head group is linked to the DAG backbone with a phosphodiester bond and can be further phosphorylated on positions 3, 4, and 5 resulting in seven distinct phosphoinositide phosphates (PIPs) (Balla, 2013). These are three monophosphorylated (PI[3]P, PI[4]P, and PI[5]P), three diphosphorylated (PI[3,4]P2, PI[3,5]P2, and PI[4,5]P2), and one triphosphorylated PIPs (PI[3,4,5]P3) (Balla, 2013). The interactome of all seven PIPs is identified by using high-resolution quantitative mass-spectrometry which is utilized as a repertory by the scientific community (Jungmichel *et al.,* in 2014).
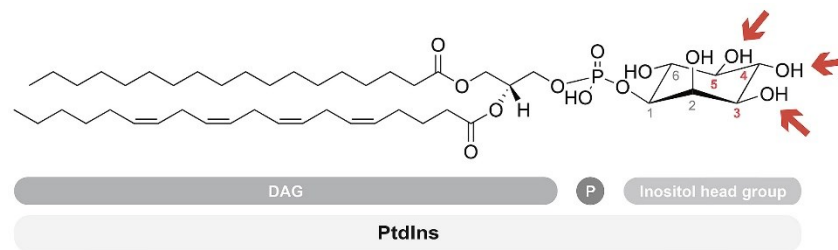


**Figure 2:** Nuclear polyphosphoinositide metabolism. Phosphatidylinositol (PtdIns) structure (18:0/20:4 [5Z,8Z,11Z, 14Z]). Hydroxyl groups that are sites of phosphorylation on the inositol head group are indicated with arrows and carbon atom numbers are shown in red. The phosphorylations of the inositol head by these three sites gives rise to seven different PIP species (Jacobsen *et al.,* 2019).

### 1.2.1. PIP binding Domains and Motifs

The tools to study lipids are limited since their hydrophobic acyl chains are embedded in the membrane and the inositol head group is the only exposed region that can be targeted. Therefore, the protein domains that interact with the inositol group are commonly used to study PIPs. The domains that show high affinity and reliable specificity are used as biosensors to detect, localize and precipitate the PIPs (Jungmichel *et al.,* 2014; Ulicna *et al.,* 2018). The PH (Pleckstrin Homology) domains are one of the most common domains that are used as tools to study PIPs. They have similar structures but low sequence conservation, therefore the PH domains of different proteins can detect different PIPs (Singh *et al.,* 2021). For example, the PH domain of Phospholipase C-delta (PLC-δ) interacts specifically with PI(4,5)P2, the PH domain of the Protein Kinase B (AKT1) interacts with PI(3,4,5)P3, and the PH domain of OSH1 interacts with PI(4)P (Singh *et al.,* 2021; Shin *et al.,* 2020). According to recent work, almost half of the proteins carrying the PH domain do not bind to any PIPs and suggests that PH-PIP interaction should be assessed in full-length protein (Singh *et al.,* 2021).

There are many other structured PIP interacting domains such as; FYVE, ENTH/ANTH (epsin n-terminal homology), PX (Phox homology), FERM, PROPPIN, TRAF, PHD finger domain, and many more (Choy *et al.,* 2017; Hammond and Balla, 2015; Kutateladze, 2010; Pemberton and Balla, 2019). The amino acid sequences of these domains commonly contain several basic amino acids carrying positive charges (mostly, K/R) that interact with the negatively charged phosphate groups on the inositol ring. Similarly, unstructured amino acid sequences can also interact with PIPs if they contain short stretches of basic amino acids such as K/R motifs (Hammond and Balla, 2015; Pemberton and Balla, 2019). In both cases, the effector proteins interact with PIPs through electrostatic interactions which regulate important functions in the cytoplasm such as controlling membrane dynamics, initiation of signaling responses, and modulating ion channels (Pemberton and Balla, 2019; Harraz *et al.,* 2020).

### 1.2.2. Phosphotidylinositol Synthesis and Export

The PIP metabolism begins with the synthesis of Phosphatidylinositol (PI) at the endoplasmic reticulum where the phospholipid composition is in constant flux due to the export of the lipids to other organelles and plasma membrane (PM). The PI synthase (PIS) is an enzyme of the endoplasmic reticulum (ER) which catalyzes the D-myo-inositol head group to cytidine diphosphate-activated DAG backbone in a single step (Pemberton and Balla, 2019). The tubular ER network extends to all organelles interfacing their membranes including PM where the lipids are exchanged by specific protein domains, and autophagosomes and peroxisome formations (reviewed in Cockcroft and Raghu, 2018). For example, the Phosphatidylinositol transfer proteins (PITPs) are shown to translocate PIs to PM and Golgi and the autophagosomes formations are initiated from the PIS-enriched subdomains of ER where PIs are ferried in vesicles (Kim *et al.,* 2011; Roulin *et al.,* 2014; Yadav *et al.,* 2015; Nishimura *et al.,* 2017; Bankaitis and Grabon, 2011).

### 1.2.3. PI(3)P

The spatiotemporal phosphorylation of the PIs and the interconversion of the PIPs are tightly regulated by specific kinase and phosphatases. PI(3)P synthesis takes place in distinct intracellular membranes such as endosomes, autophagosomes and ER (Steve Jean 2014). It is found predominantly in early endosomes and it is generated by the class II and III phosphoinositide-3-kinases (PI3KC2,3) and also by dephosphorylation of PI(3,4)P2 by Inositol polyphosphate 4-phosphatase type II (Gewinner *et al.,* 2009). It contributes to the preassembly and activity of the autophagosomes through binding to WIPI2 via proppins domain (Baskaran *et al.,* 2012). In endosomes, PI(3)P effector proteins comprise the PX and FYVE domains for the

interaction (Misra *et al.,* 2001). Recently, PI(3)P generation was shown at the primary cilium mediated autophagy (Boukhalfa *et al.,* 2020).

### 1.2.4. PI(4)P

Phosphorylation of PI by PI4–kinases produces PI(4)P which is present in PM and the membranes of Golgi and some secretory vesicles (Balla and Balla, 2006; Balla *et al.,* 2008). In Golgi, PI(4)P is required for sphingolipid biosynthesis and vesicle-mediated trafficking (Graham *et al.,* 2011). Its turnover is mediated by translocation to ER where it is dephosphorylated back to PI by Sac1 (Roulin *et al.,* 2014). It is used as a substrate in PM to generate PI(4,5)P2 by the activity of PI(4)P 5-Kinase (PIP5K) (Hammond *et al.,* 2012). Recently, Golgi-derived PI(4)P-containing vesicles were shown to drive late steps of mitochondrial division (Nagashima *et al.,* 2020). In trans-Golgi Network (TGN), the head group of the PI(4)P was shown to protonate upon glucose-dependent changes in intracellular pH which mediates the recruitment of PI(4)P effector Osh1 to TGN to regulate the cargo sorting (Shin *et al.,* 2020).

### 1.2.5. PI(5)P and PI(3,5)P2

PI(5)P have a similar abundance as PI(3)P, both representing only 0.5% of the PI pool of the mammalian cell (Zolov *et al.*, 2012; McCartney *et al.*, 2014). The exact pathway of PI(5)P generation is still elusive, but two models were proposed so far; A direct model suggests that PI(5)P is generated from PI by kinase activity of PIKfyve (Sbrissa *et al.,* 1999, 2002; Shisheva *et al.,* 2001, 2012, 2013), and the indirect model suggest dephosphorylation of PI(3,5)P2 by 3' phosphatases named myotubularins (Vaccari *et al.,* 2011; Oppelt *et al.,* 2012; Zolov *et al.,* 2012). The PI(5)P was detected at PM, ER, and Golgi (Sarkes and Rameh, 2010) as well as in early endosomes to inhibit the maturation of the endosome in case of the introduction of the virulence factor IpgD (Ramel *et al.,* 2011; Boal *et al.,* 2015). Moreover, a study reported to localize PI(5)P in autophagosomes to activate autophagy. They show that the depletion of the kinase, PI(5)P4Kγ, which generates PIP2 by phosphorylation of PI(5)P, enhanced the autophagy activity of the cell which increased the clearance of the disease-associated aggregate-prone intracytoplasmic proteins. They suggest that by autophagy induction the neurodegenerative *d*iseases, like Huntington's and Parkinson's can be targeted with specific PI(5)P4Kγ inhibition (Vicinanza *et al.,* 2015).

PI(3,5)P2 is one of the least abundant PIPs in the mammalian cell, making up only 0.05 to 0.1% of the total PI pool. It is also the least studied PIP with only a few known physiological functions. The acute elevation of PI(3,5)P2 in mammalian cells, upon insulin, and growth factor stimulations, suggests that it functions as a signaling molecule in cell homeostasis (Sbrissa *et al.,* 1999; Bridges *et al.,* 2012). The PIKfyve kinase that generates PI(3,5)P2 from PI(3)P is regulated

by Vac14 (Schulze *et al.,* 2014) and Fig4 (Edgar *et al.,* 2020) at early and late endosomes and lysosomes (Bharadwaj *et al.,* 2016). The inhibition of PIKfyve led to abnormal, enlarged endo-lysosomal compartments in mammalian cells and mouse tissues (Choy *et al.,* 2018; Saffi *et al.,* 2021; Min *et al.,* 2014, 2019). Similarly, CHMP3 also binds selectively to PI(3,5)P2 but it is not yet clear how PI(3,5)P2 regulates endosomal cargo sorting (Whitley *et al.,* 2003). Moreover, PI(3,5)P2 was shown to regulate retrograde traffic from early endosomes to the trans-Golgi network by direct binding to its effectors SNX1 and SNX2 (Carlton *et al.,* 2004; Rutherford *et al.,* 2006). Although the PROPPIN domains of WIPI 1,2,3 and 4 were shown to bind to PI(3,5)P2 with high affinity, it is not clear if these domains regulate their levels in mammalian cells (Baskaran *et al.,* 2012).

Our current knowledge is inadequate to understand the distinct roles of PI(5)P and PI(3,5)P2 in mammals. The active role of PIKfyve seems to be critical since the hypomorphic mutation was shown to exhibit neonatal lethality in mice (Ikonomov *et al.,* 2011). The mouse mutants with depleted ArPIKfyve and Fig4 showed a 50% reduction in the levels of PI(3,5)P2 and exhibited profound neurodegeneration (Jin *et al.,* 2008; Zhang *et al.,* 2007; Chow *et al.,* 2007). These observations suggest that PI(5)P and PI(3,5)P2 are crucial to maintain the functions of the nervous system (Hasegawa *et al.,* 2017).

### 1.2.6.  PI(3,4)P2 and PIP3

Cell growth and mobility is regulated by the class I phosphatidylinositol 3-kinase (PI3K)-signaling pathway which is heavily mutated in cancer (Fruman and Rommel, 2014; Mayer and Arteaga, 2016; Okkenhaug *et al.,* 2016; Thorpe *et al.,* 2015). In the PM, the growth factors stimulate PI3K to phosphorylate PIP2 from the 3' position of the inositol producing PIP3. Upon binding to PIP3 through its PH domain, Akt1 is phosphorylated and activated by the phosphoinositide-dependent kinase-1 (PDK1) and mTORC2. This signaling pathway indirectly promote anabolic growth and survival through mTORC1 complex (Dibble and Cantley, 2015; Engelman *et al.,* 2006).

The PIP3 is subsequently converted to PI(3,4)P2 by Src homology 2 domain-containing inositol phosphate phosphatase 2 (SHIP2) and INPP5 (Vanhaesebroe*c*k and Hawkins, 2012). A recent study determined the local concentration changes of PIP2, PIP3 and PI(3,4)P2 in PM by microinjection of PH domain carrying (from TAPP1), engineered sensor proteins: Upon the platelet-derived growth factor (PDGF)-induced stimulation, 0.45 mole% of the initial 1mole% of PIP2 was converted to PIP3 which then generated 0.18mole% of PI(3,4)P2 by the activity of SHIP2. The PH domain of an Akt1 isoform, Akt2 recognizes the PI(3,4P)2 and is activated by the same kinases that activates Akt1; PDK1 and mTORC2. The PI(3,4)P2 is selectively recruited to

early endosome membranes where it is converted to PI(3)P by INPP4B to terminate Akt2 activity. It is possible that the endosomal activity Akt2 might be regulating Glycogen synthase kinase-3 which has over 100 known substrates (Liu *et al.,* 2018).

The notion that PI(3,4)P2 is only produced as a by-product of PIP3 does not seem to form a consensus. A study suggests that PI(3,4)P2 is synthetized from both PIP3 and PI(4)P during cell polarization where the cortical lipid asymmetry was described with PI(3,4)P2 being the apical, and PIP3 being basolateral determinant in MDCK kidney epithelial cells (Roman –Fernandez *et al.,* 2018). A study conducted by Marat, suggests that PI(3,4)P2 is generated by phosphorylation of PI(4)P through the activity of PI3KC2β on lysosomes and late endosomes, which negatively regulates mTORC1 pathway and cell growth (Marat *et al.,* 2017). A similar finding was documented by Wang *et al.,* showing PI(3,4)P2 synthesis and turnover in endocytic pathways (Wang *et al.,* 2020). Other studies show that PI(3,4)P2 is dephosphorylated by Phosphatase and tensin homolog (PTEN) resulting in PI(4)P (Malek *et al.,* 2017; Goulden *et al.,* 2019).

The exact regulatory roles of PI(3,4)P2 and PIP3 on PI3K signaling pathway does not seem to be clear, yet, it is well-known that of both 3' phosphatases PTEN and INPP4B are tumor suppressors and their inactivation is a hallmark of many human cancers (Gewinner *et al.,* 2009; Vo and Fruman, 2015; Guo *et al.,* 2016; Song *et al.,* 2019). Consistently, PI(3,4)P2 and PIP3 were detected at invadopodium and macrophage podosomes, which are both carrying integrin receptors and F-actin filaments for cell adhesion to promote -invasive- cell migration (Zhou *et al.,* 2020; Feng and Yu*.,* 2021).

### 1.2.7. PI(4,5)P2

All seven PIPs are localized to the PM in distinct microdomains. PIP2 consist less than 1% of the cellular lipid pool and it is mainly found at the cytoplasmic leaflet of the PM (Stephens *et al.,* 1991; Tran *et al.,* 1993; Di Paolo *et al.,* 2006). Other localization of PIP2 includes, Golgi apparatus and endosomes and trace amounts in ER and mitochondria  (Watt *et al.,* 2002). PIP2 is also one of the most studied PIPs and it is involved in signaling cascades, cytoskeletal linkage, regulation of ion channels and intracellular trafficking (Balla, 2013; Picas *et al.,* 2016; Heinrich *et al.,* 2010). These processes are regulated through K/R motifs and/or the domains of the PIP2 effector proteins which include PH, BAR, ENTH/ANTH, FERM, PTB, EHD, PDZ and MARCKS (Balla*,* 2013).

