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Myosin-PIP2 interaction in the cell nucleus

Myosin-PIP2 interakce v buněčném jádře

Disertační práce

Školitel: Prof. Pavel Hozák, RNDr., DrSc.

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Prohlášení:

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V Praze, 28.03.2013

Podpis

This work was accomplished at the Department of Biology of the Cell Nucleus of the Institute of Molecular Genetics v.v.i, Academy of Sciences of the Czech Republic, under the supervision of Prof. Pavel Hozák.

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I would like to thank my family and friends whose belief in me gave me all the strength I needed. Finally, I would like to thank Johannes for being a home to me…

Yaşamak bir ağaç gibi tek ve hür ve bir orman gibi kardeşçesine...

To live! Like a tree alone and free
Like a forest in brotherhood...

Nazım Hikmet

Abbreviations

AMD: Actinomycin D

ARP2/3: Actin related protein 2/3

CaM: Calmodulin

DAG: Diacylglycerol

DFC: Dense fibrillar component

DRB: 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole

FC: Fibrillar centers

GC: Granular component

GLUT4: Glucose transporter 4

GST: Glutathione S-transferase

Ins(1,4,5)P3: Inositol 1,4,5- trisphosphate

Myo1C: Myosin 1C

NLS: Nuclear localization signal

NM1: Nuclear myosin 1

PA: Phosphatidic acid

PH: Pleckstrin homology

PI: Phosphatidylinositol

PI3K: Phosphatidylinositol 3 kinase

PI3P: Phosphatidylinositol 3- phosphate

PI4K: Phosphatidylinositol 4 kinase

PI4P: Phosphatidylinositol 4-phosphate

PI5K: Phosphatidylinositol 5 kinase

PI5P: Phosphatidylinositol 5- phosphate

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP4K: Phosphatidylinositol 5- phosphate 4 kinase

PIP5K: Phosphatidylinositol 4- phosphate 5 kinase

PITP: Phosphatidylinositol transfer proteins

PLC: Phospholipase C

PTEN: Phosphatase and tensin homolog

SHIP2: Src-homology 2-containing inositol 5'phosphatase 2

SNF2H: sucrose nonfermenting 2 homolog

SSU: Small ribosomal subunit

Star-PAP: Speckle targeted PIPKIα regulated-poly(A) polymerase

TH1: Tail homology 1

TH2: Tail homology 2

TIF-IA: Transcription initiation factor IA

WSTF: Williams syndrome transcription factor

Contents

ABSTRACT

Even though nuclear myosin 1 (NM1) and myosin 1C (Myo1C) are the products of the same gene, NM1 has additional 16 amino acids at the N-terminus due to alternative start of transcription. Studies claim that NM1 and Myo1C are nuclear and cytoplasmic proteins, respectively. Therefore, researchers thought that NM1 translocates into nucleus via nuclear localization signal (NLS) in its N-terminal extension. However, here we show that NLS is placed within second IQ domain where calmodulin (CaM) binds in a calcium- dependent manner. Since both NM1 and Myo1C have identical neck domains where NLS resides, we have confirmed that both myosin isoforms localize to nucleus.

Based on findings indicating Myo1C binding to phosphatidylinositol 4,5-bisphosphate (PIP2) via its tail domain, we tested if NM1 and Myo1C can interact with PIP2 in the nucleus. We show that both isoforms can bind to PIP2 via their tail domains, and interactions with PIP2 can recruit other nuclear proteins into this lipo-protein complex. PIP2 makes complex with a subset of Pol I transcription and processing machinery proteins and modulate their functions in the nucleolus. Moreover, PIP2 depletion results in a dramatic loss of Pol I transcription activity. NM1 and actin were already shown to promote Pol I transcription. Here, we show that myosin mobility is decreased upon inhibition of transcription and actin polymerization. PIP2 localizes to fibrillar centers together with Pol I, and UBF after the inhibition of transcription. It is known that NM1 also localizes to fibrillar centers upon inhibition of transription. Taken together, all these data indicate the cross talk between acto-myosin complex and PIP2 in the cell nucleus. Therefore, one could ask how PIP2 regulates acto-myosin complex via interactions with myosins and actin binding proteins such as profilin and gelsolin during transcription. Naturally, more studies are needed to asnwer this question.

SOUHRN

Přestože jaderný myosin 1 (NM1) a myosin 1C (Myo1C) jsou produkty stejného genu, jejich struktura není stejná. Díky alternativnímu startu transkripce obsahuje NM1 navíc 16 aminokyselin na N-konci. Podle dosavadních vědeckých studií je Myo1c lokalizován v cytoplasmě, zatímco NM1 je přítomen v jádře buněk. Proto byla za jaderný lokalizační signál (NLS) považována právě N-terminální extenze NM1. V této práci ovšem ukazujeme, že NLS je obsažena ve druhé IQ doméně, která váže kalmodulin (CaM) v závislosti na hladině vápníku. Vzhledem k tomu, že NLS je obsažen v doméně, která je společná pro NM1 i Myo1C, obě izoformy jsou schopny lokalizovat do buněčného jádra, což jsme experimentálně prokázali.

Je známo, že Myo1C v cytoplasmě váže fosfatidylinositol 4,5-bisfosfát (PIP2) ocasní doménou. Testovali jsme, zda NM1 a Myo1c mohou interagovat s PIP2 v jádře. Zjistili jsme, že obě izoformy vážou PIP2 ocasem a tato interakce umožňuje vazbu dalších jaderných proteinů do tohoto lipoproteinového komplexu. PIP2 vytváří komplex s proteiny účastnícími se transkripce polymerasou I (Pol I) a následného úpravy rRNA. Kromě toho byl pozorován významný pokles v transkripční aktivitě Pol I po snížení hladiny PIP2. NM1 a aktin jsou z literatury známy jako proteiny podporující transkripci. Z našich experimentálních dat vyplývá, že mobilita myosinu je snížena, pokud je inhibována transkripce nebo polymerizace aktinu. PIP2 po inhibici transkripce lokalizuje do fibrilárních centrech společně s Pol I a upstream binding factorem (UBF). Je známo, že NM1 také lokalizuje do fibrilárních center po inhibici transkripce. Z uvedených dat vyplývá, že v buněčném jádře dochází k aktivní komunikaci mezi akto-myosinovým komplexem a PIP2. Způsob, kterým PIP2 reguluje akto-myosinový komplex a proteiny vážící aktin, zůstává zatím nejasný jako námět k budoucím experimentům.

I. Introduction

1. Myosin structure

Myosins are ATP-dependent motor proteins mostly known for their roles in muscle contraction. These "conventional" muscle myosins contain a double motor domain. On the other hand, "unconventional" myosins, called myosin I, have only one motor domain. Based on their tail structure, myosin I class is divided into two subclasses. Subclass I myosins have long tails containing tail homology 1 (TH1), tail homology II (TH2) and SH3 domains. Subclass II myosins have only short tails containing TH1 domain. The first unconventional myosin, myosin I, was isolated from *Acanthamoeba* in the early 70s (Pollard and Korn, 1973). Decades later, Myosin 1C (Myo1C) was isolated from mammals (Wagner et al., 1992). MYOIC gene encodes three different Myo1C isoforms due to alternative start of transcription and splicing. Myo1C isoform C is the shortest form that has been thoroughly studied in cytoplasm (Wagner et al., 1992). Myosin 1C isoform B, also known as nuclear myosin 1 (NM1), has an extra 16 amino acids at the N-terminus (Nowak et al., 1997; Pestic-Dragovich et al., 2000). Recently, a new isoform with an extra 35 amino acids at the N-terminus, called isoform A, has been identified in the cell nucleus (Ihnatovych et al., 2012).

Myo1C isoforms are composed of three domains: head, neck and tail **(Fig. 1)**. Once head domain is bound to ATP, ATP is converted into ADP and inorganic phosphate (Pi). Cleavage products are released in a stepwise manner. This whole cycle is called "power stroke cycle", which is propagated by actin binding (for a review see , Olivares and De La Cruz, 2005). Neck domain of Myo1C (also called light chain binding domain) is responsible for transmitting mechanical force produced by the head to the tail. Calmodulins (CaMs) bind to short peptides called IQ motifs within the neck domain to stabilize the lever arm of Myo1C (Houdusse et al., 2006). IQ motifs were also shown to have roles in subcellular localization of Myo1C via binding to hair-cell receptors (Cyr et al., 2002). CaM binding to neck domain is negatively regulated by binding to calcium (Ca^{2+}), leading to dissociation of some CaM molecules from the neck (Manceva et al., 2007). Myo1C binds directly to acidic phospholipids, mainly phosphatidylinositol 4,5-bisphosphate (PIP2), through highly conserved basic residues within pleckstrin homology (PH) domain of β1-loop-β2 tail region (Hokanson and Ostap, 2006; Hokanson et al., 2006). Myo1C-PIP2 binding targets actin cytoskeleton to plasma membrane (Hokanson and Ostap, 2006; Tang et al., 2007). Apart from tail domain, IQ motifs in the neck domain also contribute to the plasma

mebrane attachment. Two myristoylated proteins (calcium binding protein 1 and calcium and integrin binding protein 1) interact with IQ motifs in a Ca^{2+}-dependent manner and this association provides an additional binding of Myo1C to plasma membrane via PIP2 (Tang et al., 2007), which is required for mechanical transduction and adaptation in inner ear cells (Hirono et al., 2004).

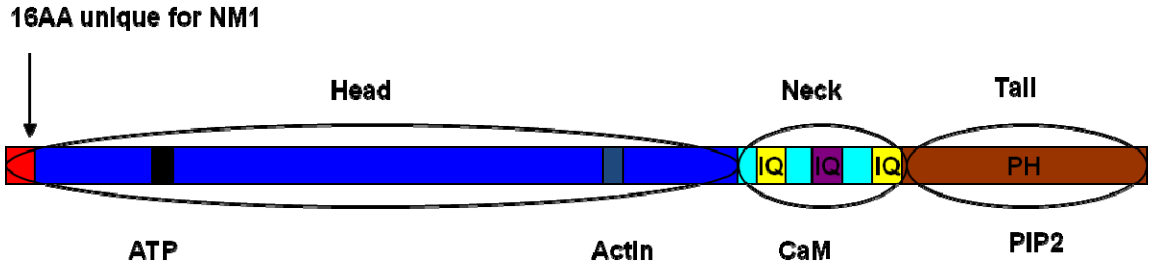

Fig. 1 Features of NM1. Three domains of NM1 and their respective binding partners are depicted here.

2. Functions of Myosin 1C and NM1

(i) Cytoplasmic functions

Since Myo1C anchors the actin cytoskeleton to plasma membrane, its functions are mostly related to membrane/ cytoskeleton dynamics. However, so far there has been no report on functions of NM1 in cytoplasm and plasma membrane.