Apart from the canonical Golgi mediated vesicular PI(4)P transfer to PM which is converted to PIP2 by PI5K, the rapid PIP2 homeostasis in PM was shown to be mediated at ER-PM junctions by the PITPs, Nir2 and Nir3. These Nir proteins were described to act as sensors

for PIP2 hydrolysis by binding to phosphatidic acid (PA) and mediate inter-organelle lipid transfer for rapid replenishment of the PIP2 levels in PM (Chang and Liou, 2015; Quintanilla *et al.,* 2022). More recently, the differential PM-ER concentrations of PIP2 (high concentrations at PM and almost no PIP2 at ER) was shown mediate the oligomerization behavior of the human serotonin transporter (hSERT) in serotonergic signaling termination (Anderluh *et al.,* 2017).

Overall, there are three possible pathways to generate PIP2 in PM; the main pathway is through the phosphorylation PI(4)P that is mediated by PIP5K. The Focal Adhesion Kinase (FAK) is activated by PIP5K which generates PIP2 at the focal adhesion (FA) sites. Subsequently, FAK is recruited to FA sites by binding to PIP2 through its FERM domain to regulate cell migration (Goñi *et al.,* 2014; Zhou *et al.,* 2015). Similarly, the talin protein is activated by PIP5K and its activity is regulated by the protonation level of PIP2 through which its FERM domain increases its affinity by three fold to acidic PIP2 (Saltel *et al.,* 2009; Moore *et al.,* 2012; Elliott *et al.,* 2010). On the PM, PIP2 bound Talin recruits and activates vinculin, localizing to adhesion complex by interacting with PIP2 for focal adhesion formation, vinculin phosphorylation and trafficking (Thompson *et al.,* 2017; Chandrasekar *et al.,* 2005). The PI5K pathway is also involved in endocytosis where PIP2 is required to end the signaling of EGFR receptors by sorting them towards Multivesicular Bodies (MVB) which are directed to lysosomes (Sun *et al.,* 2013).

The tumor suppressor PTEN is a lipid and protein diphosphatase that inhibits the above mentioned PI3K signaling pathway. This inhibition is achieved through PTEN mediated conversion of PIP3 back to PIP2. The activity of PTEN towards PIP3 is enhanced in the areas of the membrane that is enriched with PIP2, especially in polarized cells. In MDCK kidney epithelial cells, PTEN was shown to regulate phosphoinositide gradients by dephosphorylating basolateral determinant PIP3 to PIP2 (Leslie *et al.,* 2008; Roman-Fernandez *et al.,* 2018). PTEN exhibits its activity through targeting PIP2-rich areas by its intrinsically disordered, PIP2-binding K/R motif at its N-terminal and direct its catalytical activity by forming salt bridges with the membrane embedded PIP3 (Jang *et al.,* 2021).

The last and the least studied pathway that produces PIP2 is the phosphorylation of PI(5)P by PIP4K (Mandal *et al.,* 2020). PIP4K is mainly localized to ER/Golgi interface but studies showed that PI4KA also localizes to PM (Balla and Balla, 2006; Nakatsu *et al.,* 2012). The loss of PI4KA led to compensatory increase of PIPKIs that balanced the cellular levels of PIP2. Interestingly, the compensated PIP2 is not localized to PM but to the intracellular vesicles together with the proteins and lipids that are normally segregated to PM. This outcome has shown the importance of upstream events in defining the identity of the PM (Nakatsu *et al.,* 2012).

A recent work localized PI4KIIγ at the spindle pole during early mitosis which disappears at the onset of the anaphase. Although the study couldn't determine PIP2 at the spindle poles, it revealed that PIP2 inhibits the complete activation of Polo-like Kinase 1 that attenuates the activity of mitotic centromere-associated kinesin which restrains microtubule depolymerization at specific mitotic stages (Lin *et al.,* 2019). Consistently, ciliary PIP2 was shown to restrain microtubule elongation and overall ciliary length. PIP2 was determined to mediate vesicle transportation with Golgi that promoted the stability of the cilium but its constitutive increase induced ciliary fission through polymerized actin and Rho GTPase. Similarly, the stimulation of the ciliary 5HT receptors by serotonin led to increase in ciliary PIP2 and a short cilium (Stilling *et al.,* 2022).

Cytoskeletal dynamics are governed by a variety of actin binding proteins which are orchestrated by PIP2. Cofilin is phosphorylated by Rho-GTPase and LIM Kinase, and binds to PIP2 which regulates its activity (Sumi  *et al.,* 1999). In carcinoma, the sudden loss of PIP2 in membrane by EGF induction activates cofilin and lead to dynamic turnover of actin monomers (van Rheenen *et al.,* 2007). The actin capping protein gelsolin covers the barbed end of the actin filaments and prevents polymerization of the actin filaments (Tuominen *et al.,* 1999). By interacting with PIP2, the barbed end reveals enabling the elongation of the actin filament. Calcium ions promotes this interaction and helps polymerization of the monomeric actin (Honigmann *et al.,* 2013; Janmey and Stossel*,* 1987). The lateral distribution and the amount of PIP2 are important factors regulating the activity of gelsolin as well as the ATP which competes with PIP2 to bind gelsolin (Fatunmbi *et al.,* 2020; Wang *et al.,* 2016; Wang *et al.,* 2015; Szatmári *et al.,* 2018). The lateral organization of PIP2 also determines the nucleation activity of ARP2/3 complex (Bucki *et al.,* 2019). In general, PIP2 regulates the activity of many more actin binding proteins like profilin, formins, filamin and WASP mostly to promote F-actin formation and stabilization (Janmey *et al.,* 2018; Mandal, 2020).

In mammalian cells, PIP2 is the most abundant phospholipid with broader production pipeline which seems to be used as a main interchangeable PIP with many critical functions. Therefore, only recently published relevant functions of PIP2 were briefly mentioned in this work and more can be found in the following reviews; Other important roles of PIP2 includes cellular signaling processes and regulation of membrane curvature through BAR domain proteins (reviewed in Mandal, 2020), regulating ion channels (reviewed in Harraz *et al.,* 2020; Ningoo *et al.,* 2021) and HIV-1 membrane assembly in producer cells (Mücksch *et al.,* 2019).
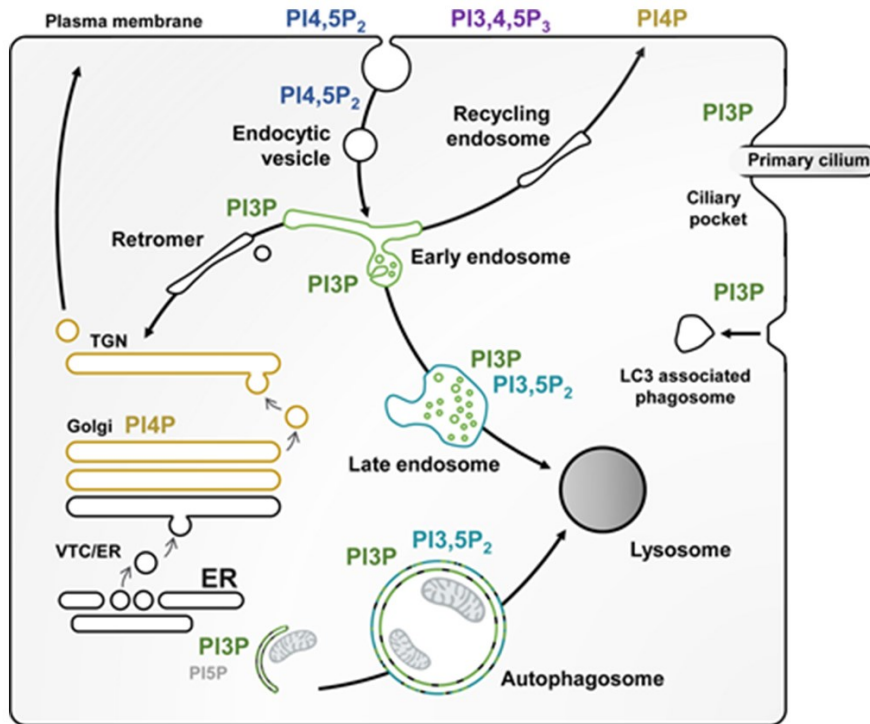
**Figure 3:** Endomembrane trafficking and phosphoinositide subcellular localization. The figure shows an overview of endomembrane trafficking in mammalian cells involving endosomes and autophagy-related organelles. Major phosphoinositides are shown in different colors. TGN, trans-Golgi network; VTC, vesiculo-tubular clusters; ER, endoplasmic reticulum (Nascimbeni *et al.,* 2016).

## 1.3. The Cell Nucleus

### 1.3.1. Nuclear Envelope

The nuclear envelope is formed of a double membrane which is continuous with the ER (Watson *et al.,* 1955). The inner and outer membranes are comprised of two phospholipid bilayers with each having a distinct lipid and protein composition that is not enriched in the peripheral ER membrane (Hetzer *et al.,* 2005). The luminal space between the two membranes is called perinuclear space and the nuclear side of the inner membrane is underlain by a lamin-rich proteinaceous meshwork (Prunuske and Ullman, 2006).

The two nuclear membranes connect at multiple sites establishing channels between nucleus and cytoplasm. These channels are gated by nuclear pore complexes which are formed by nucleoporins. The nuclear pores show eight-fold rotational symmetry with approximately 100nm outer diameter. The diameter of the central transport channel is 40nm and the phenylalanine-glycine (FG) rich repeats that are attached to the peripheral nucleoporins consist

a size barrier that allows free diffusion of molecules between cytoplasm and nucleus with a size up to 9 to 12nm (up to 60kDa) (Weis *et al.,* 2003).

The active transport requires receptor activation and, Ran and ATP regeneration system. These receptors called importins and exportins recognize proteins having the specific motifs of Nuclear Localization Signal (NLS) to enter the nucleus and Nuclear Export Signal (NES) to exit to cytoplasm (Oka and Yoneda, 2018). The active transport allows the bigger macromolecule assemblies that are up to 28nm in size to travel through the nuclear pore complexes (Weis *et al.,* 2003). These restricting features of the nuclear pores create a whole different environment inside the nucleus than of the cytoplasm which is much denser and membraneless. It is very difficult for hydrophobic molecules such as lipids to exist in such milieu. Therefore, most of the nuclear lipids reside in the nuclear envelope but there are still some lipids that locate to other nuclear compartments.

Being constituted mainly by lipids, the nuclear envelope determines the formation and maintenance of the chromosome territories as well as the DNA damage response and DNA repair mechanisms (reviewed in Moriel-Carretero, 2021). The lipid composition of the nuclear envelope is dominated by phospholipids with lesser amounts of cholesterol, sphingolipids, free fatty acids, and diacylglycerol. The ratio of Phosphatidylcholine (PC), sphingomyelin and cholesterol is determined to be crucial for gene regulation and it is maintained by the specific enzymes such as sphingomyelin synthase and PC- phospholipase. Studies have revealed that the inner nuclear membrane accommodates transcription factors which are associated with the lipid composition of the membrane (Rossi *et al.,* 2007a,b; Heessen and Fornerod, 2007). These observations led other scientists to define the ratio of these lipids in nuclear microdomains. The work of Cascianelli *et al.,* has shown that the composition of the nuclear microdomains changes during cell proliferation due to the increase in the nascent RNA levels (Cascianelli *et al.,* 2008).

### 1.3.2. Lipidation

The lipidation of the nuclear proteins is a process where lipids directly affect the nuclear homeostasis. This post-translational covalent modification impacts protein localization, catalytic activity, aggregation propensity and interacting partners. For example, the farnesylation of the prelamin A is necessary for its association with the nuclear envelope and in Progeria Syndrome, a mutation prevents the maturation of the prelamin A which remains permanently farnesylated causing misshaped nuclei and many other malfunctions (De Sandre-Giovannoli *et al.,* 2003). Another example would be the palmitoylation of the histone H4 that regulates mRNA synthesis (Zou et. al. 2011). Overall, many other lipid-based post translational modifications exist such as

myristoylation, esterifications and lipid peroxidations, however their effects on the nuclear homeostasis are yet to be elucidated (Anavi *et al.,* 2015; Moriel-Carretero, 2021).

### 1.3.3. Non-covalent Lipid Interactions

The nuclear homeostasis can be maintained by non-covalent lipid binding as well. Ceramide, for example, is a metabolite of sphingomyelin which can act as a secondary messenger. Its regulatory activity can be both stimulatory and inhibitory through -non-covalent-binding to proteins. Protein Phosphatases 1 and 2 are among these proteins which show enhanced activity and promote recovery from the inhibition of DNA replication (Bazzi *et al.,* 2010; Ferrari *et al.,* 2017). The long-chain fatty acids are transported to nuclei by binding non-covalently to Fatty Acid-Binding Proteins (FABPs). The genes that are involved in the lipid metabolism are regulated through the interaction of the long fatty acids with the ligand-dependent receptors such as the nuclear peroxisome proliferator-activated receptors, PPARs (Huang *et al.,* 2002; Gililan *et al.,* 2007; Adida and Spener, 2006). A recent work has demonstrated the importance of FABP-4 protein in type 1 and 2 diabetes, and obesity, which can be a target for future treatments to prevent these diseases (Prentice *et al.,* 2021).

Lipids can also act as chaperones and affect the nuclear dynamics. Since lipids show hydrophobic properties, they tend to compartmentalize if they are not embedded in membranes. The difference in the density between the lipidic structures and the outer environment can attract proteins and aggregates. For example, lanesterol (25-hydroxycholesterol) was shown to dissemble misfolded proteins and promote the disassembly of the aggregated proteins *in vivo* and *in vitro* (Kang *et al.,* 2018; Zhao *et al.,* 2015; Chen *et al.,* 2018). In nucleus, cholesterol was determined to promote the compaction of the chromatin by dehydration of nucleosomes (Silva *et al.,* 2017). Further studies revealed that cholesterol interaction is able bend single-stranded DNA and hide its exposed end which may have many regulatory functions especially in DNA repair (Ohmann *et al.,* 2019). On the same note, RNAs and the intrinsically disordered regions (IDR) of the proteins were defined to be attracted by lipidic structures and how these interactions effect the nuclear homeostasis is a subject of this work (Czerniak and Saenz*,* 2022; Hou *et al.,* 2018).

### 1.3.4. The Nuclear Lipid Droplets

The nuclear lipid droplets (nLDs) are formed through the active lipid metabolism of the inner nuclear membrane to store fatty acids (Barbosa *et al.,* 2019; Romanauska and Köhler, 2018). These lipid structures are major sites for PC synthesis and are composed of a hydrophobic triacylglycerol and cholesteryl ester core which is surrounded by a monolayer of polar lipids, cholesterol, and proteins (Layerenza *et al.,* 2012; Sołtysik *et al.,* 2019). PML isoform II was shown

to play a critical role in nLD formations (Ohsaki *et al.,* 2016; Lee *et al.,* 2020). Further studies found an association between PML bodies and nucleoporins that raises the possibility that nLDs might contribute to the formation of the nuclear pore complex assembly which can facilitate their insertion into the nuclear membrane (Jul-Larsen *et al.,* 2010; Lång *et al.,* 2017; Lautier *et al.,* 2021). This proposition is in agreement with the work that showed the restricted formation of nuclear pore complexes by nLDs upon stress (Lord *et al.,* 2020). Although few metabolic functions of the nLDs are somewhat elucidated, their main function and synthesis remain largely enigmatic and requires more attention (Sołtysik *et al.,* 2019; Romanauska and Köhler, 2018, 2021).

### 1.3.5. Nuclear **Phosphoinositide Phosphates**

PIPs reside in distinct nuclear compartments and they are involved in many nuclear processes (Table 1; Jacobsen *et al.,* 2019). Their interconversion occurs in nucleus by the actions of PIP-metabolizing kinases, phosphatases and phospholipases (Table 2). Due to their hydrophobic tails, they have poor solubility that requires immediate interaction with their surrounding proteins and nucleic acids (Várnai *et al.,* 2006). Therefore, how PI (or PIPs) is transported to the nucleus from their site of synthesis –ER- consist one fundamental question. It is possible that the nuclear PIPs are drifted inside the nucleus during telophase and are subsequently encased by the newly formed nuclear envelope. Indeed, during mitosis nucleolar PI(4,5)P2 remain bound to nucleolar organizing regions (NORs) (Sobol *et al.,* 2013). Similarly, nuclear speckle associated PIP2 was shown to accumulate in mitotic interchromatin granules in cytoplasm and enter into nuclei sequentially after the formation of the nuclear envelope in late telophase (Osborne *et al.,* 2001). So far, no specific nuclear transporters for phosphorylated forms of PI have been discovered. Two isoforms of phosphatidylinositol transfer proteins (PITPs), which mediate PI transport across the cytoplasmic membrane, were discovered in the nucleus (De Vries *et al.,*1995, 1996; Rubbini *et al.,* 1997; reviewed in Pemberton and Balla, 2019).

Another fundamental question about PIPs in nucleus is; How they shield their hydrophobic tails in a soluble environment? There have been several propositions but the biophysical nature of the PIPs in nucleus is not fully understood. The attempts to explain the existence of acyl chains in aqueous environment includes micellar formations, phase separation mechanisms and binding to a protein with pocket that hides the acyl chains (reviewed in Jacobsen *et al.,* 2019). Yet, there is no evidence for the existence of micellar structures in cells, and even though the PIP-containing structures such as nucleolus and nuclear speckles are formed through phase separation, more research is required to support this mechanism (Ilık *et al.,* 2020; Frottin *et al.,* 2019). The only evidence from the proposed mechanisms was provided by the Steroidogenic

factor 1 (STF-1) /NR5A1 (Blind *et al.,* 2012, 2014), and liver receptor homolog-1 (LRH-1/NR5A2) (Sablin *et al.,* 2015) to bind PIPs through a ligand-binding pocket that hide their acyl chains. However, this mechanism fails to explain how PIPs can contribute to the integrity of the $PIP_2$-rich nuclear compartments such as speckles, nucleoli, DNA repair foci and nuclear lipid islets (Kalasova *et al.,* 2016; Yu-Hsiu Wang *et al.,* 2017; Sobol *et al.,* 2018).

All PIPs, except PI(3,5)P2 were detected in nucleus (Fiume *et al.,* 2012; Shah *et al.,* 2013). Their respective sub-nuclear locations are reviewed in the Table 1.

**Table 1:** Nuclear localization of phosphoinositides.

| PIP | Sub-nuclear localization (References) |
|---|---|
| PI(3)P | Nucleolus (Gillooly *et al.,* 2000) |
| PI(4)P | Nucleus (Clarke *et al.,* 2001; Kalasova *et al.,* 2016; Vann *et al.,* 1997) <br><br> Nucleolus, Nuclear speckles (Kalasova *et al.,* 2016) (Faberova *et al.,*2020) <br><br> Nuclear envelope, nuclear lamina, nuclear speckles (Faberova *et al.,*2020) |
| PI(5)P | Nucleus and chromatin enriched fraction, detected upon stress induction (Clarke *et al.,* 2001; Jones *et al.,* 2006) |
| PI(3,4)P2 | Nuclear membrane (Yokogawa *et al.,* 2000) |
| PI(4,5)P2 | Nuclear speckles (Boronenkov *et al.,* 1998; Osborne *et al.,* 2001) <br><br> Nucleolus (Kalasova *et al.,* 2016; Osborne *et al.,* 2001; Sobol *et al.,* 2013; Yıldırım *et al.,* 2013) <br><br> Nuclear lipid islets (Sobol *et al.,* 2018) |
| PI(3,4,5)P3 | Nuclear matrix (Kumar *et al.,* 2010; Kwon *et al.,* 2010; Lindsay *et al.,* 2006) <br><br> Nucleolus (Karlsson *et al.,* 2016) |

Most of the cytoplasmic PIP-metabolizing enzymes are detected in the nucleus in discrete sub-nuclear locations where their respective PIP substrate and/or product are found. Since the work that is presented here involves nuclear speckles, the enzymes that are localized to nuclear speckles will be mentioned. Still, all the PIP metabolizing enzymes and their respective sub-nuclear locations are summarized in the table 2 with referencing the corresponding work (reviewed in Jacobsen *et al.,* 2019).

**Table 2:** Nuclear PIP-metabolizing enzymes, their substrates and nuclear localization

| Enzyme (Kinases) | Substrate | Sub-nuclear localization (References) |
|---|---|---|
| Type IIIα PI4K PI4K230 | PI | Nucleolus (Kakuk *et al.,* 2006, 2008) |
| Type IIIβ PI4K PI4K92 | PI | Nucleus (de Graaf *et al.,* 2002) <br> Nuclear speckles (Szivak *et al.,* 2006) |
| Type Iα PIPK | PI(4)P | Nuclear speckles (Boronenkov *et al.,* 1998; Mellman *et al.,* 2008) <br> Nucleolus (Chakrabarti *et al.,* 2015) |
| Type Iγ PIPK_i4 | PI(4)P | Nuclear speckles (Schill and Anderson, 2009) |
| Type IIα PIPK | PI(5)P | Nucleus (Bultsma *et al.,* 2010; Ciruela *et al.,* 2000) |
| Class I PI3K, p110β | PI(4,5)P2 | Nucleoplasm, chromatin, double stranded break foci (Kumar *et al.,* 2010, 2011; Marques *et al.,* 2009) <br> Nucleolus (Karlsson *et al.,* 2016) |
| IPMK | PI(4,5)P2 | Nucleus (Resnick *et al.,* 2005) <br> DNA Damage Foci (Yu-Hsiu Wang *et al.,* 2017) |
| Class IIα PI3K | PI, PtdIns4P | Nuclear speckles (Didichenko and Thelen, 2001) |
| Class IIβ PI3K | PI, PI(4)P | Nuclear matrix (Sindic *et al.,* 2001) <br> Nuclear lamina (Banfic *et al.,* 2009) |
| **Enzyme (Phosphatases)** | **Substrate** | **Sub-nuclear localization (References)** |
| PTEN | PI(3,4,5)P3 | Nucleus (Deleris *et al.,* 2003; Shen *et al.,* 2007; Song *et al.,* 2008) <br> Nucleolus (Li *et al.,* 2014), PTENβ (Liang *et al.,* 2017) |
| SHIP1 | PI(3,4,5)P3 | Nucleolus (Ehm *et al.,* 2015) |
| SHIP2 | PI(3,4,5)P3 <br> PI(4,5)P2 | Nuclear speckles (Deleris *et al.,* 2003; Elong Edimo *et al.,* 2011) |
| Type I PI(4,5)P2 4-phosphatase | PI(4,5)P2 | Translocates to the nucleus upon cell stress (Zou *et al.,* 2007) |
| **Phospholipase C** | **Substrate** | **Sub-nuclear localization (References)** |
| PLCβ1 | PI(4,5)P2 | Nuclear speckles (Tabellini *et al.,* 2003) |
| PLCδ1 | PI(4,5)P2 | Nucleus (Okada *et al.,* 2010) |
| PLCδ4 | PI(4,5)P2 | Nucleus (Kunrath-Lima *et al.,* 2018) |

### 1.3.5.1. Nuclear PI(3)P

Using electron and fluorescent microscopy techniques, PI(3)P was discovered in the dense fibrillar component of nucleoli through PI(3)P-binding FYVE domains (Gillooly *et al.,* 2000; Kalasova *et al.,* 2016). The PI(3)P was further identified in the membrane deprived-nuclei of the liver cells and suggested to cause oxidative stress and liver damage (Sindic *et al.,* 2001). This finding point to the fact that PI(3)P is localized to the nuclear matrix as it persisted in nucleus after rigorous nuclear extraction conditions. It is possible that PI(3)P is interacting with nucleoskeleton and involved in stress response. The recent finding reveal that cisplatin raises the nuclear presence of PI(3)P, as measured by immunofluorescent labeling, implying that PtdIns3P may play a role in the response to genotoxic stress (Choi *et al.,* 2019).

### 1.3.5.2. Nuclear PI(4)P

By incorporation of the radiolabeled phosphate, the synthesis of PI(4)P was detected in the purified liver nuclei. After the addition of exogenous PIs as the substrate, PI(4)P was synthesized in the nuclear matrix by the active PI 4-kinases (Type Iα PIPK and/or Type Iγ PIPK_i4) in the nucleus (Sindic *et al.,* 2001). PI(4)P was also found in nuclear speckles and nucleoli by antibody and OSH1-PH mediated immunofluorescence. It is then determined that the nuclear distribution pattern of PI(4)P is strikingly similar to nuclear PI(4,5)P2 (Kalasova *et al.,* 2016). Moreover, the nuclear PI(4)P levels peaked at G1 and declined through S phase of the cell cycle which was similar to the turnover of nuclear PI(4,5)P2 that peaks at the G1/S transition (Clarke *et al.,* 2001). According to these findings, nuclear PI(4)P acts as a precursor of PI(4,5)P2.

PI(4)P also resides at the nuclear envelope, where it acts as a substrate for perinuclear phospholipase C (PLC) to produce diacylglycerol (DAG) that activates nuclear protein kinase D (PKD). This pathway was determined to be involved in the regulation of cardiac hypertrophy, showing the importance of PI(4)P in the differentiated tissues (Zhang *et al.,* 2013). PI(4)P detected by the SidM-P4M domain showed rapid accumulation at DNA damage sites after UV irradiation, and the sequestration of PI(4)P by the SidM-P4M domain partially suppressed ATR-dependent DNA damage signaling, implying a role for PI(4)P in DNA damage repair (Wang *et al.,* 2017). Most recently, PI(4)P was investigated by several techniques such as electron microscopy, super-resolution florescence microscopy, and mass-spectrometry which localized PI(4)P at nuclear envelope, nuclear lamina, nuclear speckles and nucleoli while defining novel interacting partners of PI(4)P functioning in vital nuclear processes such as pre-mRNA splicing, transcription or nuclear transport (Faberova *et al.,* 2020).

### 1.3.5.3. Nuclear PI(5)P

Nuclear PI(5)P was detected for the first time in murine erythroleukaemia cells, with almost 20-fold increase at the G1 phase of the cell cycle (Clarke *et al.,* 2001). As a breakthrough, PI(5)P was shown to bind to the PHD domain of the ING2 protein which is described as a chromatin-associated protein, functioning as a nuclear phosphoinositide receptor (Gozani *et al.,* 2003). The overexpression of PIPKIIβ, which regulates the PI(5)P levels by synthetizing PI(4,5)P2, reduced the nuclear localization of ING2 (Jones *et al.,* 2006). Further studies determined that PI(5)P promoted the capacity of ING2 to bind to promoter regions of specific target genes (Bua *et al.,* 2013). Through binding to ING2, PI(5)P was shown to regulate p53 mediated apoptosis in response to UV and etoposide. The cells which carried the mutated form of the PHD domain of ING2, causing the loss of PI(5)P interaction were unable to induce apoptosis and p53 acetylation (Jones *et al.,* 2006). Moreover, the PI(5)P levels were determined to increase in the nucleus when induced by genotoxic and oxidative stress. In contrast, the antioxidant N-acetyl-cystine reduces the level of nuclear PI(5)P by alleviating the oxidative stress (Jones *et al.,* 2006). There is also evidence that the production of PI(5)P and its conversion to PI(4,5)P2 by PIPKIIβ activates the nuclear ubiquitin ligase Cul3-SPOP. The link between cellular stressors and nuclear PI(5)P levels suggests that nuclear PI(5)P plays several roles in stress signaling (Bunce *et al.,* 2008).

More recently, the interaction of all three mono-phosphorylated PIPs with the co-repressor sin3A-associated protein 30-like (SAP30L) was found to diminish PI(5)P interaction with chromatin and also its *in vitro* activity in transcriptional repression (Viiri *et al.,* 2009). The basal transcription initiation factor TFIID subunit 3 (TAF3)-regulated transcription is essential in muscle differentiation which was demonstrated to require TAF3 -PI(5)P association (Stijf-Bultsma *et al.,* 2015). Furthermore, the interaction between UHRF1 protein and PI(5)P results in a conformational change that allows UHRF1 to bind to H3K9me3 (Gelato *et al.,* 2014; Poli *et al.,* 2022; reviewed in Poli *et al.,* 2019). Latest research determined PI(5)P and PIPKIIα (which produces PI(5)P) in globin gene regulation in KU812 basophil cells as a promising therapeutic target to treat hemoglobinopathies, such as in sickle cell disease and beta thalassemia (Malimpensa *et al.,* 2021).

### 1.3.5.4. Nuclear PI(3,4)P2

There hasn't been much investigation on the nuclear functions of PI(3,4)P2. Its nuclear presence was shown at the nuclear envelope by using a specific monoclonal antibody (Yokogawa *et al.,* 2000). The staining of nuclear PI(3,4)P2 was shown to increase at nuclear envelope by hydrogen peroxide treatment which might indicate a role in oxidative stress response (Yokogawa *et al.,* 2000). PI(3,4)P2 was shown to be synthesized *de novo* in nuclear extracts by

detecting the conversion of radiolabeled P(3,4,5)P3 to PI(3,4)P2 (Deleris *et al.,* 2003). The presence of SHIP2 in nuclear speckles and SHIP1 in nucleoli indicates that PI(3,4)P2 might also be present in these sub-nuclear compartments (Deleris *et al.,* 2003; Ehm *et al.,* 2015). In 2016, Kalasova *et al.,* detected PI(3,4)P2 in nuclear speckles and in nucleoplasmic foci, however due to cross-reacting commercial antibodies and unspecific PIP-binding domains, this localization of PI(3,4)P2 was not validated. Recently, PARP1 was determined to colocalize with PI(3,4)P2 in the nucleoplasmic loci and shown to interact with PI(3,4)P2 *in vitro* (Gavgani *et al.,* 2021). Overall, there is not enough research done on this nuclear phosphoinositide and its nuclear functions are yet to be defined.