Myo1C involvement in slow adaptation of the hair cell response to sustained stimuli was studied thoroughly. A bunch of stereocilia called hair bundle serves as a sensor for stimuli at the apical surface of hair cells. In case of mechanical stimuli, hair bundle moves back and forth, leading to increase and decrease in tension of the tip links. During these changes in tension, Myo1C moves up and down along the actin filaments to ensure new balance (Gillespie, 2004; Gillespie and Cyr, 2004). It was shown that PIP2 and cadherin 23 play roles in hair cell adaptation via interactions with Myo1C (Cyr et al., 2002; Hirono et al., 2004; Phillips et al., 2006). According to a model proposed by Hirono et al., 2004, during hair cell adaptation Myo1C binds to plasma membrane via its tail domain and when CaMs disassociate from the second and third IQ domains, first IQ domain bound to CaM is brought closer to plasma membrane where high surface potential increases Ca^{2+} concentration. Thus, Myo1C binds to PIP2 at two different sites, leading to a tighter interaction with plasma membrane **(Fig. 2)**.

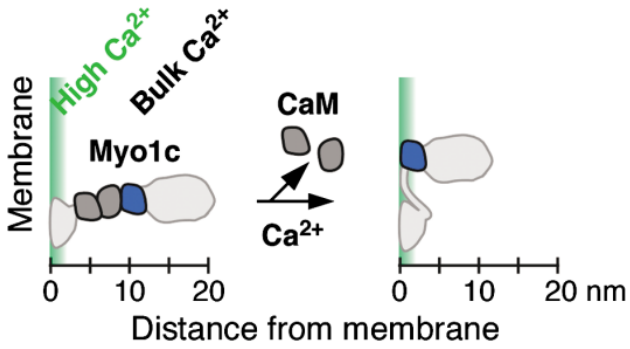

Fig. 2 Regulation of slow adaptation through PIP2-Myo1C interaction. Differential interactions of Myo1C with plasma membrane due to differential exposures of PIP2 binding sites. Figure is taken from Hirono et al., 2004.

Myo1C and phosphatidylinositol 3-kinase (PI3K) were shown to be involved in glucose transport regulation **(Fig. 3)**. Upon stimulation with insulin, glucose transporter GLUT4 is translocated from perinuclear regions to plasma membrane. Myo1C interaction with plasma membrane is required for proper membrane fusion of the GLUT4-containing vesicles, since dominant negative tail construct impaires glucose transport (Bose et al., 2002). Inhibition of PI3K was also shown to result in the impairment of exocytosis of GLUT4-containing vesicles (Bose et al., 2004).

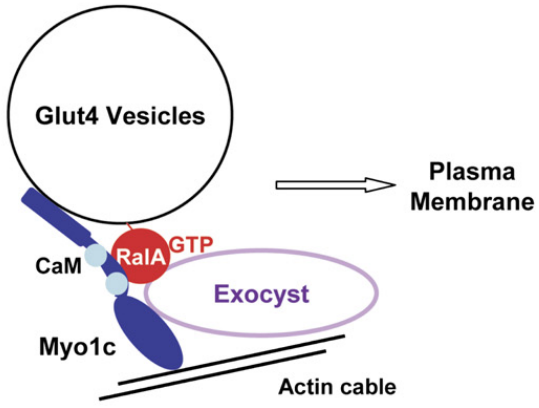

Fig. 3 Regulation of exocytosis of GLUT4 vesicles via Myo1C. In adipocytes, upon insulin stimulation, Myo1C tethers GLUT4-containing vesicles to actin cable and aids fusion of these vesicles with plasma membrane. Figure is taken from Chen et al., 2007.

(ii) Nuclear functions

NM1 was heavily studied in the processes taking place in the nucleus. However, there is no study on the nuclear functions of Myo1C. One of the most studied nuclear process that NM1 is

involved in is transcription. NM1 was found to colocolize with actin and RNA polymerase I (Pol I) in the transcriptionally active sites of nucleoli (Fomproix and Percipalle, 2004). In parallel, studies showed that NM1 promotes Pol I transcription *in vitro* and *in vivo*. Blocking NM1 with anti-NM1 antibodies results in a decrease in transcription. Similarly, addition of purified NM1 or NM1 overexpression causes an increase in the transcription of ribosomal RNA genes (Pestic-Dragovich et al., 2000; Philimonenko et al., 2004). Furthermore, NM1 presence on the promoter and on the coding region of rDNA was detected (Percipalle et al., 2006; Philimonenko et al., 2004). Some findings support the notion that NM1 can interact with naked DNA and RNA (Hofmann et al., 2006a; Obrdlik et al., 2010). NMI interaction with the Pol I transcription complex is regulated by the phosphorylation of TIF-IA, which is a transcription initiation factor (Philimonenko et al., 2004). According to a model **(Fig. 4)** proposed by Grummt, 2006, actin-NM1 interaction brings together TIF-IA and Pol I in order to assemble transcription complexes (Philimonenko et al., 2004; Ye et al., 2008).

Fig. 4 Recruitment of Pol I transcription machinery by NM1 and actin interaction. Since NM1 binds to actin through its head domain, NM1 binding to actin can recruit transcription factor TIF-IA and Pol I to promoter bound to UBF and SL1. Figure was taken from Grummt, 2006.

The step of Pol I transcription modulated by NM1 is still a matter of debate. NM1 is required for the early step of transcription, since it is involved in the formation of first phosphodiester bond during Pol II transcription (Hofmann et al., 2006b). On the other hand, it was reported that NM1 is also needed for the later stages of Pol I transcription, such as promoter escape or elongation (Percipalle et al., 2006).

NM1 has also impact on the status of chromatin by interacting with chromatin remodelling proteins **(Fig. 5)**. NM1 makes a complex with SNF2h and WSTF chromatin remodelling proteins and this complex is called B-WICH (Percipalle and Farrants, 2006; Percipalle et al., 2006). During replication, SNF2h and WSTF keep the chromatin in open conformation to facilitate the

transmission of epigenetic status to the newly synthesized DNA (Poot et al., 2005). NMI-WSTF-SNF2h complex also contains 45S ribosomal RNA, 5S ribosomal RNA and 7SL RNA; and reduction in WSTF levels by siRNA knockdown leads to a reduction in 5S and 7SL transcription (Cavellan et al., 2006). WSTF-SNF2h-NMI complex also associates with Pol I (Percipalle et al., 2006).

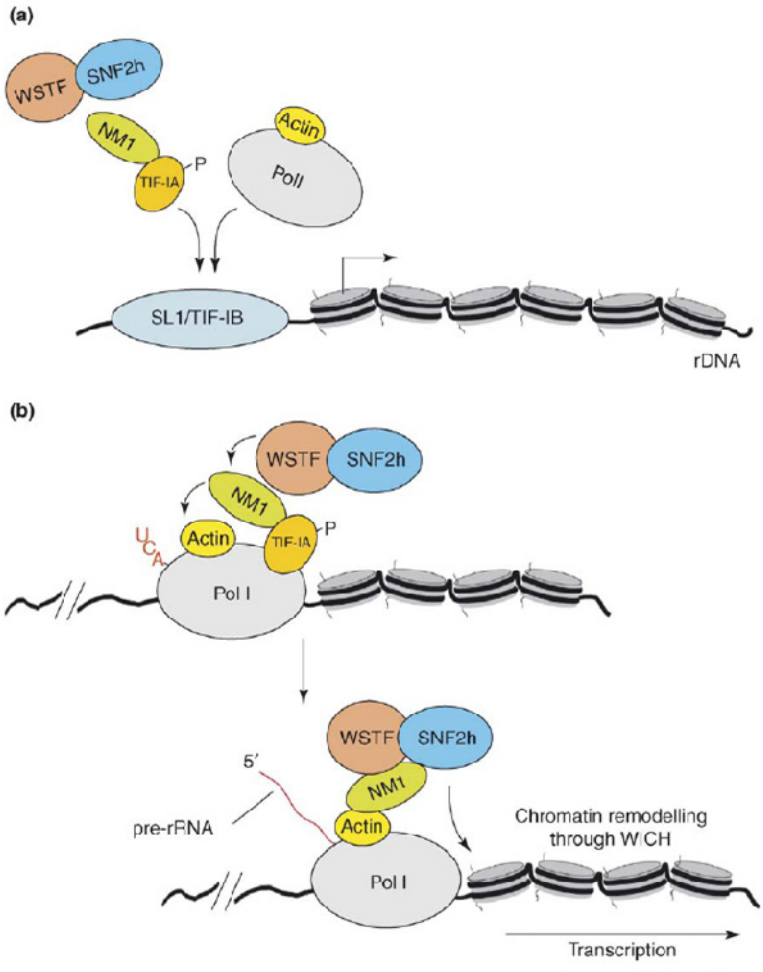

Fig. 5 A model of the function of WICH complex during ribosomal RNA gene transcription. (a) Assembly of transcription initiation factors by NM1-actin interaction. (b) B-WICH is recruited to the Pol I transcription machinery via association with NM1 to modulate the state of the chromatin for efficient transcription. Figure was taken from Percipalle and Farrants, 2006.

NM1 and actin were shown to facilitate the movement of small ribosomal subunits (SSUs) from nucleolar periphery towards nuclear pores (Obrdlik et al., 2010). Also, addition of anti-NM1 and actin antibodies caused a retention of SSU in the nucleus (Cisterna et al., 2006; Cisterna et al., 2009).

Chromosomal movements are shown to be regulated by acto-myosin complex (Chuang et al., 2006; Hu et al., 2008; Mehta et al., 2010). Upon activation, artifical gene loci move toward center of nucleus, and this movement is impaired when the cells overexpress mutant NM1 lacking motor activity (Chuang et al., 2006).

3. Canonical phosphoinositide cycle

In eukaryotic cells, a wide range of cellular processes are modulated by phosphoinositides. Phosphatidylinositol (PI) is a negatively charged lipid that can be anchored to the membrane via its acyl chains leaving its inositol head group exposed and accessible for phosphorylation by the family of PIP kinases. Phosphorylation of PI can generate seven different phosphorylated PIPn species, which act as direct messengers and precursors to messengers (Clarke, 2003). Phosphoinositide signaling cycle **(Fig. 6)** was discovered the in the early 50s (Hokin and Hokin, 1953). Upon extracellular stimulus, spatially regulated phosphoinositide signals are generated in specific subcellular compartments. Because of its role as a precursor of second messangers, most studies focused on the PIP2, which comprises about 1% of plasma membrane phospholipids.

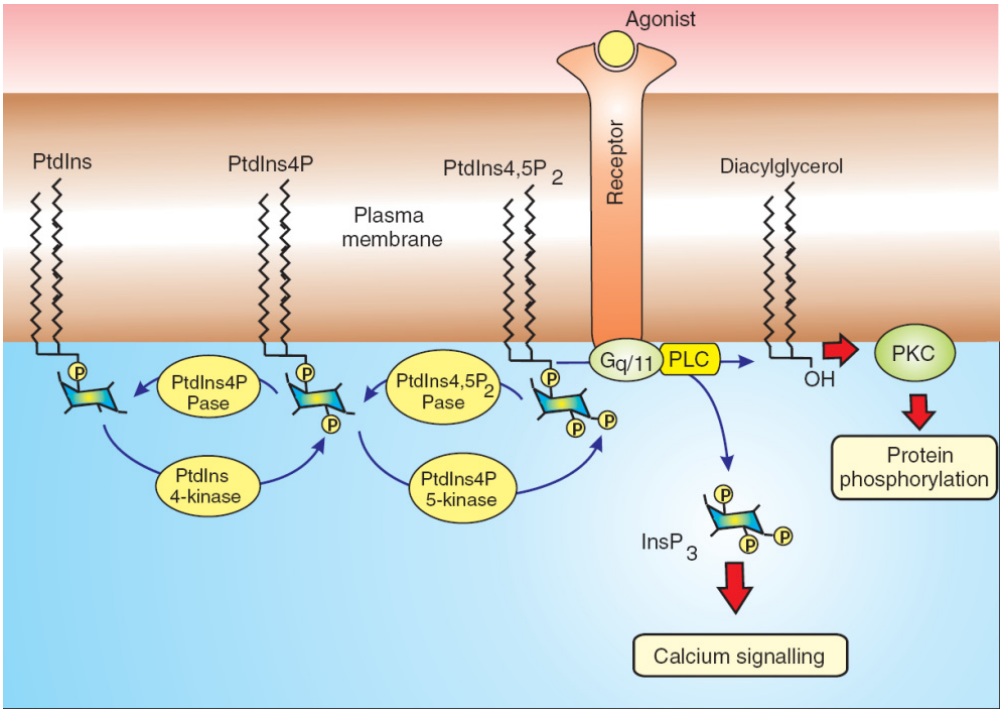

Fig. 6 Canonical phosphoinositide signaling. Phosphoinositides are embedded in the inner leaflet of the plasma membrane, and successively phosphorylated by specific kinases. Activated cell-surface receptors are coupled through the G protein Gq/11 to phospholipase C (PLC) which

hydrolyses PIP2 to release second messengers inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] and diacylglycerol (DAG). Upon generation of Ins(1,4,5)P3, Ca^{2+} is released from endoplasmic reticulum to the cytoplasm. DAG generation recruits protein kinase C (PKC) to the plasma membrane to initiate protein phosphorylation. Figure was taken from Berridge, 2012.

4. Nuclear phosphoinositide metabolism

When nuclei of rat liver cells (Smith and Wells, 1983) and human erythroleukemia cells (Cocco et al., 1987) were incubated with [γ^{32}P]-ATP, radioactivity was incorporated into phosphatidic acid (PA), PI4P and PIP2, indicating a distinct nuclear phosphoinositide cycle. Recent studies show that PI lipids together with the enzymes that synthesize them, form an intranuclear phospholipase C (PI-PLC) signaling system that generates DAG and Ins(1,4,5)P3. Generation of DAG within the nucleus is believed to recruit PKC to the nucleus to phosphorylate intranuclear proteins. Generation of Ins(1,4,5)P3 may mobilize Ca^{2+} from the space between the nuclear membranes and thus increase nucleoplasmic Ca^{2+}.

There are 13 isozymes of PI-PLC that are divided into six subfamilies – phospholipase C- β (1,2,3,4), -γ (1,2), -δ (1,3,4), -ε, -ζ, and –η (1,2), and each isozyme has more than one alternative splicing variant. Even though several PI-PLC isozymes were detected in the cell nucleus, most of the studies have focused on PI-PLCβ_1, since this isozyme is predominantly localized to the nucleus. PI-PLCβ_1 localizes to the nucleus of quiescent Swiss 3T3 fibroblasts (Martelli et al., 1992), and contains nuclear localization signal (NLS) in its C terminus (Kim et al., 1996). Also other subtypes of PI-PLCβ (PI-PLCβ_2, β_3 and β_4) and other isozymes such as PI-PLCγ_1, PI-PLCδ_1, PI-PLCδ_4 and PI-PLCζ have been confirmed to localize to the cell nucleus. PI-PLCγ1 does not contain any known NLS, thus its presence in the nucleus (Bertagnolo et al., 1995) might be facilitated by piggy-back mechanism via interaction with Vav protein (Bertagnolo et al., 1998). PI-PLCδ1 contains a leucine-rich nuclear export sequence (NES) (Yamaga et al., 1999) and putative NLS-like signal (Okada et al., 2002), therefore shuttles between the nucleus and the cytoplasm. On the other hand, PI-PLCδ4 is dominantly present in cell nucleus (Liu et al., 1996). PI-PLCζ isozyme contains an NLS between X and Y domains but its nuclear function still remains to be investigated (Visnjic and Banfic, 2007).

Other groups of enzymes involved in phosphoinositide signaling are PI phosphate kinases (PIPK) and phosphates. PI4P can be generated from PI via phosphorylation of the 4' position by PI4K,

and it was shown that nuclei of CHO and yeast cells (de Graaf et al., 2002) show PI4Kβ activity. Moreover, PI4K230, a PI4K isoform, was also found to be present in the nucleoli of mammalian cells (Kakuk et al., 2006). However, the exact function of PI4P in nucleus is still unknown. PI5P can be generated from PI by PI5K and nuclear PI5P levels elevate 20-fold during G1 phase of the cell cycle (Clarke et al., 2001). Different research groups showed the presence of PIP2 in nucleus by various methods (Boronenkov et al., 1998; Mellman et al., 2008; Osborne et al., 2001). Two classes of PIPK synthesize PIP2; type I kinases (PIP5KI) utilize PI4P, whereas type II kinases (PIP4KII) use PI5P as a substrate (Barlow et al., 2010), and isoforms of both types were found in the cell nucleus. PIP5KIα localizes to nuclear speckles (Boronenkov et al., 1998; Mellman et al., 2008). PIP5KIγ_i4 is present not only in the cytoplasm but also localizes to the nucleus where it colocalizes with markers of nuclear speckles (Schill and Anderson, 2009). Type II PIPK kinases, PIP4KIIα and PIP4KIIβ, also localize to nuclear speckles in the cell nucleus (Boronenkov et al., 1998). PIP4KIIβ is linked to the nuclear stress pathway, which upon induction results in the inhibition of PIP4KIIβ by p38 MAPK. This results in the accumulation of PI5P in the cell nucleus (Barlow et al., 2010). At the 3' position, phosphoinositides are phosphorylated by phosphatidylinositol 3-kinases (PI3K). PI3P localization to nucleoli of human fibroblast cells was shown by PI3P-specific double FYVE probe (Gillooly et al., 2000). In parallel, activation of Class II PI3K C2β increased PI3P levels within the nucleus in a cell-cycle dependent manner (Visnjic et al., 2003). Moreover, Class II PI3K C2α has NLS sequence and localizes to nuclear speckles (Didichenko and Thelen, 2001). Using a specific antibody, the presence of PI(3,4)P2 was shown to be at the nuclear membrane (Yokogawa et al., 2000). PI(3,4)P2 can be generated in the cell nucleus via phosphorylation by Class II PI3K C2α and Class II PI3K C2β, or dephosphorylation by Src-homology 2-containing inositol 5'phosphatase 2 (SHIP-2). SHIP-2 hydrolyzes phosphate group at 5' position of Ins(1,3,4,5)P4 and PI(3,4,5)P3 (Damen et al., 1996; Lioubin et al., 1996), and SHIP-2 colocalizes with a speckle specific marker SC35 in nuclear speckles (Deleris et al., 2003). Class IA PI3Kβ and Class IB PI3Kγ were shown to localize into the nucleus, where they can generate PI(3,4,5)P3 from PIP2. In addition, PI(3,4,5)P3 can also be generated by phosphorylation of PI(3,4)P2 by PIP5KIα. PI(3,4,5)P3 interacts with nuclear protein B23 and modulates anti-apoptatic effects of neural growth factor by inhibiting DNA fragmentation via blocking endonuclease DFF40/CAD activity (Ahn et al., 2005).

Phosphatidylinositols are reversely dephosphorylated by phoshatidylinositol phosphatases. Phosphatase and tensin homolog (PTEN) which dephosphorylates phosphoinositides at 3-position (Vanhaesebroeck et al., 2001) was shown to localize to the cytoplasm but also to the nucleus (Deleris et al., 2003; Gimm et al., 2000; Lachyankar et al., 2000; Torres et al., 2001). However, a recent study showed that PTEN does not dephosphorylate PI(3,4,5)P3 in the nucleus (Lindsay et al., 2006). Moreover, type I phosphatidylinositol-4,5-bisphosphate 4-phosphatase I (PIP2-4Ptase I), which dephosphorylates PIP2 to PI5P (Ungewickell et al., 2005), was observed to translocate into the nucleus upon cellular stress (Zou et al., 2007). **Fig. 7** shows the PIs and their effector enzymes detected in the cell nucleus.

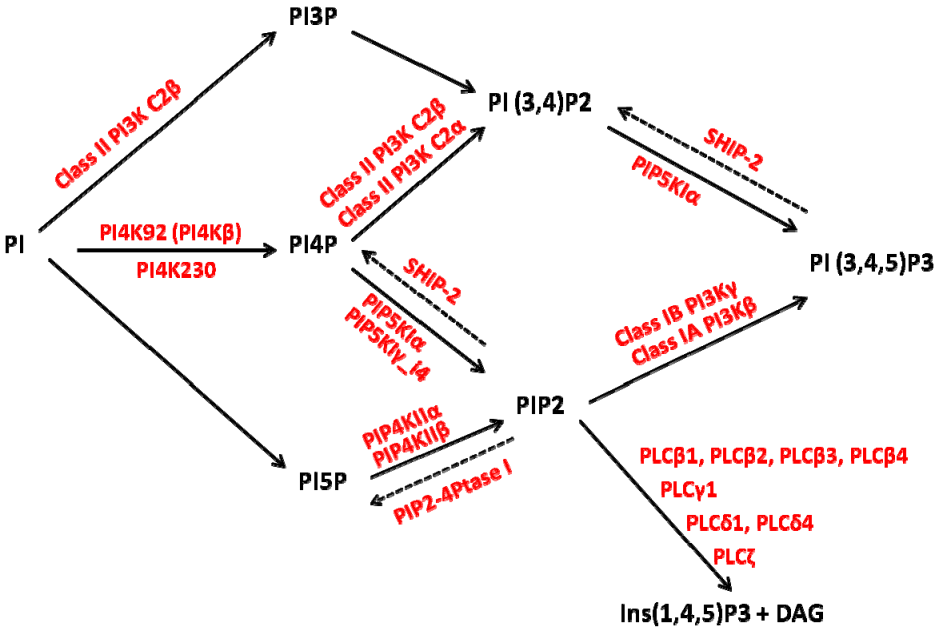

Fig. 7 Major metabolism of phosphoinositide in the nucleus. Nuclear phosphoinositides and their effector enzymes that are involved in the nuclear PI pathway are depicted here. Figure was modified from Ye and Ahn, 2008.