### 1.3.5.5. Nuclear PIP3

The first indication of the nuclear presence of the PIP3 was observed by Deleris *et al.,* in 2003, where the dephosphorylation of radiolabeled of PIP3 was determined in membrane-depleted nuclei. While this observation suggested that PIP3 localizes to nuclear matrix, the same work also showed colocalization of SHIP2 with the splicing factor SC-35 that indicated nuclear speckles as another sub-nuclear location for PIP3 (Deleris *et al.,* 2003). The first detection of nuclear PIP3 was performed by using the purified PH domain of the Grp1 protein under electron microscopy and showed PI(3,4,5)P3 in the nuclear matrix (Lindsay *et al.,* 2006). Finally, in 2016 the PIP3 has been revealed in the nucleoplasm and nucleoli by immunofluorescence yet, the presence of PIP3 in nuclear speckles is still ambiguous (Karlsson *et al.,* 2016; Kalasova *et al.,* 2016).

PIP3 is known to interact with STF-1 (Blind *et al.,* 2012, 2014), which affects the transcription of genes involved in lipid and steroid metabolism, as well as the regulation of cytoskeleton dynamics, cell cycle, and apoptosis (Lalli *et al.,* 2013). Both PI(4,5)P2 and PIP3 bind to the sterol-binding pocket of STF-1 through their acyl chains which stabilizes its tertiary structure. PIP3 bound STF-1 has substantially higher affinity for a coactivator peptide than when bound to PI(4,5)P2 (Blind *et al.,* 2012, 2014). Therefore, it is possible that the activity of PTEN phosphatase or IMPK kinase can alter the STF-1 pathway and its target gene expression (Blind *et al.,* 2012; reviewed in Castano *et al.,* 2019).

PIP3 also plays a role in anti-apoptotic signaling. The nerve growth factor (NGF) promotes the direct binding of PIP3 to nucleophosmin (B23) that translocate AKT1 to nucleus n a PI3K-dependent manner (Borgatti *et al.,* 2003; Ahn *et al.,* 2004, 2005; Nguyen *et al.,* 2006). The AKT protein protects B23 from degradation and the PIP3-B23 complex inhibits the caspase activated DNase from fragmenting the DNA (Lee *et al.,* 2008; Ahn *et al.,* 2005). Both SHIP-2 and PTEN

phosphatases, as well as the overexpression of a PIP3-binding mutant of B23, can suppress NGF-induced anti-apoptotic activities (Ahn *et al.,* 2004, 2005). However, PTEN does not dephosphorylate PIP3 in the nucleus (Lindsay *et al.,* 2006) and therefore it is unclear whether PTEN can inhibit the NGF pathway by lowering PIP3 levels or by another mechanism. The NGF might also be mediating PIP3-Aly interaction, regulating mRNA nuclear export. IPMK-produced PIP3 was demonstrated to modulate Aly-mediated recognition of specific mRNAs for nuclear export, especially those implicated in homologous recombination DNA repair (Wickramasinghe *et al.,* 2013). Moreover, long intergenic non-coding RNA for kinase activation (LINK-A) was shown to interact directly with AKT and PIP3, promoting AKT-PIP3 binding and leading to hyper-activation of AKT that is associated with tumorigenesis (Lin *et al.,* 2017).

The functions of nucleolar PIP3 is not thoroughly investigated. Yet, a study identified that an AKT interactor, EBP1 to bind to PIP3 in the nucleolus through a C-terminal polybasic motif. Although EBP1 protein is involved in growth regulation by binding to rRNAs, this study was not able to define the exact nucleolar process that PIP3 was involved in (Karlsson *et al.*, 2016). Further investigation identified an increase in nucleolar PIP3 levels and also the expression of the catalytic subunit (p110β) of the PI3K in nucleolus, in endometrial cancer cells with high amounts of pre –rRNA (Karlsson *et al.,* 2017; Gavgani *et al.,* 2021a). Therefore, it is possible that the PIP3 regulates nucleolar PI3K signaling cascade and contributing cell proliferation.

Most recently, the quantitative mass-spectrometry experiment has revealed interactors of PIP3 that are involved in RNA processing/splicing, cytokinesis, protein folding, and DNA repair (Gavgani *et al.,* 2021b). The half of these interactors were identified as nucleolar proteins. For example, the PARP1 protein, that is colocalized with PI(3,4)P2 in nucleoplasmic foci, was also identified as an interactor of nucleolar PIP3 which also colocalize in nucleolus (Gavgani *et al.,* 2021b). Since PARP1 plays a key role in DNA repair, PIP3 might also be regulating this mechanism. Indeed, the PIP3, that was identified by the Btk-PH domain, was shown to accumulate at DNA damage sites, and its sequestration by the Btk-PH domain reduced ATR-dependent DNA damage signaling, implying a function for PIP3 in DNA damage repair (Wang *et al.,* 2017).

### 1.3.5.6.    *Nuclear PI(4,5)P2*

As its cytoplasmic counterpart, the nuclear PIP2 is also extensively studied due to its versatile functions in genome regulation. Its nuclear localization is also well described. In nuclear matrix, de novo synthesis of PIP2 was detected by incorporation of radiolabeled phosphate and its conversion was shown to be promoted by addition of exogenous PI(4)P (Sindic *et al.,* 2001).

The sub-nuclear localization of PIP2 is resolved by using specific antibodies which is defined as nuclear speckles, nucleoplasm and nucleoli (Osborne *et al.,* 2001; Kalasova *et al.,* 2016; Sobol *et al.,* 2018). Moreover, the work of Sobol *et al.,* determined the ratio of the nuclear PIP2 pool in these compartments as; 68% in nuclear speckles, 28% in nucleoplasm, and 4% in nucleoli (Sobol *et al.,* 2018).

One of the most important feature of both cytoplasmic and nuclear PIP2 is the ability to regulate actin dynamics by interacting with actin binding proteins. In nucleus, PIP2 was determined to associate with actin for chromatin remodeling. In this mechanism, SWI/SNF-like BAF complex binds to PIP2 and interacts with the actin pointed ends, stabilizing actin filaments (Zhao *et al.,* 1998; Rando *et al.,* 2002). Since BAF complex is a chromatin remodeling complex, PIP2 interaction is expected to play a role in this mechanism, however the exact functional consequence is not yet clear. Since the cytoplasmic PIP2 is able to nucleate actin filaments and regulate its dynamics, it is reasonable that PIP2 preserves the similar functions in nucleus; especially, if we consider that most of the PIP2-interacting actin regulators also exist in the nucleus, such as cofilin, formin, filamin, profilin, ARP2/3 and many others (Wiggan *et al.,* 2017; Du Toit *et al.,* 2013; Deng *et al.,* 2012; Söderberg *et al.,* 2012; Schrank *et al.,* 2018; Kalendová *et al.,* 2014). Therefore, the PIP2- and also other nuclear interactions of actin are crucial for numerous nuclear processes and they consist an important part of this work where they will be mentioned and discussed in further topics.

PIP2 also contributes to chromatin remodeling by directly binding to H1 and H3 histones through their C-terminal that alleviate H1-induced inhibition of RNAPII transcription (Yu *et al.,* 1998).  In contrary, PIP2  was shown to repress chromatin acetylation by a mechanism involving myristoylation of the transcriptional corepressor, BASP1. PIP2 mediates the complex formation by binding to BASP1 via its myristoyl moiety and facilitating BASP1 interaction with histone deacetylase 1 (HDAC1). This complex is then recruited to target gene promoters, where HDAC1 deacetylates histones and reduces promoter accessibility for RNAPII transcription machinery (Toska *et al.,* 2012). Recently, the PIP5K1A and its product PIP2 was determined to interact with the tumor suppressor p53, increasing its stability during cellular stress. Upon DNA damage, PIP5K1A interacts with p53 to generate PIP2, which then binds to p53 via its C-terminal domain (CTD). This binding promotes the recruitment of the small heat-shock proteins to p53, promoting its stability. Authors suggest that this pathway represents a promising therapeutic target in cancer (Choi *et al.,* 2019).

The colocalization of PIP2 with speckle markers such as the splicing factor SC-35 or Sm proteins suggests that PIP2 localizes to nuclear speckles (Boronenkov *et al.,* 1998; Osborne *et al.,* 2001, Kalasova *et al.,* 2016). Nuclear speckles are dynamic nuclear compartments that are hubs for pre-mRNA splicing factors, determining the dynamics of the gene expression machinery (Galganski *et al.,* 2017; Spector and Lamond, 2011). The PIP2 at the nuclear speckles interacts with the small nuclear ribonucleoproteins (RNPs), RNAs such as U1-U6 snRNAs, and the hyper-phosphorylated form of RNAPII (Osborne *et al.,* 2001). PIP2 also associates with other components of the nuclear speckles such as Aly that regulates the nuclear export of activities and cell proliferation (Okada *et al.,* 2008). In addition, a study discovered a non-canonical poly(A) polymerase called nuclear speckle targeted PIPKI regulated-poly(A) polymerase (Star-PAP), and demonstrated that its polyadenylation activity is directly stimulated by PIP2 (Mellman *et al.,* 2008). Research from the same group also showed that an element of the Star-PAP complex, Casein kinase I, also requires PIP2 to regulate the 3'-end mRNA processing activity of the Star-PAP complex (Gonzales *et al.,* 2008).

The nucleolar functions of PIP2 started to reveal with the work of Yıldırım *et al.,* in 2013 where the group showed that PIP2 and RNAPI binding is required at the promoter region of the rDNA for reliable transcription. The depletion of PIP2 was reduced RNAPI transcription that is rescued by exogenous PIP2 addition. PIP2 binding to the upstream binding factor (UBF) and fibrillarin was shown modulate their interaction with DNA and RNA, respectively (Yıldırım *et al.,* 2013). As UBF is involved in the formation of pseudo-NORs, PIP2 was determined to regulate the formation of NORs by modulating the binding of UBF to rDNA (Mais *et al.,* 2005; Sobol *et al.,* 2013). The interaction of PIP2 with the subunits of RNAPI and UBF indicates a structural role for PIP2 that maintains the NOR formation (Sobol *et al.,* 2013). Furthermore, the regulation of rRNA gene expression by PIP2 was shown at epigenetic level. The interaction of PIP2 and PHF8, through its C-terminal K/R-rich motif, triggers a conformational change in PHF8 that represses its H3K9me2 demethylase activity and regulates expression of rRNA genes (Ulicna *et al.,* 2018).

The recent discovery of the nucleoplasmic PIP2 structures describe novel nuclear compartments called as nuclear lipid islets (NLIs). These structures are 40 to 100nm in diameter and exist in great numbers in nucleoplasm that shows a granular pattern under light microscope. At the core of these structures carbon-rich compounds were observed which are most likely the acyl chains of the PIP2 molecules. The periphery of these structures contains nucleic acids and proteins; including RNAPII large subunit (RPB1), transcription factors, and nuclear myosin 1 (NM1). The nascent transcripts are also prominent in the periphery of these structures with RNA being indispensable for the integrity of NLIs. It is therefore suggested that NLIs can serve as a

scaffolding platform which facilitates the formation of the transcription factories that contributes to the nuclear architecture (Sobol *et al.,* 2018).

### 1.3.6. RNAPII Transcription

The RNAPII transcribes the DNA into pre-mRNAs, small nuclear RNAs (snRNAs) and regulatory RNAs such as micro RNAs (Cramer*,* 2019). RNAPII is a 550 kDa complex which has 12 subunits in mammals with largest being the DNA-directed RNA polymerase II subunit RPB1 (Myer and Young, 1998). This subunit of the RNAPII comprises a domain called carboxy terminal domain (CTD) that comprises a 52-repetitive heptad sequence with the consensus amino acid sequence being; Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. The predominant phosphorylation state of this heptad correlates with different steps of the RNAPII transcription cycle and coins the term "CTD code" (Harlen and Churchman, 2017).

The RNAPII transcription cycle can be divided into four steps; Initiation, promoter-proximal pausing, elongation, and termination. Transcription begins at the transcription start site, at the downstream of the promoter where there is a specific DNA sequence that stabilizes the transcriptional machinery. RNAPII requires general transcription factors to recognize the specific promoter sequences and the assembly of the pre-initiation complex (PIC) is the first step of the RNAPII transcription. The PIC consists of many factors but mainly includes the polymerase, mediator and the general transcription factors (GTFs); TFIIA -B, -D, -E, -F, and -H. The GTFs are important for promoter recognition and transcription initiation (Liu *et al.,* 2012; Petrenko *et al.,* 2019; Schier and Taatjes, 2020). After the assembly, PIC opens the DNA and RNAPII transcribes around 20 to 60 base pairs before pausing for a regulatory stage.

At the second step, known as promoter-proximal pausing, the active site of the polymerase, that involves the DNA:RNA hybrid, goes through a conformational change that prevents the addition of NTPs. The additional factors such as the negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) stabilize and prolong the paused duration. During this step, the RNAPII complex interacts with many regulatory factors that define the expression of the gene (Core and Adelman, 2019). The release from this stage requires the activity of the positive transcription elongation factor b (P-TEFb) complex, which phosphorylates DSIF (Spt5 subunit), NELF, and serine 2 of the CTD (Czudnochowski *et al.,* 2012; Eick and Geyer, 2013; Core and Adelman, 2019). The transcription factors, Mediator, and coactivators recruit P-TEFb to promoters (Li *et al.,* 2018) and the subsequent phosphorylation activity leads NELF to dissociate from RNAPII. The phosphorylation of Spt5 converts DSIF from a repressor to an activator of transcription (Ivanov *et al.,* 2000; Yamada *et al.,* 2006). Spt5, which remains attached

to the RNAPII complex, acts as a scaffolding platform for other factors involved in RNAPII elongation and RNA processing. The pause-release also correlates with Tyr1 phosphorylation of the CTD which was shown to alter the specificity of P-TEFb from phosphorylating Ser5 CTD to Ser2 CTD (Mayfield *et al.,* 2019; Czudnochowski *et al.,* 2012).

After the release from the promoter-proximal region, the RNAPII enters the elongation stage that defines two important aspect of the transcription: Processivity and Speed. The processivity describes if the entire gene or a portion of the gene is transcribed. The premature termination of the transcription is usually an unstable RNA that is degraded rapidly. However, the stable premature RNAs might also give rise to a non-coding RNA with a regulatory function or even translated into a protein with a different function than its full-length version (Kamieniarz-Gdula and Proudfoot, 2019). The speed of the elongation is defined by the number of the nucleotides that are generated per unit of time and it is determined by number of factors such as the DNA sequence and nascent RNA folding, gene structure and chromatin landscape, and many other transcription factors that travel with RNAPII during elongation (reviewed in Muniz *et al.,* 2021).