5. Functions of PIP2

(i) Cytoplasmic functions

PIP2 transmits signals from plasma membrane to cytoplasm via binding to proteins through negative charges in its head domains. Protein surfaces that interact with PIP2 contain either unfolded basic amino acid clusters or folded domains. One of the main mechanisms which influence cell shape, cytokinesis, motility is actin polymerization. PIP2 promotes actin

polymerization through various mechanisms. For instance, N-WASP is activated upon binding to PIP2 together with small GTPase Cdc42. This activation results in ARP2/3-mediated actin nucleation (Rohatgi et al., 2000). PIP2 also facilitates actin polymerization by releasing the capping proteins (gelsolin, capZ) from the plus ends and binding to profilin to liberate G-actin monomers (for a review, see Yin and Janmey, 2003).

PIP2 is generated by PIPK type 1γ at the sites of cell adhesion to activate adaptor proteins such as ezrin/radixin/moezin at the sites of cell-cell or cell-matrix contacts (Ling et al., 2002). Moreover, PIP2-enriched microdomains are involved in microtubule capture to stimulate leading edge motility in the cells (Golub and Caroni, 2005). PIP2 marks the plasma membrane for fusion with secretory granules, thus PIP2-enriched microdomains colocalize with these organelles (Martin, 2001). PIP2-mediated calcium responses also affect exocytosis by triggering secretion. PIP2 has an important role in endocytosis, since it binds to endocytic clathrin adaptors and recruits them selectively to plasma membrane (Owen et al., 2004; Wenk and De Camilli, 2004). In phagocytosis, PIP2 is required for actin nucleation to create cell protrusions to engulf large particles by cells (Botelho et al., 2004). Many ion channels are activated by PIP2, and when PIP2 is not present these channels are in a sleeping state (Robertson, 2007). **Fig. 8** summarizes the various cytoplasmic processes that PIP2 is involved in.

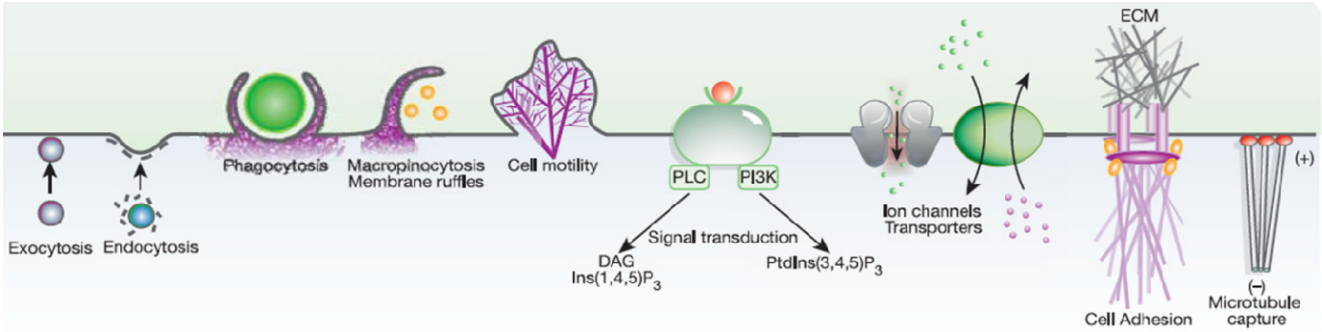

Fig. 8 Roles of PIP2 at the plasma membrane. PIP2-regulated processes at the plasma membrane are depicted here. Figure was modified from Di Paolo and De Camilli, 2006.

(ii) Nuclear functions

Two questions still puzzle researchers regarding nuclear PIs:

1. What is the physico-chemical form of nuclear PIs?
2. What is the mechanism of PI translocation into the nucleus?

PIP2 was shown to be present in the inner leaflet of nuclear envelope and accessible to nuclear PLCs to generate DAG and Ins(1,4,5)P3 (Tran et al., 1993). Another study showed that invaginations of nuclear envelope contain Ins(1,4,5)P3 receptors and can mobilize Ca^{2+} and translocate PKC to nuclear envelope (Echevarria et al., 2003). However, there is also an intranuclear PIP2 pool that is resistant to detergent extraction (Vann et al., 1997). Studies using anti-PIP2 antibody or PIP2-specific pleckstrin homology (PH) domain showed PIP2 localization on nuclear speckles and nucleoli, although there was no signal for PIP2 in the nuclear envelope and endoplasmic reticulum (Osborne et al., 2001; Watt et al., 2002). These results suggest that proteins with specific PIP2-binding modules or basic amino acids bind and sequester PIP2 within the nucleus.

Even though a conclusive study on the transport of PIs into nucleus is missing, it has been suggested that phosphatidylinositol transfer proteins (PITP) can bind and transfer PIs into nucleus. Once bound to PIs, PITBs are in a closed conformation, completely shielding the hydrophobic parts of PIs from hydophilic environment, thus facilitating the transport of PIs between different compartments of the cell (Rubbini et al., 1997; Shadan et al., 2008; Snoek, 2004). Since both isoforms (α and β) of PITBs localize to the nucleus (De Vries et al., 1996), PIP2 can be transported into nucleus via interactions with PITBs.

PIP2 was reported to have several roles in the cell nucleus. PIP2 is present in nuclear speckles (Boronenkov et al., 1998) where it makes complexes with proteins involved in mRNA splicing (Osborne et al., 2001). Another nuclear process that PIP2 has a role is 3`end processing of pre-mRNAs in eukaryotes. PIP2 was shown to interact with an enzyme called Star-PAP (speckle targeted PIPKIα regulated-poly(A) polymerase) in nuclear speckles and promote the processivity of the enzyme resulting in longer poly(A) tails (Mellman et al., 2008). RNA export is also regulated by interaction with PIP2 or higher inositol phosphates. PIP2 cleavage by PLC generates Ins(1,4,5)P3, which is used as a precursor for higher inositol phosphates. Inositol (1,2,3,4,5,6)P6 was shown to be required for efficient RNA export in yeast (York et al., 1999). Moreover, PIP2 was shown to bind to mRNA export protein Aly, and disruption of PIP2 binding caused a reduction in its mRNA export activity (Okada et al., 2008).

PIP2 was implicated in nuclear actin regulation. Even though actin does not have NLS, it was suggested that actin is transported into nucleus via piggy-back mechanism (Vartiainen, 2008). PIP2 facilitates the synthesis of filamentous actin in the nucleus by interfering with BRG1-actin

binding at the C-terminus of BRG1 (Rando et al., 2002), which is analogous to uncapping of actin via PIP2 in cytoplasm (Yin and Janmey, 2003). PIP2 also promotes the binding of SWI-SNF like BAF chromatin remodeling complex to nuclear matrix/chromatin in actin-dependent manner (Zhao et al., 1998). Arp2/3 complex, which interacts with PIP2, is involved in transcription by Pol II (Yoo et al., 2007). Moreover, PIP2 binding to histone H1 reverses the inhibitory effect of histone H1 on transcription (Yu et al., 1998). In *Drosophila*, a nuclear PIP5K, called SKTL, is shown to have an impact on transcriptionally active chromatin maintenance via reducing histone H1 hyperphosphorylation (Cheng and Shearn, 2004). PIP2 interaction with only the hyperphosphorylated active form of Pol II (Osborne et al., 2001), indicates the possible involvement of PIP2 in Pol II transcription. PIP2 functions in the cell nucleus are shown in **Fig. 9**.

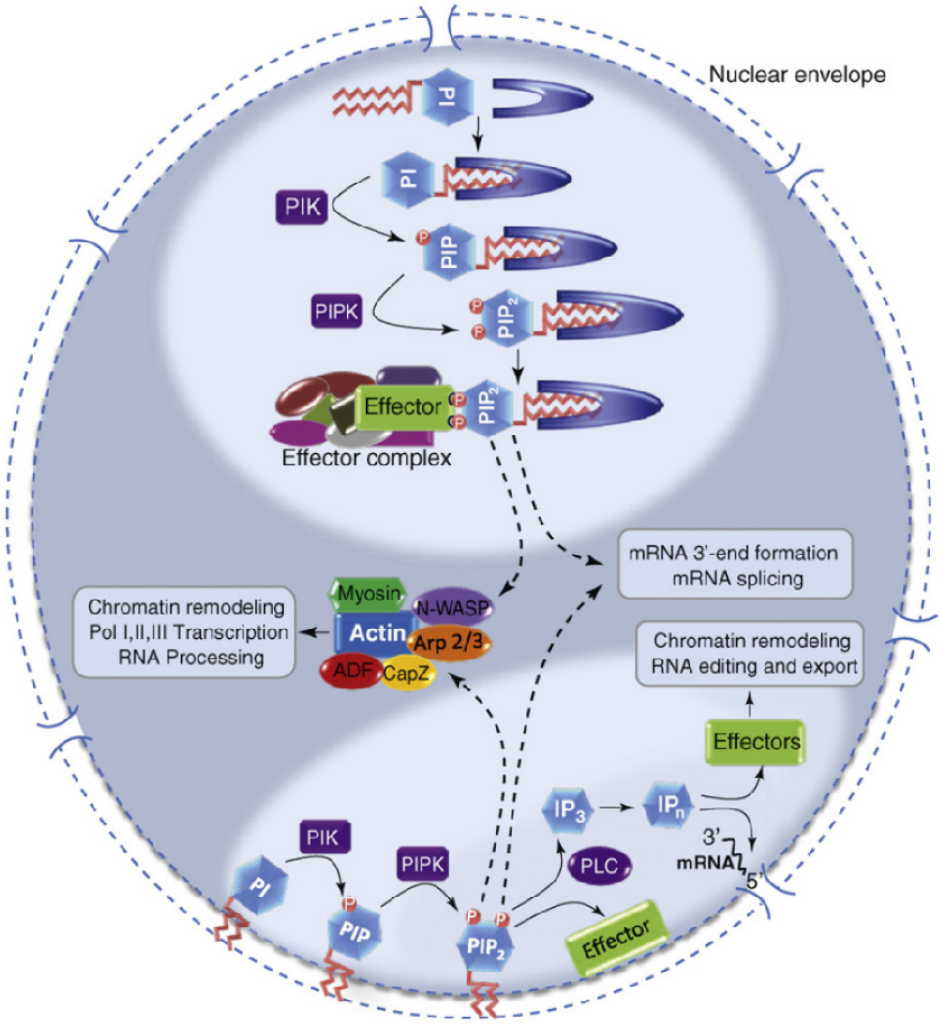

Fig. 9 Roles of PIP2 in the cell nucleus. PIP2-regulated nuclear processes are depicted here. Figure was taken from Barlow et al., 2010.