The termination step of transcription begins with RNAPII reaching the end of the gene where the poly(A) site is recognized by cleavage and polyadenylation complex. Two components of the cleavage and polyadenylation specificity factor mediates an endonuclease to cleave the nascent pre-mRNA and the cleaved 3' end is then polyadenylated. The rest of the RNA that is synthetized after the poly(A) site is degraded from its unprotected flagging 5' end by the Xrn2 5'–3' exonuclease (Eaton and West, 2020). The Protein Phosphatase 1 slows down the post-termination transcribing RNAPII by dephosphorylating Spt5 which induces an allosteric change in the elongation complex (Cortazar *et al.,* 2019). The stagnated elongation complex allows Xrn2 endonuclease to catch-up with RNAPII and dislocate it from the DNA; resulting in the transcription termination (Cortazar *et al.,* 2019; Eaton *et al.,* 2020). Overall, the RNAPII transcription cycle is a tightly regulated mechanism that is orchestrated by a myriad of enzymes that leads to different phosphorylation states of the CTD. Nonetheless, the activity of RNAPII is also dependent on other factors such as the formation of phase-separated structures (Guo *et al.,* 2019).
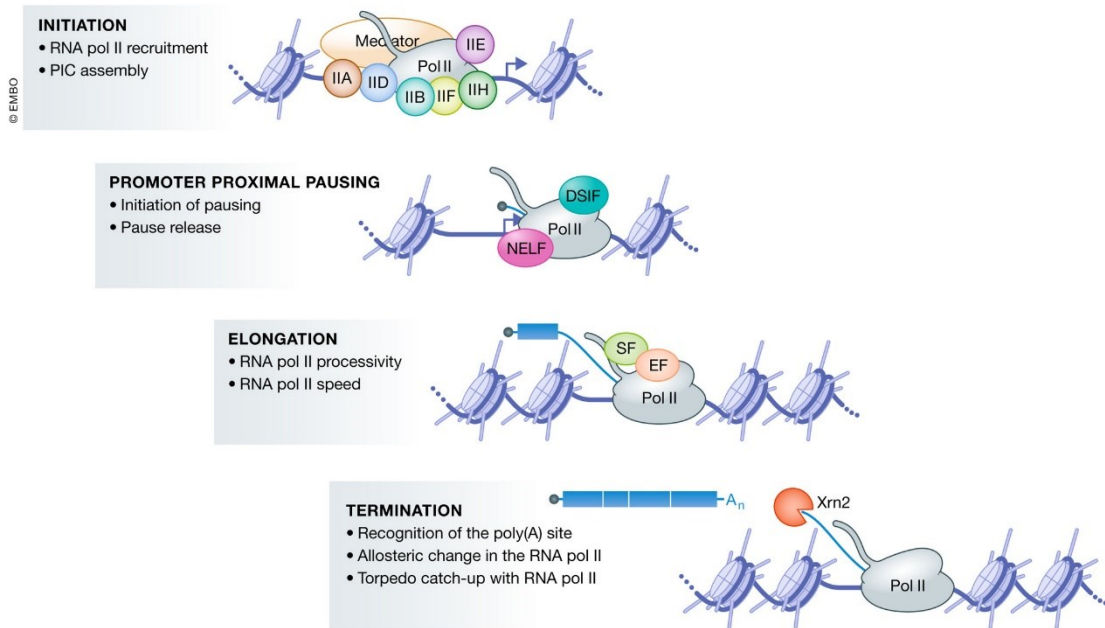
**Figure 4:** Schematic representation of the stages of RNAPII transcription. The transcription cycle of RNAPII can be divided into four major regulated phases. First, transcription initiation begins with the recruitment of RNAPII to the promoter and the formation of the pre-initiation complex (PIC). After initiation, RNAPII pauses at the promoter-proximal region. A conformational shift in the RNA:DNA hybrid in the polymerase active site precludes the addition of incoming NTPs via conventional base pairing. In many metazoans, NELF and DSIF stabilize and extend the lifetime of the paused complex. After pausing, RNAPII enters into productive elongation by multi-kinase activity of P-TEFb. Elongation factors (EF) and Splicing Factors (SF) help coordinate pre-mRNA processing. Finally, transcription termination involves poly(A) site recognition by the cleavage and polyadenylation complex which cleaves the nascent pre-mRNA that is then polyadenylated. Transcription of the poly(A) site induces an elongation slow-down which promotes transcription termination upon catch-up of RNA Pol II by the exonuclease Xrn2, which degrades the RNA synthesized beyond the poly(A) site from its 3' end (Muniz *et al.*, 2021).

### 1.3.7. Liquid-Liquid Phase Separation in the Cell Nucleus

Cells form membraneless internal compartments by a process known as liquid–liquid phase separation (LLPS). These chemically different compartments known as condensates result from weak multivalent interactions between biological macromolecules. In materials science, the phase behavior of a system depends on the temperature, the concentration and the interaction energy (Strom and Brangwynne, 2019). By controlling the concentration (protein expression, molecular crowding) and the interaction strength (post-translational modifications) of the biological macromolecules, the cell can form and dissolve the condensates. As these

condensates can be nucleated by other structures, they can also be formed through weak multivalent interactions between the proteins which possess low complexity sequences such as intrinsically disordered regions (IDRs).

In nucleus, the interactions among the components of DNA, RNAs and proteins can trigger condensation of the phase-separated compartments (Banani *et al.,* 2017; Shin and Brangwynne, 2017). For example, during mitosis most of the nuclear bodies dissolve and then reform reliably at the correct location in every cell cycle (Rai *et al.,* 2018). After mitosis, the NORs determine the location of reforming nucleoli by seeding the nucleation of proteins that condense and delineate nucleoli. When the rRNA transcription is inhibited at NORs, the nucleoli were reformed at random locations (Shevtsov and Dundr, 2011; Berry *et al.,* 2015; Falahati *et al.,* 2016). Other nuclear structures such as Cajal bodies (Kaiser *et al.,* 2008; Sawyer *et al.,* 2016; Wang *et al.,* 2016), non-nucleolar transcription assemblies (Hnisz *et al.,* 2017) and possibly the nuclear speckles are formed through condensation by seeded nucleation (Spector and Lamond, 2011; reviewed in Strom and Brangwynne, 2019). Thus, the seeded nucleation might be a common mechanism for the formation of the nuclear bodies that ensures their number and localization.

The protein condensation requires proteins to contain specific domains such as IDRs that can form of ionic, multipolar, hydrophobic, cation-pi, and pi-pi interactions (Chong *et al.,* 2018; Vernon *et al.,* 2018; Brangwynne *et al.,* 2015; Bremer *et al.,* 2022). The IDRs are type of sequences that do not possess a defined secondary structure. Therefore, these regions rarely have aliphatic or aromatic amino acids that usually build the core of the folded domains that stabilizes the structures in a low energy state. In contrast, IDRs do not stabilize in a single energy state; instead, they can get into a range of conformations with similar energy states. This flexibility of IDRs enables them to interact with each-other, forming condensates through transient multivalent bonds that enables them to behave like water molecules. These condensates are recognized by their spherical shape which reforms after a force removal such as fusions, fission, etc. They also show rapid fluorescence recovery after photobleaching (FRAP) and a sensitivity to 1,6-hexanediol that impairs their phase separation characteristic. They exist both in cytoplasm and nucleus and are dynamic liquid-like structures in physiological conditions. Notably, these condensates can solidify and aggregate leading to several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease amyotrophic lateral sclerosis, and frontotemporal dementia (Polymenidou and Cleveland, 2011; Ling *et al.,* 2013).

### 1.3.7.1.     *Liquid-Liquid Phase Separation in RNAPII Transcription*

The mammalian transcription mechanism is formed through the condensation of the transcription factors, co factors and Polymerase itself. These condensates can be found at the typical enhancers and super enhancers, having higher density of transcription factors and coactivators to establish a cell identity (Sabari *et al.,* 2018; Whyte *et al.,* 2013). In general, the transcriptional factors carry transactivation domains which contain a disordered region to facilitate their condensation. A recent study has shown that a naturally occurring product, procyanidin C-13,3',3"-tri-O-gallate (PCG),  binds specifically to the transcriptional co-activator BRD4 and inhibits its condensation at enhancers, shutting down specifically BRD4-dependent transcription (C. Wang *et al.,* 2021). Furthermore, the molecular crowding agents was shown to promote RNAPII to phase separate by itself (Boehning *et al.,* 2018). The largest subunit of RNAPII, RPB1, phase separates through its CTD domain which is also an IDR. Therefore, the condensation of RNAPII is directly linked with the CTD code wherein the addition of phosphate groups largely impacts the LLPS propensity of RNAPII (Guo *et al.,* 2019; reviewed in Portz and Shorter, 2020).

At the beginning of the transcription, the Mediator subunit Med1 and BRD4 are recruited to super enhancers and form phase-separated condensates (Sabari *et al.,* 2018). At these condensates, Mediator complex and the GTFs facilitate the recruitment of RNAPII to the promoter region which is mostly unphosphorylated. The BRD4 on the other hand, recruits P-TEFb to the condensate which phosphorylates the Ser5 residues of the CTD. The Ser5 phosphorylation marks the initiation stage where RNAPII is visualized as clusters that are bright spots of large accumulations in the nucleus (Cisse *et al.,* 2013; Boehning *et al.,* 2018). This phenotype of RNAPII is not observed when the phosphorylated Ser2 residues are visualized. Since Ser2 phosphorylation marks the elongation of RNAPII, it indicates that the RNAPII condensates are prevalent during the two steps of the transcriptional cycle; the initiation and the promoter-proximal pause. Indeed, recent progress indicates that NELF and P-TEFb regulates promoter-proximal pausing through a phase separation mechanism (Lu *et al.,* 2018; Rawat *et al.,* 2021).

NELF and DSIF pause transcription through a physical interaction with RNAPII. The regulatory subunit of P-TEFb called cyclin T1 comprises a histidine-rich domain which is a low complexity region that promotes interaction with the CTD. This interaction allows CDK9, the catalytic domain of P-TEFb, to phosphorylate CTD and NELF to separate the elongation complex from its condensate (Lu *et al.,* 2018). NELF undergoes phase separation through its disordered region, NELFA. When cells are in stress P-TEFb becomes unable to phosphorylate NELFA,

promoting the phase separation of NELF that inhibits the transcription at promoter proximal pause (Rawat *et al.,* 2021). This averted-transcription of housekeeping genes is required under stress conditions to ensure cell survival (reviewed in Wang B. *et al.,* 2021).

As a feedback mechanism, the products of the transcription condensates – RNAs - can also regulate the formation of the transcription condensates (Henninger *et al.,* 2021). Low levels of RNA (or short polymers) were found to promote the formation of the condensates whereas the increasing RNA levels (or long polymers) started to dissolve these condensates. This mechanism is considered like a poly-electrolyte system where the negatively charged RNA molecules promotes the interaction between the positively charged proteins when both charges are equal. However, the increase in the RNA levels causes negative charges to repulse each other and dissolves the transcriptional condensates (reviewed in B. Wang *et al.,* 2021). Basing on this, micro-emulsions were shown to lead phase separation into segregated microdomains (Hilbert *et al.* 2021). In the model of Hilbert *et al.,* two phases are described as one being transcriptionally inactive chromatin (long polymer) and the other being RNA and RNA-binding proteins. The RNAPII is considered as an amphiphile that connects the two phases and promotes the formation of larger RNA-RBP phases by synthetizing RNA (short polymer). This model bridges both polymer-polymer and liquid-liquid phase separation that provides a physical principal of how transcription can organize the euchromatin. Recently, as a chromatin remodeler, actin was also determined to promote the phase separation of the amphiphilic RNAPII (Mahmood *et al.* 2021; Wei *et al.,* 2020).

### 1.3.8.  The Nuclear Actin

Actin is an essential component of the cell cytoskeleton, providing structural and regulatory functions in a variety of crucial cellular processes. The presence of actin in nucleus is widely known for several years and increasing evidence highlight its importance in nuclear processes such as transcription (Percipalle *et al.,* 2013; Hofmann *et al.,* 2004).

Actin does not contain an NLS signal for active transport into the nucleus and the size of actin (42kDa) is quite large for passive diffusion through nuclear pore complexes. Yet, actin constantly shuttles between nucleus and cytoplasm (McDonald *et al.,* 2006). Studies demonstrated the transportation of monomeric actin occurs through binding to its cofactors, cofilin and profilin, which shuttle in and out of the nucleus by active transportation (Dopie *et al.,* 2012; Stüven *et al.,* 2003).

Overall, the nuclear actin is involved in many aspects of the gene transcription. It is present in chromatin remodeling complexes (Kapoor *et al.,* 2014); it participates the

transcriptional machinery of all three polymerases (Percipalle *et al.,* 2013; Hofmann *et al.,* 2004; Philimonenko *et al.,* 2004); it regulates the activity of the serum response factor (SRF) cofactor MAL (Vartiainen *et al.,* 2007); it binds to heterogeneous nuclear ribonucleoproteins (hnRNPs) to regulate transcription (Percipalle *et al.,* 2001; Kukalev *et al.,* 2005; Obrdlik *et al.,* 2008) and it is involved in DNA damage repair (Belin *et al.,* 2015; Schrank *et al.,* 2018; Caridi *et al.,* 2018; Okuno *et al.,* 2020), chromatin organization (Chuang *et al.,* 2006; Dundr *et al.,* 2007; Baarlink *et al.,* 2017) and genomic integrity (Saha *et al.,* 2016).

### 1.3.9. Nuclear Actin Polymerization

The polymerization of actin was generally considered cytoplasmic and its nuclear presence was found controversial for a long time due to the methodological difficulties of its visualization. Now, it is well-established that nuclear filamentous actin (F-actin) can be present both in physiological and stress induced conditions (Grosse and Vartiainen, 2013; Plessner and Grosse, 2019; Caridi *et al.,* 2019; Wei *et al.,* 2020). Although the cytoplasmic actin is mainly monomeric and filamentous, the nuclear actin was shown in multiple states as monomeric, dimeric, oligomeric or even liquid –like forms (Schoenenberger *et al.,* 2005; Jockusch *et al.,* 2006; Weirich *et al.,* 2017). However, the roles of the forms of actin other than the monomeric and filamentous are still elusive.

The nuclear functions of actin depend on its nuclear concentration which is mainly regulated by the polymerization status of both nuclear and cytoplasmic actins that sequestrates the monomeric actin into filaments (Miralles *et al.,* 2003; Huet *et al.,* 2012; Saha *et al.,* 2016). Indeed, the nuclear F-actin formations disrupted actin–RNAPII interaction and impaired RNA polymerase II localization that reduced global transcription levels and inhibited cell proliferation (Serebryannyy *et al.,* 2016a). Therefore, the cell utilizes the actin concentration between the nucleus and cytoplasm as a communication channel through which it regulates the transcriptional activity (Sharili *et al.,* 2016). In agreement with this, the serum stimulation, that affects the actin cytoskeleton organization (Schratt *et al.,* 2002), was shown to induce actin polymerization in nucleus through the activity of diaphanous-related formins (mDia1/2) (Baarlink *et al.,* 2013; Sidorenko and Vartiainen, 2019). Studies show correlation between the serum stimulation and changes in RNAPII cluster formation (Cisse *et al.,* 2013; Cho *et al.,* 2016) which might be due to the alterations in the SRF-MAL signaling pathway that regulates gene expression (Baarlink *et al.,* 2013). More recently, the serum induction was suggested to promote RNAPII clusters through N-WASP and Arp2/3 branched F-actin formations (Wei *et al.,* 2020).

### 1.3.10. Actin and Myosins in RNAPII Transcription

The roles of actin and myosin in transcription have been investigated for decades. Both are involved in the transcription machinery of all three RNA polymerases (Fomproix and Percipalle, 2004; Hofmann *et al.,* 2004; Hu *et al.,* 2004; Kukalev *et al.,* 2005; Philimonenko *et al.,* 2004) with actin being bound to their common subunits RPABC2 and RPABC3 (Hu *et al.,* 2004). Actin also interacts with the CTD domain of RNAPII which is not present in other polymerases (Kukalev *et al.,* 2005). Pre-initiation complex formation at promoters of the expressing genes requires actin for transcription (Hofmann *et al.,* 2004, Obrdlik *et al.,* 2008). The functional interaction between actin and RNAPII was shown *in vivo* and *in vitro* by anti-actin antibodies which inhibited the transcription (Hofmann *et al.,* 2004; Hu *et al.,* 2004). These evidences supported the role of actin as a transcriptional factor which is first suggested when RNAPII transcription was stimulated by exogenously added actin (Scheer *et al.,* 1984).

The promoter-proximal escape requires the activity of the CDK9, a subunit of P-TEFb, which phosphorylates the Ser2 residues of the CTD of RNAPII. CDK9 binds to the monomeric G-actin through its conserved Thr186 in the T-loop and promotes the transcriptional elongation (Qi *et al.,* 2011). After the promoter-proximal escape actin remains bound to the hyper-phosphorylated CTD and interacts with hnRNPU for H3K9 acetylation to regulate transcription elongation (Percipalle *et al.,* 2001). Actin might be polymerizing throughout elongation stage by the activity of cofilin (Obrdlik and Percipalle, 2011).

Similarly, the Nuclear Myosin 1 (NM1) was identified as an interactor of RNAPI and RNAPII (Pestic-Dragovich *et al.,* 2000) where the *in vitro* studies revealed the function of NMI in RNAPII complex as to mediate the formation of the first phosphodiester bond (Hofmann *et al.,* 2006b). Through its PH domain, NMI was found to be interacting with the PIP2 rich-nuclear lipid islets (NLIs) which are shown to facilitate RNAPII transcription (Sobol *et al.,* 2018). Further, NMI was described to shuttle in and out of the nucleus and its nuclear localization was determined to be dependent on its PIP binding ability (Nevzorov, 2018). NMI also has a role in nucleosome organization through interacting with the B-WICH complex that acetylates the downstream nucleosomes to promote gene expression (Almuzzaini *et al.,* 2015).

Myosin VI (MVI) was described as another actin-based molecular motor that drives the RNAPII transcription (Vreugde *et al.,* 2006). Its interaction with NDP52 unfolds the tail domain of MVI which enables its cargo binding domain to bind DNA (Fili *et al.,* 2017; Fili and Toseland, 2019). It was shown that the inhibition of its motor activity by small molecule TIP (Heissler *et al.,* 2012) reduced *in vitro* RNAPII transcription around 75%, indicating that MVI is required for full

activation of RNAPII (Cook *et al.,* 2018). Based on these evidences, it is suggested that NDP52 promotes dimerization and activation of MVI in nucleus which then binds to actin on the RNAPII complex and also to DNA to facilitate the movement of RNAPII on DNA (Fili *et al.,* 2017).

MVI was determined to localize on nuclear actin filaments where it moves along them for several micrometers. This movement, which requires the activity of ATPase, was not affected by the inhibition of RNAPII transcription but rather associated to long range chromatin arrangements (Große-Berkenbusch *et al.,* 2020). Yet, MVI suggested to mediate promoter-proximal release of RNAPII machinery, as allelic pairing of the tumor necrosis factor (TNF) required MVI at its locus to be released from the promoter-proximal pause within TH1 cells (Zorca *et al.,* 2015). Similarly, the most recent study on MVI demonstrated the importance of MVI in transcription initiation by facilitating the phase separation of RNAPII that determines its nuclear dynamics (Hari-Gupta *et al.,* 2022).

### 1.4. Myosin Phosphatase Rho-Interacting Protein

The Myosin Phosphatase Rho-Interacting Protein (MPRIP) is a myosin interactor and an F-actin regulator. It was first identified in murine vascular smooth muscle cells where it localized to the acto-myosin stress fibers (Surks *et al.,* in 2003). When bound to these stress fibers, MPRIP recruits both RhoA and MYPT1 to regulate MLCP (Myosin Light Chain Phosphatase, catalytic subunit: PP1) holoenzyme activity, facilitating myosin dephosphorylation (Surks *et al.,* 2003; Mulder *et al.,* 2004; Koga and Ikebe, 2005; Riddick *et al.,* 2008). Depletion of MPRIP leads to an increased number of stress fibers in smooth muscle cells through stabilization of actin fibers, whereas its overexpression leads to disassembly of stress fibers in neuronal cells (Mulder *et al.,* 2004; Surks *et al.,* 2005; Koga and Ikebe, 2005; Riddick *et al.,* 2008). The overexpression of MPRIP was also determined to inhibit RhoA-induced SRF activation without affecting RhoA-GTP levels (Mulder *et al.,* 2005). The C-terminal of MPRIP was shown to self-oligomerize and the disruption of this oligomerization did not affect its inhibitory activity towards SRF (Mulder *et al.,* 2005). Furthermore, MPRIP mRNA expression was shown to be reduced when JNK1 kinase expression was knocked-down by siRNAs. It is then suggested that EGF-induced JNK1 can upregulate MPRIP expression and regulate cell motility through actin turnover, facilitating an invasive phenotype in cancers (Ono *et al.,* 2008). In addition, NUAK2 kinase was determined to bind to MPRIP through its C-terminal at the same region that binds to MYPT1. However, the NUAK2-MPRIP association was shown to inhibit myosin light chain dephosphorylation and contribute to the actin stress fiber maintenance in HeLa cells (Vallenius *et al.,* 2010). Recent work determined MPRIP in regulation of DDR1 receptor activation which is involved in collagen migration and contraction through the actomyosin associated integrins (Coelho *et al.,* 2020).

MPRIP is a relatively large protein (~120 kDa) and it comprises two PH domains (PH1: 44-153; PH2: 387-484) as putative binding sites to PIP2 (Surks *et al.,* 2003). In between the two PH domains, it contains an IDR. The first PH domain at the N-terminal region is covered by an F-actin binding domain (2 – 383), which might suggest that this region binds exclusively either to actin or PIP2, determining the nuclear localization and function of MPRIP. The C-terminus of MPRIP is a disordered region (Balaban *et al.,* 2021) with exception of three short coiled-coil domains (CC1: 672-707; CC2: 728-878; CC3: 900-974) that are known to mediate binding to RhoA and MYPT1 (Surks *et al.,* 2003).  There are three known isoforms of this protein, two of them lacking a small portion at the tail of the protein (1017-1025) (Surks *et al.,* 2003), where the isoform 3 also lacks a region (346-383) at the IDR in between the two PH domain corresponding the last thirty-seven amino acids of the F-actin binding domain. Overall, these structural features of MPRIP suggests that it can be involved in a range of processes, both in nucleus and in cytoplasm, that are regulated by PIP2, F-actin and phase separation.

## 2. AIMS

Nuclear PIP2 is a versatile regulator and a determinant of the nuclear architecture. It is involved in one of the most essential cellular processes; the RNAPII transcription. However, the regulatory pathway showing how PIP2 affects the RNAPII transcription is still elusive. Therefore, with this work, we first aim to define the characteristics of the PIP2-interactome by a novel mass spectrometry approach. This lead us to discover an F-actin-binding protein (MPRIP) which constitutes a link between RNAPII and PIP2. We then sought to elucidate the nuclear functions and LLPS propensity of MPRIP in relation to PIP2-containing nuclear structures. Finally, we aim to reveal the molecular mechanism underlying the regulatory axis of PIP2 and actin dependent-RNAPII transcription machinery.

- To identify the nuclear PIP2 interactome and to define some of the functional interactions of PIP2.

- To elucidate nuclear functions and LLPS characteristics of MPRIP in relation to PIP2 and nuclear architecture

- To reveal the molecular mechanism underlying the regulatory axis of PIP2 and actin dependent-RNAPII transcription machinery

# 3. RESULTS & DISCUSSION

In this section, the experimental work of each publication was first described and then, the obtained results were discussed. The figures for each published work are presented at the Section 7; Publications and Manuscripts.

## 3.1. Limited Proteolysis-Coupled Mass Spectrometry Identifies Phosphatidylinositol 4,5 Bisphosphate Effectors in Human Nuclear Proteome

### 3.1.1. A Novel Workflow to Identify Nuclear Effectors of PIP2

The Limited Proteolysis-Coupled Mass Spectrometry (LiP-MS) method was first described in 2014 by Feng *et al.,* to analyze the structural changes in complex proteomes (Feng *et al.,* 2014). Originally, this approach applies a double digestion with two different proteinases. The first digestion includes a broad-specificity protease (such as proteinase K, subtilisin and thermolysin) which is applied for a short time and at a low enzyme substrate ratio to create large protein fragments; hence, the limited-proteolysis. By utilizing control beads as a binding surface and applying trypsinization for a short-time, we were able to create the large protein fragments for MS analysis. This method is also applicable to other PIPs and ligands that are covalently bound to agarose beads. Currently, the LiP-MS approach is gaining more recognition as it is a label-free method which enables peptide level resolution, offering broad applicability in a native-like environment and proteome-wide scale (Schopper *et al.,* 2017; Pepelnjak *et al.,* 2020; Zhang and Shah, 2021; Shuken *et al.,* 2022).

In our study, a novel quantitative mass-spectrometry workflow was employed to identify the nuclear effectors of PIP2 and to characterize the functional interactions that associates with nuclear PIP2. The experimental workflow was based on Limited proteolysis-coupled quantitative mass spectrometry (LiP-qMS) where two different protocols were applied prior to MS analysis in order to determine two types of PIP2-binding regions of the nuclear proteins. In one set of experiments, the trypsinization was applied simultaneously as the pull-down with PIP2 beads, whereas in the other set of experiments the pull-down was performed after the trypsinization. Since PIP2 binding domains and motifs might require a conformational change to be exposed, the trypsinization before the PIP2 pull-down revealed these "hidden" binding regions. Therefore, the PIP2 effectors were divided into two groups; the effectors, containing regions that are readily available for PIP2 interaction are named "Exposed", and others that are not primarily accessible are named "Hidden".

One main obstacle in this protocol was the heat inactivation of the trypsin, which is eliminated by a high efficacy protease inhibitor mix (Figure 1A). Our protocol that allows preparation of highly concentrated nuclear lysates, which is required for the subsequent MS analysis, showed very low cross-contamination from cytoplasm (Figure 1B). Furthermore, the covalent PIP2-conjugated agarose beads enabled us to perform the pull-down after trypsin inactivation, defining the "hidden" PIP2-effectors.

### 3.1.2. The Functional Analysis of the Nuclear Effectors of PIP2

Our PIPsLiP-qMS experimental approach identified in total of 515 PIP2-effector proteins, of which 191 were identified as "exposed" and 324 were identified as "hidden" with a threshold of 2-fold increase in abundance (Figure 2).

The GOCC (gene ontology cellular component) analysis revealed four prominent categories that are associated with the "exposed" PIP2-effectors (Figure 3). One category contains cytoskeleton regulatory proteins which are known to translocate to the nucleus. The other two corresponds to the two PIP2-containing nuclear bodies; nuclear speckles and nucleolus. The fourth category "non-membrane-bounded organelle" reflects proteins associated to an organized structure of distinctive morphology and function, without possessing a membrane. Altogether, their associated biological processes connected with regulation of RNAPII mediated transcription, mRNA processing, and actin organization. This shows that the exposed effectors are highly involved in processes that are related to gene expression. In contrast, the "hidden" effectors are associated with nucleoplasm, nuclear envelope and nuclear pores, with their associated biological processes being at the downstream of the exposed effectors such as mRNA transport and RNA localization and also, peripheral processes connected to protein import and nuclear envelope disassembly.

We next sought to analyze the hydropathy index of the PIP2-effectors. The PIP2 bound peptides of the "exposed" group showed the highest hydrophilicity at their side chains, suggesting these regions are not composing the core of the globular protein domains. On the other hand, the PIP2 binding regions of the "hidden" group showed lower hydrophilicity as expected, since these regions are not readily available for PIP2 interaction (Figure 4).

### 3.1.3. K/R Motifs are Common in PIP2-effectors

Our analysis on the amino acid sequence of the PIP2-effectors revealed that only few of them contain canonical PIP-binding domains. However, the substantial part of the PIP2-effectors contains a K/R motif (Supplementary Tables S4 and S5). The K/R motifs consist of short stretches of basic amino acids and they presumably provide less-stable interactions compare to the

structured binding domains (Jacobsen *et al.,* 2019). In addition, the poly-K/R motifs are also common in IDRs of the RNA-binding proteins (Zaharias *et al.,* 2021). This is also explained with the linker and sticker theory; where the specific regions of the IDR are acting as stickers–such as K/R motifs-, promoting phase separation by increasing the cohesive interactions and the linkers are providing a space for flexibility between stickers (Harmon *et al.,* 2017, 2018). Therefore, it is possible that these positively charged residues (K/R motifs) are promoting phase separation of the IDRs by increasing their presence in the proximity of negatively charged RNAs and PIP2, providing the liquid-like nature of the LLPS structures.