I. Motivation and aims

Even though a distinct nuclear phosphoinositide cycle was shown 30 years ago (Cocco et al., 1987; Smith and Wells, 1983), little is known about the physico-chemical properties and functions of these intranuclear phosphoinositides. Since there are no visible membranous structures inside of the nucleus, researchers suggested that intranuclear phosphoinositides are anchored via interaction with hyodphobic amino acids of nuclear proteins (for a review, see Irvine, 2003).

PIP2 is a minor membrane phospholipid which is involved in cytoplasmic signal transduction. Interestingly, recent studies showed that PIP2 can also localize to nucleoplasm, nuclear speckles and nucleoli (Boronenkov et al., 1998; Mellman et al., 2008; Osborne et al., 2001; Yildirim et al., 2013). It is crucial to investigate the composition of PIP2-containing complexes to elucidate the mechanism of intranuclear PIP2 retention. PIP2 is known to bind directly to Myo1C and tether it to the plasma membrane. Since Myo1C and NM1 share the same tail domain responsible for PIP2 binding, we tested if PIP2 can be retained within the nucleus by making lipo-protein complexes with Myo1C and NM1. We also aimed to identify the binding partners of these intranuclear lipo-protein complexes.

There are hints in the literature sugggesting the involvement of PIP2 in Pol II transcription. PIP2 addition was shown to promote Pol II transcription (Yu et al., 1998) and the binding of chromatin remodeling complex to DNA (Zhao et al., 1998). NM1 was shown to make complexes with Pol I and Pol II transcription machineries (Hofmann et al., 2006b; Philimonenko et al., 2004; Ye et al., 2008). However, it is not known if the association of NM1 with Pol I and Pol II transcription machineries is PIP2-dependent. Since PIP2 involvement in Pol I transcription has not been investigated yet, we focused on the roles of PIP2 in Pol I transcription in which the actomyosin complex is a modulator.

In particular, we aimed to aswer following questions:

1. Which part of the Myo1C/NM1 is responsible for nuclear localization?
2. Does Myo1C/NM1 bind to PIP2 in the nucleus? What are the binding partners of this lipo-protein complex?
3. Is PIP2 involved in Pol I transcription?
4. Is the localization of nucleolar PIP2 connected with transcriptional activity of the cell?

III. Research papers

1. Actin complexes in the cell nucleus: new stones in an old field

Castano E, Philimonenko VV, Kahle M, Fukalová J, Kalendová A, **Yildirim S**, Dzijak R, Dingová-Krásna H, Hozák P.

Histochemistry and Cell Biology. 2010 Jun;133(6):607-26. doi: 10.1007/s00418-010-0701-2. Epub 2010 May 5.

Impact factor: 2.588 (2011)

S.Y. wrote the gelsolin and villin superfamily part in the article.

Actin complexes in the nucleus range from very dynamic chromatin-remodeling complexes to structural elements of the matrix with single partners known as actin-binding proteins (ABPs). This review summarizes the recent findings of actin-containing complexes in the nucleus. Particular attention is given to key processes like chromatin remodeling, transcription, DNA replication, nucleocytoplasmic transport and to actin roles in nuclear architecture. Understanding the mechanisms involving ABPs will definitely lead us to the principles of the regulation of gene expression performed via concerting nuclear and cytoplasmic processes.

2. Specific nuclear localizing sequence directs two myosin isoforms to the cell nucleus in CaM-sensitive manner

Dzijak R, **Yildirim S**, Kahle M, Novák P, Hnilicová J, Venit T, Hozák P.

PLoS One. 2012;7(1):e30529. doi: 10.1371/journal.pone.0030529. Epub 2012 Jan 25.

Impact factor: 4.092 (2011)

S.Y. designed and performed mutagenesis experiments.

NM1 differs from the "cytoplasmic" myosin 1c only by additional 16 amino acids at the N-terminus of the molecule. This amino acid stretch was therefore suggested to direct NM1 into the nucleus. We investigated the mechanism of nuclear import of NM1 in detail. Using over-expressed GFP chimeras encoding for truncated NM1 mutants, we identified a specific sequence that is necessary for its import to the nucleus. This novel nuclear localization sequence is placed

within calmodulin-binding motif of NM1, thus it is present also in the Myo1c. We have shown that the novel specific NLS brings to the cell nucleus not only the "nuclear" isoform of myosin I (NM1 protein) but also its "cytoplasmic" isoform (Myo1c protein). This opens a new field for exploring functions of this molecular motor in nuclear processes, and for exploring the signals between cytoplasm and the nucleus.

3. Phosphatidylinositol-4,5-bisphosphate serves as intranuclear anchor for nuclear myosin 1 and myosin 1C

Yildirim S, Castano E, Dzijak R, Kalendová A, Kalasová I, Novák P, Sýkora J, Hof M, and Hozák P.

Manuscript.

S.Y. designed and performed experiments (FRAP, FCS, plasmid construction, mutagenesis, immunofluorescence, pull-downs) and wrote the manuscript.

Myo1C is known to tether to plasma membrane via binding to PIP2 through its putative pleckstrin homology (PH) and IQ domains. Since membranous structures have not been seen inside of the nucleus, PIP2 should reside in the nucleus in a different form than the classical bilayer membrane, presumably forming complexes with specific nuclear proteins. We explored therefore PIP2 interactions with Myo1C isoforms in the cell nucleus. We show that both NM1 and Myo1C bind to PIP2 via their PH domains also in the nucleus. PIP2 interaction with NM1 and Myo1C recruits lamin A and farnesylated proteins to the lipo-protein complex. Our study also showed that inhibition of transcription and inhibition of actin polymerization result in a decrease in NM1 mobility, suggesting that NM1 might drive the transcription by interacting with polymerized actin. Moreover, several lipid molecules were also found to associate with nuclear PIP2. In addition, nuclear proteins involved in chromatin regulation, transcription, splicing, ribosome synthesis and genomic stability were also found to interact with NM1. These findings suggest that NM1 and Myo1C are tethered in nucleus via PIP2 possibly nucleating lipo-protein complexes which function in various nuclear processes.

4. Involvement of PIP2 in RNA Polymerase I transcription

Yildirim S, Castano E, Sobol M, Philimonenko V. V, Dzijak R, Venit T and Hozák P.

Journal of Cell Science. 2013 In press.

Impact factor: 6.111

S.Y. designed and performed experiments (immunofluorescence, protein expression and purification, gel shift, direct binding assays, pull-downs) and wrote the manuscript.

Pol I transcription is essential for the cell cycle, growth, and overall protein synthesis in eukaryotes. We found that PIP2 is a part of the protein complex on the active ribosomal promoter during the transcription. PIP2 makes a complex with Pol I and Pol I transcription factor UBF in the nucleolus. PIP2 depletion reduces Pol I transcription which can be rescued by the addition of exogenous PIP2. In addition, PIP2 also binds directly to the pre-ribosomal RNA processing factor, fibrillarin, and co-localizes with nascent transcripts in the nucleolus. PIP2 binding to UBF and fibrillarin modulates their binding to DNA and RNA, respectively. In conclusion, PIP2 interacts with a subset of Pol I transcription machinery, and promotes Pol I transcription.

5. Transcription-dependent PIP2 distribution in the nucleolus

Yildirim S, Sobol M, Philimonenko V.V, Castano E and Hozák P.

Manuscript.

S.Y. designed and performed experiments (immunofluorescence) and wrote the manuscript.

The nucleolus is a non-membrane bound structure composed of three distinguishable regions: fibrillar centers (FCs), dense fibrillar component (DFC), and granular component (GC). ribosomal RNA transcription takes place at the boundary of the FC and DFC, while ribosomal subunits assemble in GC of the nucleolus. Recent studies indicate the presence of PIP2 within the nucleolus. Here we studied the connection between transcriptional activity of the cell, sub-nucleolar localization of PIP2, and ribosomal RNA transcription machinery. We have shown that PIP2 colocalizes with Pol I and UBF in a transcription-independent manner, however PIP2-fibrillarin colocalization is dependent on transcription. These results indicate that PIP2 binds to

Pol I transcription machinery regardless of active transcription, and to fibrillarin only upon synthesis of ribosomal RNA.

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REVIEW

Actin complexes in the cell nucleus: new stones in an old field

E. Castano · V. V. Philimonenko · M. Kahle · J. Fukalová · A. Kalendová · S. Yildirim · R. Dzijak · H. Dingová-Krásna · P. Hozák

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Abstract Actin is a well-known protein that has shown a myriad of activities in the cytoplasm. However, recent findings of actin involvement in nuclear processes are overwhelming. Actin complexes in the nucleus range from very dynamic chromatin-remodeling complexes to structural elements of the matrix with single partners known as actin-binding proteins (ABPs). This review summarizes the recent findings of actin-containing complexes in the nucleus. Particular attention is given to key processes like chromatin remodeling, transcription, DNA replication, nucleocytoplasmic transport and to actin roles in nuclear architecture. Understanding the mechanisms involving ABPs will definitely lead us to the principles of the regulation of gene expression performed via concerting nuclear and cytoplasmic processes.

Keywords Actin · Actin-binding proteins · Cell nucleus · Chromatin · Transcription · Replication · Nucleocytoplasmic transport · Nucleoskeleton · Regulation

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Introduction: the history of actin and actin-binding proteins in the nucleus

The relevance of actin in the nucleus has been demonstrated during the last few decades, changing the status from a controversial finding to an understanding that actin is a key protein required in several nuclear processes (Chen and Shen 2007; Schleicher and Jockusch 2008). However, the precise structure and mechanism of action of nuclear actin is still enigmatic. In the cytoplasm, actin forms a highly versatile and dynamic filamentous network, which is involved in shaping the cell, distributing the cellular organelles, cellular motility and adhesion just to name some key functions. In the nucleus, however, the situation is different: the notoriously known forms of polymeric actin existing in the cytoplasm have not been found (with some exceptions mentioned below) and actin forms, at most, short stretches of fibers which may represent atypical structures since they do not bind to phalloidin—a hallmark of filamentous actin (Cooper 1987). In addition to actin, there are several well-known actin-binding proteins (ABPs), which are of relevance in a myriad of processes in the cytoplasm (for reviews, see e.g. Insall and Machesky 2009; Pollard and Cooper 2009). Thus, ABPs anchor the actin network to the plasma membrane and other cellular structures, move cargo along the filaments, promote growth of actin fibers by capping the growing ends and shielding them from disassembly, promote the network arboring by branching the filaments, increase the strength of the filaments by bundling them or prune the network by severing and depolymerizing the filaments. Since most of these processes involve polymeric actin, it is quite interesting to note that many of these ABPs are also found in the nucleus. In recent years, the nuclear form of actin and its binding partners have been shown to take part in several key

cellular mechanisms. Table 1 shows a comprehensive list of ABPs that have been found in the nucleus. The large number of proteins fulfilling diverse functions in the cell is indicative of the complex function of nuclear actin. In this review, we will focus on the involvement of ABPs in some of the key nuclear processes: chromatin remodeling, transcription, DNA repair, DNA replication and formation and maintenance of nuclear structure. The facts about nuclear ABPs will be summarized and discussed in separate chapters according to these principal nuclear functions, and the activities of ABPs in the nucleus are schematically depicted in Figs. 1, 2.