The work of Ilık *et al.,* showed that the deletion of the IDRs of both SRRM2 and SON proteins lead to near-complete dissolution of nuclear speckles (Ilık *et al.,* 2020). Our experimental data identified SRRM2 as an "exposed" effector of PIP2 and the hydropathy index of SRRM2 is calculated as -1.358 which is highly hydrophilic compare to the rest of the PIP2-interactors (average being -0.31). It is noteworthy that phase-separating proteins have hydrophilic side chains due to their IDR regions (Uversky, 2019). Moreover, the SRRM2 protein contains several K/R motifs at the N- and C-terminal, as putative binding regions to PIP2 and a long IDR region covering almost its entire sequence. The nuclear pattern of the SRRM2 protein show resemblances to the nuclear pattern of NLIs, which might as well be formed by the condensation of this protein. The dehydrated core of the SRRM2 condensate can sequestrate the acyl chain of PIP2, whereas the K/R motifs might support its stability and promote other protein-PIP2 interactions. In such case, it should be considered if NLIs are originated from nuclear speckles and able to recycle, as both are PIP2-containing dynamic structures, participating to RNAPII transcription.

### 3.1.4. Actin-Binding PIP2-effectors Regulate RNAPII transcription

Our experimental analysis identified actin binding proteins such as Cofilin-1 (exposed), Profilin (Exposed), which are known to translocate to the nucleus by active transportation (Dopie *et al.,* 2012; Stüven *et al.,* 2003). These proteins are known to interact with PIP2 in cytoplasm to regulate F-actin (Mandal, 2020) and it is possible that they might be regulating the nuclear actin dynamics or expression of the specific genes by interacting with the nuclear PIP2. For example, Cofilin was recently described as a mechanosensitive regulator of RNAPII transcription that increase its nuclear presence and promote RNAPII transcription elongation, when cells are grown on a soft surface (Domingues *et al.,* 2020). Furthermore, we have identified nuclear myosins as PIP2-effectors some of which are known transcription factors such as NMI and Myosin VI (Hofmann *et al.,* 2004; Hari-Gupta *et al.,* 2022). As a myosin interactor, MPRIP protein was determined to be an exposed PIP2-effector with 2-fold enrichment. Its two PH domains are

the putative binding sites for PIP2 which can be hidden when bound to F-actin (Singh *et al.,* 2021). The hydropathy index of - 0.971 reveals the highly hydrophilic structure of MPRIP which is mostly due to its C-terminal IDR (-0.995). In addition, MPRIP comprises six K/R motifs, five of them corresponding to its IDRs. Our further analysis determined MPRIP as an interactor of NMI constituting a link between RNAPII and PIP2. Therefore, we have studied the phase characteristics of MPRIP on the regulatory axis of PIP2, RNAPII and F-actin.

## 3.2. The F-Actin-Binding MPRIP Forms Phase-Separated Condensates and Associates with PI(4,5)P2 and Active RNA Polymerase II in the Cell Nucleus

### 3.2.1. MPRIP is a Nuclear Protein Which Associates with PIP2 and RNAPII

MPRIP is a known F-actin interactor, regulating the turn-over of the actin stress fibers in the cytoplasm (Surks *et al.,* in 2003; Mulder *et al.,* 2004; Koga and Ikebe, 2005). By this work, we have provided evidence for the presence of MPRIP in the cell nucleus of human cells (Figure 1). Since MPRIP is a large protein of 120kDa, it would need active transport to be translocated to the nucleus. Our bioinformatics analysis predicted an NLS motif that was covering the amino acids in position 155 to 164 (Figure S1). The two PH domains of MPRIP are putative sites for interaction with PIP2. Therefore, we have tested whether MPRIP binds to PIP2 specifically. All seven PIP variants were used to pull-down MPRIP from HeLa nuclear lysates. This revealed that MPRIP binds exclusively to PIP2 (Figure 2) and further indicated that MPRIP might reside at PIP2-rich nuclear structures. To determine the sub-nuclear localization of MPRIP, we have employed the STED microscopy that localized MPRIP and PIP2 with 28nm resolution in U2OS nuclei (Figure 3A). The subsequent statistical analysis revealed that MPRIP localizes to the proximity of the PIP2 signal from the nuclear speckles and NLIs (Figure 3B-G). As NLIs and nuclear speckles are involved in the regulation of the active RNAPII transcription, we have tested if MPRIP might constitute a link between PIP2 and RNAPII. The immunoprecipitation experiment, using HeLa nuclear cell lysates, identified MPRIP in the same complex with active RNAPII CTD and transcriptional factor, NMI (Figure 4).

### 3.2.2. Overexpression of GFP-MPRIP Forms LLPS Structures that Interact with F-actin

To verify the IF results, we overexpressed the GFP-tagged MPRIP in U2OS cells. The transfected cells showed three different nuclear patterns for MPRIP. The most common was a diffused granular pattern resembling IF with specific antibody. A second phenotype showed microscale-sized globular structures, and the last and least observed phenotype showed fibrous

structures (Figure 5). We have subjected the cells that show globular structures to 1,6-Hexanediol that dissolved them completely after 2 minutes of incubation. Yet, the 2,5-Hexanediol did not lead to this complete dissolution (Figure 6). This indicated that these MPRIP structures are formed through LLPS. We have then recorded the condensates for 8 hours by a live-cell imaging experiment. The condensates showed dynamic behavior as they were able move, merge and divide while preserving their globular shapes (Figure 7A and Video S1). The combination of our experiments (Hexanediol treatment, live cell imaging and FRAP) provides evidence that MPRIP forms condensates through LLPS. Furthermore, our live-cell imaging captured the formation of the fibrous formations, which were originating from the globular structures (Video S1). This dynamic behavior of LLPS structure presumably occurs due to surface condensation that is nucleated by a polymer (described in; Renger *et al.,* 2022; Morin *et al.,* 2022). Therefore, we wanted to test whether nuclear F-actin formation was driving the formation of the fibrous structures. To that end, we have performed Fluorescence Recovery After Photobleaching (FRAP) experiments and determined the diffusion kinetics of each MPRIP structures. Both globular and fibrous structures showed high mobility for MPRIP molecules, however an immobile GFP-MPRIP fraction was determined in fibrous structures, as the recovery percentage of the fibers were almost half of the globular structures. This indicated that the fibrous structures are LLPS condensates that are bound to a nuclear structure. Furthermore, to determine if the fibrous structures were bound to nuclear F-actin, we have stained the GFP-MPRIP overexpressing cells with phalloidin. As expected, our microscopy imaging revealed F-actin structures which are covered with MPRIP condensates (Figure 9). Finally, to determine the region of MPRIP which is responsible for phase separation, we have split MPRIP in two fragments; the fragment 1 (f1) is the N-terminal region, carrying structured binding domains (two PH and one F-Actin binding domain) and the NLS sequence, and fragment 2 (f2) is the C-terminal region which is mostly disordered (Figure S3) but also carrying low complexity coiled coils for binding to F-actin interacting proteins such as RhoA, NUAK2 and MYPT1 (Surks *et al.,* in 2003; Mulder *et al.,* 2004; Koga and Ikebe, 2005; Vallenius *et al.,* 2010). The overexpression of f1 did not cause any LLPS structures in nucleus, supporting the presence of the predicted NLS. The f1 also found to be bound to the stress fibers in nucleus, which is mediated by its F-actin binding region. In contrast, the f2 was not translocated to nucleus and formed both globular and fibrous LLPS structures in cytoplasm, conforming that IDR of MPRIP is mediating its phase separation.

### 3.2.3. MPRIP as an Actin Binding Protein in the Cell Nucleus

In cell lysates, MPRIP is observed in two different forms (Figure 1), which are presumably correspond to either an isoform or a different post-translational modification (PTM) of the protein. The predicted PTM sites showed that MPRIP is mostly phosphorylated from its F-actin binding region and also from its C-terminal IDR region (Figure S4). The larger form was observed exclusively in the nuclear fraction which suggests a different function for MPRIP in nucleus (Figure 1). In addition, the heavier form might also be necessary for translocation of MPRIP to nucleus. Since the NLS motif of MPRIP is covered by its F-actin binding region, a specific phosphorylation at this region might prevent the binding of MPRIP to F-actin, exposing its NLS motif. Moreover, the F-actin binding region also hinders the first PH domain of MPRIP. Therefore, in nucleus, MPRIP is more likely to interact with PIP2-containing structures, unless a stimulus induces nuclear F-actin formation (Figure 3, 9). The actin binding protein, MRTF-A, has a similar molecular mechanism (Vartiainen *et al.,* 2007; Pawłowski *et al.,* 2010; Mouilleron *et al.,* 2011). MRTF-A is predominantly cytoplasmic and its NLS motif is covered by its actin-binding RPEL domain. Upon RhoA-dependent actin polymerization, the cytoplasmic actin concentration decreases and exposes the NLS motif of MRTF-A which is then translocated to nucleus to regulate the transcription of the cytoskeletal genes (reviewed in Ulferts *et al.,* 2021). The identification of MPRIP in the active RNAPII complex also indicates a role in the transcription. Therefore, depending on the cytoplasmic actin filament turnover, MPRIP can increase its nuclear concentration to regulate RNAPII transcription in close proximity of NLIs and nuclear speckles.

### 3.2.4. Condensation of MPRIP

The transcriptional factors such as NELF and BRD4 tend to phase separate in order to regulate the RNAPII transcription (Rawat *et al.,* 2021; B. Wang *et al.,* 2021). Similarly, the phase separation of MPRIP might consist a crucial step in this mechanism. It is possible that the exposed PH domains localize MPRIP to NLIs and nuclear speckles and nucleate its condensation. Yet, it should be determined if phase separation of MPRIP occurs in physiological or pathological conditions. The nuclear F-actin formations need to be induced by serum stimulation or heat shock treatment to be visualized (reviewed in Serebryannyy *et al.,* 2016b). The stable cell lines that were expressing GFP-MPRIP showed globular nuclear condensates where the fibrous formations are induced when cells are kept in 41°C for one hour (Figure S6). This provides further evidence that the globular MPRIP condensates can reshape and bind to the nuclear F-actin structures. This is an important finding showing a novel behavior for the LLPS structures, including the nuclear bodies. This experiment demonstrates that the form of the LLPS condensates can be modulated by a region that binds to a cellular structure.

Many neurodegenerative disorders are associated with the solidification of the phase-separated condensates such as the tau protein in Alzheimer's disease (Kanaan *et al.,* 2020). We have also observed the time-dependent solidification of the MPRIP condensates. In addition, MPRIP was shown to fuse with three kinases causing fusion kinase cancers with increased expression levels (Vaishnavi *et al.,* 2013; Stransky *et al.,* 2014; Naumann *et al.,* 2015; Fang *et al.,* 2019). The fusion kinase cancers that fuses with the C-terminal of the MPRIP (such as ALK1, Fang *et al.,* 2019) are prone to form LLPS condensates due to the increased expression levels. The cancer treatments that are targeting LLPS condensates are already drawing attention (Jiang *et al.,* 2020; Klein *et al.,* 2020). Therefore, the phase separation of MPRIP and its involvement in RNAPII transcription (described and discussed in section 3.3) might be critical in some cancers and it might offer new approaches for treatment of these diseases.

## 3.3. RNA Polymerase II Condensation and Transcription is Regulated by the Association of the Transcriptional Complex with PI(4,5)P2

### 3.3.1. MPRIP Localization Correlates with RNAPII and Determines the Transcriptional Output

The identification of MPRIP in the cell nucleus, in the same complex with RNAPII prompt us to further investigate its role in transcription. To that end, we first aim to visualize the localization of MPRIP in comparison to three different phosphorylated forms of RNAPII CTD (Ser5P-CTD, Tyr1P-CTD, Ser2P-CTD) , which mark different stages of the transcription. The Ser5P-CTD marks the initiation, the Tyr1P-CTD marks the transition to elongation (also called, initiation-release) and Ser2P-CTD marks the elongation stage of the transcription (Mayfield *et al.,* 2019). The spatial distribution of MPRIP in regard to the three phosphorylated forms of RNAPII-CTD showed significant correlations, where the Tyr1P- CTD marked the highest significance (Figure 1).

The transition to elongation which is labelled by Tyr1P-CTD, is a critical regulatory stage in transcription and the defects at this stage can abort transcription and inhibit new transcription initiation, leading to decreased transcriptional output (Mayfield *et al.,* 2019; Shao and Zeitlinger, 2017; Gressel *et al.,* 2017, 2019). Therefore, we have determined the transcriptional activity of the cells that were MPRIP depleted (Figure 2A). To measure the transcriptional activity, we have quantified the signal from the 5-Fluorouridine (5-FU) that is incorporated to the nascent transcripts (Jao and Salic, 2008). The siRNA-mediated depletion of MPRIP caused a substantial decrease in the signal from the incorporated 5-FU, whereas the signal level of PIP2 remained constant (Figure 2B). The statistical analysis of the spatial distributions of 5-FU and PIP2 revealed

that the depletion of MPRIP affected the 5-FU incorporation level at nucleoplasm (NLIs) and at nuclear speckles (Figure 2C). The decrease of spearman coefficient between PIP2 and 5-FU signal in MPRIP depletion, indicates that the reduction of 5-FU incorporation into nascent RNA occurs in correlation to PIP2. This strengthens the importance of PIP2 in RNAPII transcription.

### 3.3.2. The Initiation Condensate Formation Correlates with the Association between RNAPII and PIP2

In the initiation stage, the Ser5-phosphorylated RNAPII complex associates with phase-separated condensates (Guo *et al.,* 2019; Hilbert *et al.,* 2021). Studies revealed that the defects in the initiation stage cause an accumulation of Ser5-phosphorylated RNAPII at the promoter-proximal region of the gene (Hou *et al.,* 2019; Steurer *et al.,* 2018; also reviewed in Core and Adelman, 2019). Similarly, we have determined that the MPRIP depletion increases the number of the initiation condensates that are marked with Ser5P-CTD in U2OS cells. This result suggests that MPRIP affects the formation of the initiation-condensates, providing an explanation for the decreased transcriptional output (Figure 2, 3). Furthermore, we assessed whether MPRIP have an impact on the association between RNAPII and PIP2 in different stages of transcription. For this, we used PIP2-conjugated agarose beads to perform pull-down experiments from control and MPRIP depleted lysates, and we measured the association of different forms of RNAPII to PIP2 by using anti-Ser5P, Tyr1P and Ser2P-CTD specific antibodies (Figure 4). Since we performed the pull-downs from the total cell lysate, the determined protein interactions can be either direct or indirect. Bearing this in mind, we have determined an increased interaction between Ser5P-CTD and PIP2 when MPRIP was depleted. This observation might further explain the increased number of the initiation condensates, where the increased association between Ser5P-CTD and PIP2 reduces the dissolution of the initiation condensate. Furthermore, the markers for the later stages of the transcription (Tyr1P- and Ser2P-CTD) showed diminished association between RNAPII and PIP2, which is in accordance with the previous suggestion that the transcription might be aborted at promoter-proximal pause. In addition, the overall levels of the all three forms of RNAPII remained same in depletion of MPRIP, suggesting that MPRIP do not alter the phosphorylation state of RNAPII-CTD but affects its association with PIP2.