ABPs in chromatin remodeling

Modification of chromatin is a key process during cell differentiation, DNA duplication and repair, as well as gene expression. These chromatin modifications are achieved by multi-subunit complexes that exhibit two kinds of activity. They are able to either move and/or replace nucleosomes on DNA strands (remodel chromatin) or mark histones or DNA with covalent modifications. Interestingly, actin and ABPs are part of both types of these complexes and this association is conserved from yeast to humans. In addition to actin, actin-related proteins (Arps) are very prominent components of chromatin-modifying complexes. Arps are actin-derived proteins, which share the same three-dimensional structure of ATP-binding pockets known as the "actin fold". They are classified into 11 subfamilies where Arp 1 is the most similar in sequence to conventional actin, and Arp 11 the least similar (Muller et al. 2005). Arps 4–9 are located in the nucleus and they have all been found to physically associate with chromatin-remodeling complexes. Out of four classes of chromatin remodelers SWI/SNF, ISWI, INO80 and Mi-2/CHD (Bao and Shen 2007), actin together with Arps are prominent parts of two of them: SWI/SNF and INO80. Originally, the association of actin and Arps with SWI/SNF-related remodelers was reported. Later actin and ARPs were found also in INO80 and related SWR1-associated complexes as well as in some histone acetyltransferases (reviewed by Chen and Shen 2007).

Arp4 (in humans one of the Brg1-associated factors, Baf53) is the most widely distributed Arp in chromatin-modifying complexes (for a review, see Meagher et al., 2009). It is present, depending on species, in SWI/SNF and INO80 complexes as well as in various histone acetyltransferases. Arp4 was found as a heterodimer with actin in SWI/SNF of Drosophila and humans, while in yeast SWI/SNF Arp 4/actin is replaced by Arp7/Arp9 dimer. The depletion of Arp4 by siRNA led to the expansion of chromosome territories and decompaction of the chromatin, reduction of H3-K9 dimethylation and an increase of H3-K79 methylation followed by cell cycle arrest. This suggests that Arps are essential for maintenance of global chromatin structure contributing to chromatin fiber folding and/or interactions with non-histone architectural components (Lee et al. 2007a). Arp4 accumulates in the nucleus prior to the onset of mitosis and associates with mitotic chromosomes suggesting that it might be involved in chromatin architecture transition during chromosome decompaction (Lee et al. 2007b). Furthermore, one of the histone acetyltransferases that contribute to chromatin remodeling and subsequent transcription—NuA4 (Doyon and Cote 2004)—is also found in complex with actin and Arp4 (Harata et al. 2002). In fission yeast, a point mutation in a conserved region of Arp4 homolog Alp5 lead to mitotic arrest caused by chromosome missegregation. This phenotype could be rescued by the addition of trichostatin A—an inhibitor of histone deacetylases of classes I and II. Fission yeast Arp4 forms a complex with the histone acetyltransferase Mst1. It was suggested that Arp4 is required for proper function of the kinetochore through its contribution to histone acetylation (Minoda et al. 2005).

In *Saccharomyces cerevisiae*, a second, more abundant SWI/SNF-like complex exists, which is known as RSC complex. Although RSC complexes contain SWI/SNF-like ATPase activity derived from the Sth1 subunit, biochemical studies suggest that SWI/SNF and RSC complexes regulate expression of distinct sets of genes (Szerlong et al. 2003). Arp7 and Arp9 are components of the RSC complex in yeast but homologs can also be found in plants. Arp7 is an essential gene in Arabidopsis required for normal embryo development and survival. Arp7 knockdown by siRNA affected plant architecture and considerably delayed abscission of floral organs (Kandasamy et al. 2005). Immunocytochemical studies showed nuclear localization of Arp7, while aberrant plant embryos did not have detectable levels of Arp7 protein in the nucleus. This data suggested the involvement of Arp7 in plant chromosomal rearrangement during cell development.

Arp6 is a component of INO80 class remodelers that seem to be involved in heterochromatin maintenance and transcription inhibition. In yeast, Arp6 binds to telomere DNA as shown by CHIP analysis (Ueno et al. 2004). Drosophila Arp4/Arp6 co-localizes with heterochromatin protein 1 (HP1) at the pericentric heterochromatin (Kato et al. 2001), and direct interaction of chicken and human Arp6 with HP1 was shown by pull-down experiments and yeast two-hybrid assays (Ohfuchi et al. 2006). On the other hand, Arp6 was also implicated in activation of transcription. For example, in plants, Arp6 has been shown to be required for high expression of FLOWERING LOCUS C in both FRIGIDA-containing lines and in autonomous

Table 1 Overview of actin-binding proteins in the nucleus

Name(s)	MW (kDa)	Organism	Structural domains	Relation to actin	Function in cytoplasm	Nuclear localization	Nuclear (expected) function
c-Abl	125	Animals	Alternative domain, Src-homology domain, SH3, SH2 domains, modular domain, tyrosine kinase, ATP binding, NLS, DNA binding, G- and F-actin binding, NES	Bundling, crosslinker	Signal transduction via non-receptor kinase activity (Van Etten 1999), actin bundling (Van Etten et al. 1994)	Diffuse nucleoplasmic (Nihira et al. 2008)	DNA damage response (regulation of cell cycle, apoptosis) (Wang 2000) proposed possible effect on transcription (Baskaran et al. 1993)
CABP14	260	*Caenorhabditis elegans*	Not determined		Participate in remodeling of cytoskeleton in early development of oocyte, cytokinesis (Aroian et al. 1997)	Along the cleavage furrow of dividing cells (Aroian et al. 1997)	Possible role in cell division (Aroian et al. 1997)
MAL cofactor	92	Eucaryotes	Cytoplasmic localizing domain, actin binding, NLS	Actin inhibits interaction of MAL with SRF	Signaling molecule (Miralles et al. 2003)	Diffuse nucleoplasmic (Posern et al. 2004)	Coactivator of SRF transcription factor (Vartiainen et al. 2007)
Thymosin $\beta 4$	4.9	Mammals	N-terminal helix, G-actin-binding domain, capping helix	Sequestering	Actin-sequestering, regulation of actin polymerization (Zoubek and Hannappel 2007)	Diffuse nucleoplasmic (Huff et al. 2004)	Actin-sequestering (Hannappel 2007)
Arp2	44	Eucaryota	Actin fold	Nucleation	De novo actin filament formation	Diffuse/punctuate nucleoplasmic and cytoplasmic (Yoo et al. 2007)	Possible bridge with N_WASP and PSF-NonO with RNA pol II (Yoo et al. 2007)
Arp3	49	Eucaryota	Actin fold	Nucleation	De novo actin filament formation	Diffuse/punctuate nucleoplasmic and cytoplasmic (Yoo et al. 2007)	Possible bridge with N_WASP and PSF-NonO with RNA pol II (Wu et al. 2006; Yoo et al. 2007)
Arp4	53	Eucaryota	Actin fold	Dimerizes with actin	Not described	Diffuse/punctate nucleoplasmic	Forms part of SWI2/snf2, INO80. INO80, SWR1, NuA4, dBAP, dPBAP, hSWI/SNF, hPBAF, hWINAC, hSRCAP,hp400, hTip60 Remodeling chromosome territories and decompactation of chromatin. Arp4 is the only known nuclear Arp that has been shown to have ATP-binding ability dependent on the conserved ATP/ADP-binding pocket (Chen and Shen 2007; Lee et al. 2003, 2007a, b)
Arp5	68	Eucaryota	Actin fold		Not described	Diffuse (Grava et al. 2000)	Forms part of INO80. Involve in replication (Shimada et al. 2008)
Arp6	50	Eucaryota	Actin fold		Not described		Forms part of SWR1, dISWI, hSRCAP (Chen and Shen 2007; Martin-Trillo et al., 2006)
Arp7	53	Fungi and plants	Actin fold		Not described		Forms part of RSC and SWI/SNF (Chen and Shen 2007)
Arp8	100	Eucaryota	Actin fold		Not described	Diffuse/punctuate nucleoplasmic during mitosis stains the chromosomes (Aoyama et al. 2008)	Forms part of INO80, depletion causes defects in chromosome misalignment during mitosis (Aoyama et al. 2008)
Arp9	51	Fungi and plants	Actin fold		Not described		Forms part of RSC and SWI/SNF chromatin-remodeling complexes (Chen and Shen 2007; Kandasamy et al. 2005; Szerlong et al. 2003)

Table 1 continued

Name(s)	MW (kDa)	Organism	Structural domains	Relation to actin	Function in cytoplasm	Nuclear localization	Nuclear (expected) function
N-WASP	54	Vertebrates	Verprolin-homology domain, a cofilin-homology domain, and an acidic region		Induce filopodium formation	Strong diffuse nucleoplasmic stain and cytoplasmic (Wu et al. 2006)	Interaction with RNA pol II ARP2/3, PSF-NonO (Linardopoulou et al. 2007; Rebowski et al. 2008; Wu et al. 2006)
WASP	52	Eucaryota	Verprolin-homology domain, a cofilin-homology domain, and an acidic region		Regulate actin filament organization, actin polymerization	Diffuse/punctuate nucleoplasmic (Moulding et al. 2007)	Chromatin remodeling
WAVE1,2,3	61, 54, 55, 92 in plants	Eucaryota	Verprolin-homology domain, a cofilin-homology domain, and an acidic region		Involved in actin reorganization in neural tissue, controls depolarization-induced trafficking of the mitochondria to dendritic spines WAVE2 regulating actin polymerization in response to T cell activation, particularly in the formation of the immunologic synapse		Chromatin remodeling
Actinin	100	Eucaryota	N-terminal actin-binding domain, two calponin homology (CH) domains, four spectrin repeats, four EF-hand calcium-binding domains, C-terminal calmodulin-like domain	Crosslinker	A-actinin 1,4 mediate membrane attachment at adherens-type junctions; A-actinin-2,3 are major structural components of Z-disk and analogous dense bodies where they cross-links actin filaments form adjacent sarcomeres	Regions of decondensed chromatin in nucleoplasm and in the granular component of nucleoli in HeLa cells; at interchromatin granules and nucleoli in human lymphocytes (Dingova et al. 2009)	Actinin 4 regulates DNaseY activity during apoptosis (Liu et al. 2004). A-actinin-4 co-localizes with p65/RelA subunit of NF-kappa B during cell activation by epidermal growth factor (EGF) (Babakov et al. 2008). Possibly involved in transcriptional regulation
Filamin	240–280	Eucaryota	N-terminus with two calponin homology domains (CH1 and CH2) followed by a rod region comprised of numerous repeat segments. C-terminal domain for dimerization	Crosslinker	Participate in stability of actin filaments at the cell periphery and link them to the plasma membrane, provide anchor of transmembrane receptors to the actin cytoskeleton and serve as a scaffold for many intracellular signaling molecules	Condensed and decondensed chromatin and nucleoli in HeLa cells, perichromatin in human lymphocytes (Dingova et al. 2009)	Interacts with the androgen receptor (AR) and inhibited transcriptional activity of AR (Loy et al. 2003; Ozanne et al. 2000). Plays an important role in Smad-mediated signaling (Sasaki et al. 2001). Associate with tumor suppressor protein BRCA2 (Meng et al. 2004) and FOXC1 (Berry et al. 2005)
Paxillin	68	Eucaryota	The N-terminus contains five aspartate-rich LD motifs and several SH2-binding domains. The C-terminus consists of four LIM domains	Binds actin	Adaptor protein that recruits a number of signal transducers to focal adhesions and transducing signals from integrins and growth factors to downstream regulation of cell migration and gene expression	Around interchromatin granules in HeLa cells; nucleoplasm in human lymphocytes (Dingova et al. 2009)	Assists in the translocation from the nucleus to focal adhesions for proteins such as Abl and STAT3 (Brown and Turner 2004). Expression of nuclear-localized paxillin LIM domains stimulateDNA synthesis and cell proliferation (Dong et al. 2009)
Spectrin II				Crosslinker		Nuclear in HepG2 hepatocytes	Regulation of Smad localization in the TGF-b signaling pathway (Kitisin et al. 2007)

Table 1 continued

Name(s)	MW (kDa)	Organism	Structural domains	Relation to actin	Function in cytoplasm	Nuclear localization	Nuclear (expected) function
Spectrin IIS	246–270	Eucaryota	Spectrin repeats, actin-binding domain (CH-domains), a pleckstrin homology (PH) domain, a Src homology 3 (SH3) domains, and a calmodulin-like domain	Crosslinker	Control membrane organization, stability and shape, participate in the trafficking of organelles along the secretory pathways, and play a role in regulated secretion	DNA-repair foci or diffuse nuclear in lymhoblastoid cells	Involved in DNA repair and act as scaffolding protein for DNA-repair proteins (Sridharan et al. 2003)
Spectrin IVS5				Crosslinker		PML nuclear bodies and the nuclear matrix (Tse et al. 2001)	Scaffolding for PML body proteins (Tse et al. 2001)
Myo16b	210	Vertebrates	N-terminal ankyrin repeat, motor domain, single IQ domain, and long tail with several polyproline stretches	Motor	Unknown	Punctuate nucleoplasmic (Cameron et al. 2007)	Unknown
Myo18b	285	Vertebrates	Motor domain, single IQ motif, and long tail with predicted coiled-coil regions, ERM domain, AAA domain, and NLS (Salamon et al. 2003)	Motor	Unknown	Cytoplasmic in myoblasts, diffuse nuclear in myocytes (Salamon et al. 2003)	Tumor suppressor (Nishioka et al. 2002)
Myo1c/NM1	120	Vertebrates	Motor domain, three IQ domains, PIP2-binding tail domain	Motor	Adjusting of the hearing sensors, exocytosis, axon growth	Diffuse/punctate nucleoplasmic	Transcription (Hofmann et al. 2006; Pestic-Dragovich et al. 2000; Philimonenko et al. 2004; Ye et al. 2008), chromatin remodeling (Percipalle et al. 2006)
Myo5a	200	Eucaryotes	Motor domain, 6 IQ domains, dilute domain	Motor	Vesicle transport and anchoring, spindle pole alignment and RNA translocation	Nuclear speckles (Pranchevicius et al. 2008)	Not known
Myo6	150	Metazoa	Motor domain, single IQ domain	Motor	Hearing, endocytosis, Golgi complex maintenance, cell motility	Punctate, colocalizes with transcription	Transcription (Vreugde et al. 2006)
Myopodin	80–95	Vertebrates	NLS	Bundling	Unknown	Diffuse nuclear excluding nucleoli in myoblasts, Z-discs in myocytes (Faul et al. 2007; Weins et al. 2001)	Tumor suppressor (Lin et al. 2001)
Syne/nesprin (NUANCE, enaptin)	1,014	Vertebrates	Interacts with chromatin structure regulator barrier-to-auto integration factor, presumably via the LEM domain	Bundling	ENAPTIN belongs to a family of recently identified giant proteins that associate with the F-actin cytoskeleton as well as the nuclear membrane		Integrate the cytoskeleton with the nucleoskeleton
Bpag1	630	Vertebrates	Multi-domain proteins that interacts with microtubules, actin filaments and intermediate filaments, as well as proteins found in cellular junctions		The dystonin/Bpag1 cytoskeletal interacting proteins play important roles in maintaining cytoarchitecture integrity in skin and in the neuromuscular system		Structural
Protein 4.1	80	Eukaryota		Binds actin	Stabilizes the spectrin-actin network and anchors it to the plasma membrane		Structural

Table 1 continued

Name(s)	MW (kDa)	Organism	Structural domains	Relation to actin	Function in cytoplasm	Nuclear localization	Nuclear (expected) function
Anillin	124	Eucaryota	ATPase domain	Binds actin	Bind septins and is a component of the cytokinetic ring	Diffuse/punctate nucleoplasmic	Localizes to the nucleus during interphase, the cortex following nuclear envelope breakdown, and the cleavage furrow during cytokinesis
Lamin A	75	Eucaryota		Binds actin		Nuclear envelope	Nuclear architecture
Emerin	34	Eutheria-mammals	LEM domain, transmembrane domain (Cai et al. 2007), NLS	Capping (Holaska et al. 2004)	Not described	Nuclear envelope (Fairley et al. 1999)	Maintenance of nuclear envelope
Enaptin	1,014 (predicted)	Bilateria-vertebrates and insects	N-terminal alpha actinin-type actin-binding domain, coiled coil rod - spectrin repeats, leucine zipper, C-terminal transmembrane domain, several NLSs	Bundling	Bundling of actin filaments, attachment of nucleus to the cytoskeleton	Nuclear envelope (Padmakumar et al. 2004)	Not known
Plastins	About 64	Eucaryota	EF-hands, Calponin homology actin-binding domains, NES (Delanote et al. 2005)	Crosslinking	Bundling of actin filaments	Diffuse nucleoplasmic	Not known
Tropomodulin	40	Vertebrates	Unstructured N-terminal domain, C-terminal domain contains several leucine repeats, NLS (Kong and Kedes 2004; Kong and Kedes 2006)	Capping	Stabilizing the pointed end of actin filament (Fischer and Fowler 2003)	Diffuse nucleoplasmic	Stabilizing pointed ends of actin filament?
CapG	38	Vertebrates	3 gelsolin-like repeat domains, NLS	Capping	Reversibly capping or blocking actin monomer exchange at the fast growing-barbed ends of actin filaments (Southwick and DiNubile 1986)	Diffuse nuclei and nucleoli (Onoda et al. 1993)	Promotes collagen invasion via modulation of transcription (De Corte et al. 2004)
Flightless I	145	Higher eucaryotes	NLS, NES, 6 gelsolin like repeat domains, 16 leucine-rich repeats (LRR)	Severing	Possessing filamentous actin severing activity via its gelsolin like repeat domains (Liu and Yin 1998)	Diffuse nuclear in fibroblasts (Davy et al. 2001)	Chromatin remodeling, nuclear receptor activation (Lee et al. 2004)
Gelsolin	90	Vertebrates	6 gelsolin-like repeat domains (LRR), 16 leucine-rich repeats	Severing, capping, nucleating	Severing actin filaments and capping the barbed ends, nucleating actin polymerization in a calcium dependent manner (Burtnick et al. 1997)	Diffuse nuclear staining in endothelial cells (Salazar et al. 1999)	Chromosome decondensation by severing actin in the nucleus (Ocampo et al. 2005). Androgen receptor co-activator (Nishimura et al. 2003)
Supervillin	205	Vertebrates	6 gelsolin like repeat domains, NLS, Headpiece domain (HD), Protein kinase A phosphorylation sites	Bundling	Inhibition of cell spreading by binding to myosin IIA and IIB, formation of actin filament bundles (Wulfkuhle et al. 1999)	Diffuse/punctuate nuclear (Pestonjamasp et al. 1997)	Nuclear receptor induced transcription, enhancing of vitamin D receptor activation (Ting et al. 2005)

Table 1 continued

Name(s)	MW (kDa)	Organism	Structural domains	Relation to actin	Function in cytoplasm	Nuclear localization	Nuclear (expected) function
Profilins	14–17	Eucaryotes vaccinia virus		Nucleotide exchange factor, increases polymerization	Regulation of actin dynamics at plasma membranes during cell locomotion, cytokinesis, embryonic development, morphogenesis. Intracellular vesicle trafficking. Endocytosis nad exocytoses at synapses (Jockusch et al. 2007)	Fine granular nucleoplasm staining (Rawe et al. 2006); nuclear gems (Sharma et al. 2005), speckles, Cajal bodies (Skare et al. 2003)	Cofactor of actin export from the nucleus mediated by exportin 6 (Stuven et al. 2003). Regulates the activity of Myb-related transcription factor p42POP (Lederer et al. 2005). Possible involvement in splicing (Skare et al. 2003). Tumor suppressor (Wittenmayer et al. 2004)
ADF/cofilin	15–21	Eucaryotes	ADF-homology (ADF-H) domain	Severing and pointed-end depolymerization	Cell motility, cytokinesis, endocytosis, exocytosis, phagocytosis, regulation of myofibril assembly. Involvement in actin-based motility of pathogenic bacteria (Ono 2007)	Localizes in intranuclear actin rods under stress conditions (Ono et al. 1996; Pendleton et al. 2003)	Chaperoning actin into the nucleus (Pendleton et al. 2003). Repressor of the glucocorticoid receptor (Ruegg et al. 2004)
CAP2		Eucaryotes		Sequestering	Mediator of a signal transduction pathway to actin cytoskeleton. Cell migration, endocytosis, embryonic development (Hubberstey and Mottillo 2002)	Diffuse excluding nucleoli (Peche et al. 2007)	Unknown

pathway mutants. In addition to FLOWERING LOCUS C, Arp6 regulates additional flowering repressors (Martin-Trillo et al. 2006). Arabidopsis Arp6 is located at specific regions of the nuclear periphery as demonstrated by confocal microscopy, and its subnuclear localization is different from that of TFL2, a plant homolog of HP1 (Choi et al. 2005). The mechanism of transcriptional regulation by Arp6 in plants seems to be via promoting both histone acetylation and methylation (Martin-Trillo et al. 2006).