### 3.3.3. MPRIP Mediates the Association between RNAPII Complex and PIP2

To determine the predominant phosphorylated form of RNAPII-CTD that associates with MPRIP, we have performed a GFP-Trap experiment. The two GFP tagged fragments of MPRIP (f1 and f2, from Balaban *et al.,* 2021; see section 7.2) were expressed in U2OS cells and pulled-down by GFP-covered beads to determine which form of RNAPII-CTD associates with which region of MPRIP. The Tyr1P-CTD was revealed to associate with MPRIP through its N-terminal region (f1),

which comprises the two PH domains, an F-actin binding region and the NLS motif (Balaban *et al.,* 2021). We have also determined the transcriptional factor, NMI and beta-actin at this region. This result shows that Tyr1P-CTD is the predominant phosphorylated form of RNAPII that interacts with MPRIP and the presence of the initiation factors such as NMI and actin suggests that MPRIP interacts with the RNAPII initiation-complex. Regarding the Tyr1 phosphorylation of the CTD peaks at promoter proximal region (Harlen and Churchman, 2017), it is possible that MPRIP interacts with the RNAPII complex at the promoter-proximal region and affects its affinity to PIP2 to regulate the initiation-release of transcription.

To test whether MPRIP is mediating the association between PIP2 and RNAPII complex, we have performed another pull-down in the same setting as GFP-trap experiment but this time using the PIP2-conjugated agarose beads. The GFP-tagged MPRIP fragments that are pulled-down by PIP2 revealed that MPRIP, especially its N-terminal (f1), is crucial for recruitment of Tyr1P-CTD to PIP2 (Figure 6). Finally, to complement our biochemistry experiment, we compared the *in situ* localization of Tyr1P-CTD in regard to PIP2-containing nuclear structures, in control *versus* MPRIP depleted cell nuclei (Figure 7). The statistical analysis revealed a significant decrease in Tyr1-CTD localization to both PIP2-rich structures where the impact of the depletion was more prominent at the nucleoplasm (NLIs). This is in accordance with our biochemistry experiment and suggests that MPRIP regulates transcription through affecting the affinity of RNAPII to nuclear PIP2-containing structures.

### 3.3.4. MPRIP Associates with Nuclear-PIP2 Which is Involved in the Initiation of the Transcription

MPRIP was shown to localize to PIP2-containing structures in nucleus by STED microscopy (Balaban *et al.,* 2021). In this study, we have detected the presence of PIP2 signal localizing to RNAPII condensates which mark the initiation of the RNAPII transcription (Figure 3B, C). Accordingly, the Ser5-phosphorylated RNAPII complex was detected in PIP2-immunoprecipitations and the nuclear PIP2 was reported to localize with the nascent transcripts (Sobol *et al.,* 2018). The initiation factors, NMI and the transcription factor II D (TFIID), were also shown to associate with the nuclear PIP2 (Sobol *et al.,* 2018). It is noteworthy that the TFIID was recently shown to enable the promoter-proximal pausing (Fant *et al.,* 2020).

NMI interacts with the transcription complex through polymerase-associated actin to form the first phosphodiester bond of the transcript and maintain the active RNAPII transcription (Sarshad and Percipalle, 2014; Pestic-Dragovich *et al.,* 2000; Hofmann *et al.,* 2004). As NMI carries a PH-like domain, it was previously suggested that it can mediate the interaction between RNAPII and PIP2 (Sobol *et al.,* 2018). Similarly, we identified NMI in the same complex

with MPRIP and active RNAPII (Balaban *et al.,* 2021). Then, we determined the association of NMI and RNAPII with the N-terminal of MPRIP (Figure 5). Taken together, these results suggest that MPRIP associates with nuclear PIP2 that is involved in the regulation of the early stages of the RNAPII transcription.

### 3.3.5. Tyr1-Phosphorylation of the CTD at the Promoter-Proximal Pause

The study by Descostes *et al.,* determined that Tyr1 phosphorylation of the CTD peak at the initiation of the transcription (Descostes *et al.,* 2014). Another study showed that this transient increase in Tyr1 phosphorylations occurs during the promoter-proximal pause and alters the specificity of P-TEFb from phosphorylating Ser5 to Ser2 CTD that triggers the release of RNAPII (Mayfield *et al.,* 2019). During the promoter-proximal pause many regulatory proteins function in cohort that either abort the transcription or proceed it to the productive elongation (Muniz *et al.,* 2021). The Tyr1-phosphorylation of CTD by c-Abl kinase was shown to be one of the regulatory steps of the promoter-proximal pause (Mayfield *et al.,* 2019). Our data indicates that MPRIP is a regulatory protein that promotes the recruitment of Tyr1-phosphorylated RNAPII complex to PIP2, presumably controlling the release of RNAPII (Figure 6).

### 3.3.6. RNAPII Condensation During Promoter-Proximal Pause

One of the crucial determinants that regulates the transcription initiation is the propensity of RNAPII to form phase-separated condensates (Boehning *et al.,* 2018). Our findings further support this by showing decreased levels of RNAPII transcription that is correlated with an increase in the initiation condensate formation (Figure 2 and 3). This is in accordance with the study that shows accumulation of Ser5 at the promoter-proximal region due to the lack of a regulatory protein that is known to control pause-release (Paf1C; Hou *et al.,* 2019). Moreover, the RNAPII condensate formation and the transcription initiation were determined to be regulated by nuclear myosin VI (MVI) (Hari-Gupta *et al.,* 2022). The interaction between MVI and RNAPII was shown to be through actin (Fili *et al.,* 2017) and the cargo-binding domain of MVI is determined to bind DNA that might offer a force to burst RNAPII from its condensate to regulate the pause-release through short F-actin formations (Zorca *et al.,* 2015). This could be a combined effect with that of P-TEFb which phosphorylates many transcription factors, and Ser2-CTD to release RNAPII from the initiation condensate (Lu *et al.,* 2016).

The nuclear F-actin formations are important regulators of the RNAPII transcription which can affect condensation of RNAPII (Wei *et al.,* 2020). These formations can be induced upon serum-stimulation, effecting the nuclear actin concentration. The fluctuations in the concentration of an initiation factor such as actin, can determine the transcription activity. Indeed, studies has documented that the F-actin formations can both activate and repress the expression of

different genes (Hyrskyluoto and Vartiainen, 2020; Yamazaki *et al.,* 2020). In addition, discrete RNAPII condensate formations were observed in serum stimulation that induced nuclear actin polymerization, through the activity of actin-binding proteins (Wei *et al.,* 2020). Considering MPRIP is a negative F-actin regulator and its depletion increases the number of RNAPII condensates; it is possible that MPRIP plays a role in the regulation of nuclear F-actin that impacts RNAPII transcription. Nonetheless, further research is required to elucidate the exact role of actin in the MPRIP-associated RNAPII machinery.

In summary, we show that MPRIP represents a novel regulator of RNAPII transcription that defines the number of initiation-condensates and the transcriptional output. This regulatory action of MPRIP provides molecular insights into PIP2-associated RNAPII transcription mechanism. We determine that MPRIP interacts with Tyr1P-CTD and mediates its association with PIP2. Our immunofluorescence experiment supports this data, providing an *in situ* evidence for the nuclear function of MPRIP. Considering the literature on Tyr1P-CTD, several indications point that MPRIP is involved in the regulation of pause-release of RNAPII. Finally, we suggest that MPRIP might be involved in F-actin associated regulation of RNAPII transcription. Taken together, we demonstrate that MPRIP regulates RNAPII condensation and transcription by providing the association between the initiation complex and nuclear PIP2.

# 4. SUMMARY & CONCLUSIONS

## 4.1. Nuclear PIP2-effector proteins can be classified in two groups each associating with distinct nuclear localizations and functions that point to a structure-dependent molecular function

We have developed a novel quantitative Mass Spectrometry workflow that classified the PIP2-effectors in two groups as "exposed", and "hidden". The exposed effectors are associated with PIP2-containing nuclear bodies and involved in processes that are related to these structures, whereas the hidden effectors were associated with the processes that are related to nuclear periphery. This difference in the ontology of the PIP2 effector proteins indicates structure-dependent molecular processes where the hidden proteins are associated with the downstream processes of the exposed effectors.

## 4.2. K/R motifs are common in nuclear PIP2-effectors but they differ in hydropathy index

The analysis of the PIP2-effectors showed that only a small portion of them contains a structural PIP2-binding domains whereas almost all of them contains a K/R motif that is known to provide binding to the negatively charged phosphate head group of the PIP2. The hydropathy index further indicated that the exposed effectors are more hydrophilic than the hidden effectors, which might be due to the hydrophilic structure of the IDR containing domains that are presumably more common in the exposed effectors.

## 4.3. The PIP2-effector protein MPRIP is a nuclear protein that localize to NLIs and nuclear speckles where it associates with RNAPII

MPRIP was considered as a cytoplasmic protein. We have showed that MPRIP localizes to nucleus and possesses a predicted NLS motif. MPRIP is then determined to bind specifically to PIP2 and our super-resolution microscopy experiment localized MPRIP at NLIs and nuclear speckles. The immunoprecipitation experiment revealed MPRIP in the same complex with Ser5-phosphorylated RNAPII CTD, suggesting a function in transcription.

## 4.4. MPRIP can form phase-separated structures in nucleus that can interact with nuclear F-actin

GFP-MPRIP overexpression led to the formation of globular nuclear structures. We have confirmed that these structures are formed through LLPS by 1,6-Hexanediol treatment, FRAP and live cell imaging experiments. Surprisingly, these structures preserved their ability to bind F-actin structures taking a fibrous shape, when F-actin disappeared, the structures

revert their shape back to globular ones. We then showed that these highly dynamic structures are formed through the C-terminal IDR of the MPRIP.

## 4.5. MPRIP is a transcriptional regulator that mediates the association between RNAPII and PIP2

We show that the depletion of MPRIP promotes the formation of the initiation condensates and increases the affinity of Ser5-phosphorylated RNAPII complex to PIP2. We then revealed that N-terminus of MPRIP mediates the association between Tyr1-phosphorylated RNAPII complex and PIP2. This domain of MPRIP also carries an F-actin binding domain which suggest a role for F-actin in the MPRIP-mediated association of RNAPII to PIP2. Finally, we show that MPRIP determines the localization of the Tyr1P-CTD to nuclear speckles and NLIs. Our data indicates that, in MPRIP depletion, the RNAPII transcription might be impaired due to a defect at promoter-proximal pausing that leads to decreased transcriptional output.

# 5. FUTURE PROSPECTS

## 5.1. What is the IDR content of the proteins that are PIP2-effectors?

The nucleolus and nuclear speckles are two PIP2-containing nuclear bodies that are formed by phase separation. The proteins that are associated with the formation of these nuclear bodies contain IDR regions that promotes the phase separation. To identify the PIP2-effectors that have high IDR content would further enable us to determine the proteins that are susceptible to contribute to the formation of all the PIP2-containing nuclear bodies. This can be possible via subjecting the effector proteins to bioinformatics tools that detect the IDR domains of the proteins.

## 5.2. Does MPRIP phase-separate in pathological conditions?

We have determined that MPRIP has the ability to form LLPS condensates by its C-terminal IDR region. The formation of the condensates is important to understand the molecular mechanisms of many cellular processes in health and disease. In physiological conditions, phase separation of MPRIP might be induced by a stimulus that might alter the RNAPII transcription. In addition, MPRIP is overexpressed in many cancers including fusion kinase cancers (BCR-Abl1 is a type of fusion kinase cancer). The LLPS condensates are promising targets as cancer therapeutics. Therefore, determining if MPRIP phase-separates in these diseases, might offer new approaches for their treatment.

## 5.3. What is the exact molecular mechanism of MPRIP in the transcription machinery?

Our results demonstrate that MPRIP is a transcriptional factor that regulates the association of Tyr1 phosphorylated RNAPII complex and PIP2. Even though, many indications point that MPRIP affects the transcription at the promoter-proximal pause, Chip-Seq experiments can provide further evidence. In such experiment, RNAPII would be pulled-down in control and MPRIP depleted cells where the comparative analysis would show the location of RNAPII accumulation on a gene body. To define the molecular mechanism, the binding partners and the regions that are responsible of these interactions should also be determined.

## 5.4. How nuclear F-actin formations affect the allocation of MPRIP to the transcription machinery?

MPRIP contains an F-actin binding domain and localizes to actin stress fibers in nucleus. In our live cell imaging experiments, the cells that were overexpressing GFP-MPRIP were

localized to the nuclear F-actin structures. It is also known that nuclear F-actin formations alter RNAPII transcription. Therefore, it is important to determine whether nuclear F-actin formations affect the allocation of MPRIP to the RNAPII transcription machinery. To determine this, the RNAPII complex should be investigated by immunoprecipitation experiments in conditions when nuclear F-actin is induced.

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# 7. PUBLICATIONS & MANUSCRIPTS

## 7.1. Limited Proteolysis-Coupled Mass Spectrometry Identifies Phosphatidylinositol 4,5 Bisphosphate Effectors in Human Nuclear Proteome

Martin Sztacho, Barbora Šalovská, Jakub Červenka, <u>Can Balaban</u>, Peter Hoboth and Pavel Hozák

Cells. 2021, 10(1), 68; doi:10.3390/cells10010068

IF: 7.666 (2021)

C.B. prepared the nuclear fractions for the PIP2 pull-down assays and revised the manuscript.

## 7.2. The F-Actin-Binding MPRIP Forms Phase-Separated Condensates and Associates with PI(4,5)P2 and Active RNA Polymerase II in the Cell Nucleus

<u>Can Balaban</u>, Martin Sztacho, Michaela Blažíková and Pavel Hozák

Cells. 2021, 10(4), 848; doi:10.3390/cells10040848

IF: 7.666 (2021)

C.B. designed and performed all of the experiments (Confocal and STED microscopy imaging, molecular cloning, generating stable cell lines, heat shock treatments, cellular fractionation, immunoprecipitation, pull-down assays, immunofluorescence experiments, FRAP experiments, live cell imaging, western blotting and data analysis), wrote and revised the manuscript.

## 7.3. RNA Polymerase II Condensation and Transcription is Regulated by the Association of the Transcription Complex with PI(4,5)P2

<u>Can Balaban</u>, Martin Sztacho and Pavel Hozák

Manuscript

C.B. designed and performed all of the experiments (Confocal and STED microscopy, transfections,  cellular fractionation, pull-down assays, western blotting and data analysis), wrote and revised the manuscript.

## 7.4. Nuclear Phosphoinositides and Phase Separation: Important Players in Nuclear Compartmentalization

Martin Sztacho, Margarita Sobol, <u>Can Balaban</u>, Sara Escudeiro Lopes, Pavel Hozák

C.B. wrote the section "Actin-NM1-PIP2 interactions in RNAPII transcription"