To date, the structural organization and function of actin and Arp molecules in the chromatin remodeling and acetyltransferase complexes is unknown. It is possible that actin together with a myosin partner [Nuclear Myosin 1 (NM1), Myo1C or Myo6] may be needed as a motor for chromatin rearrangement or for the relocation of multi-complex transcription machinery. There is some evidence for the involvement of actin-based myosin motors in chromatin remodeling. During active transcription, 10% of cellular NM1 associates with the chromatin-remodeling complex Williams syndrome transcription factor (WSTF)-SNF2h (Cavellan et al. 2006; Percipalle et al. 2006). Together with several other nuclear proteins, WSTF-SNF2h and NM1 form a large 3-MDa complex referred to as B-WICH. SNF2h is a member of the ISWI family of chromatin-remodeling ATPases, and its functions depend largely on the protein complex in which it is contained. For instance, it partners with TIP5 to form nucleolar remodeling complex (NoRC), a chromatin-remodeling complex that sits on the promoters of unused ribosomal genes and inhibits their transcription (Santoro and Grummt 2005). WSTF associates with both ISWI and SWI/SNF chromatin-remodeling complexes. Together with SWI/SNF chromatin-remodeling complex, it is involved in regulation of vitamin D coupled transcription (Kitagawa et al. 2003). WSTF binds acetylated histones, mainly H3 acetylated at lysine 14, through its bromodomain and tethers the chromatin-remodeling/modification complexes to the promoter of vitamin D regulated genes (Fujiki et al. 2005). Because WSTF associates with two different chromatin-remodeling complexes, it was suggested that it acts as a platform tethering the complexes to chromatin (Kitagawa et al. 2003). NM1 was shown to co-purify and co-immunoprecipitate with WSTF-SNF2h. The complex also contained RNAs: Pol I transcript 45S rRNA and Pol III transcripts 5S rRNA and 7SL RNA (part of signal recognition particle SRP). NM1 was also found on rRNA, 5S and 7SL RNA genes by ChIP. Intriguingly, siRNA knockdown of WSTF decreases 5S and 7SL transcription (Cavellan et al. 2006). RNA Pol I transcription is inhibited by WSTF knockdown only on chromatin templates but not on naked DNA (Percipalle et al. 2006). It was possible to immunoprecipitate Pol I with the WSTF-SNF2h-NM1 complex, but only after in vivo crosslinking, which indicates that the association of the

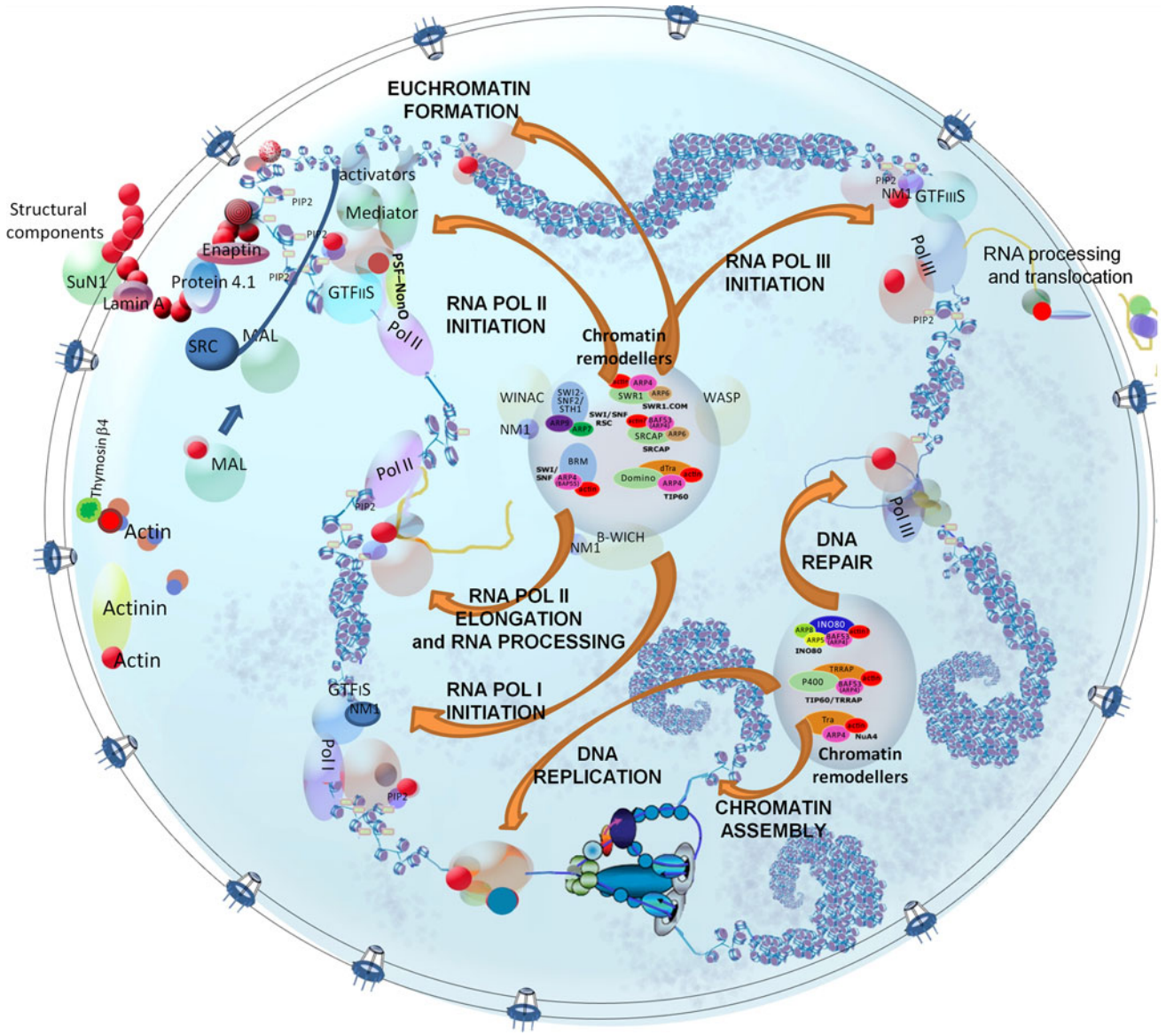

Fig. 1 The model depicts the nuclear processes involving actin and actin-binding proteins and actin complexes in chromatin remodeling, transcription initiation and elongation, DNA repair and replication. The center of the model shows a pool of complexes for chromatin remodeling that may interchange factors depending on the process. Actin may function as a key protein for interchanging such factors by bringing them together like a network that can be used in several processes in the nucleus

complex with RNA Pol I is relatively weak or very dynamic (Percipalle et al. 2006). Moreover, both activation and repression of ribosomal genes could be achieved by similar mechanisms. Ribosomal genes are repressed by NoRC, where TIP5 binds to transcription termination factor 1 on rDNA and brings SNF2h. SNF2h mobilizes histone deacetylases and DNA methyl transferases that modify the chromatin (Santoro and Grummt 2005). Conversely, WSTF could bring the SNF2h and associated factors to the rDNA to activate transcription, and the recruitment of WSTF itself could occur via NM1 or other ABP and the Pol I-bound actin (Grummt 2006; Percipalle and Farrants 2006).

A question remains how myosins would interact with nuclear actin to fulfill their motor function as they require a stretch of polymeric actin, and conventional F-actin is not readily formed in most nuclei. One possibility is that a "twisted" conformation of actin filament (so-called T-actin) is present in nuclei (Egelman 2003). This conformation is more favorable to binding of ADF/cofilin and does not allow tropomyosin binding with subsequent myosin recruitment. However, a few tropomyosin isoforms have been found to interact with actin filaments in the presence of cofilin (Kuhn and Bamburg 2008). It is yet to be explored whether these complexes also allow myosin

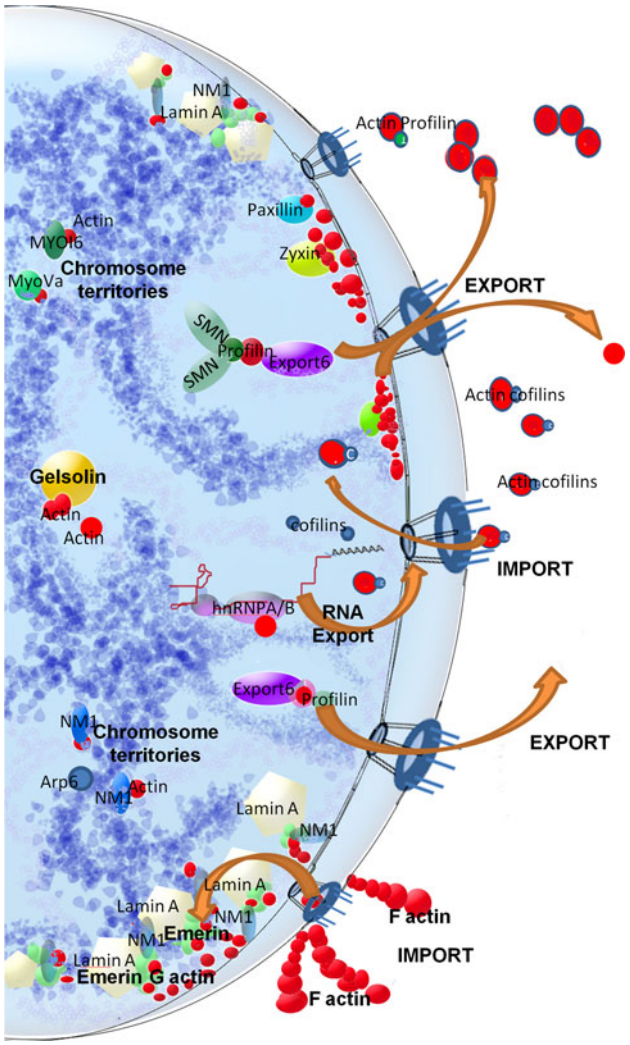

Fig. 2 The model shows actin and actin-binding proteins involved in nuclear structure, nucleocytoplasmic transport as well as chromosomal rearrangements. Actin is well known for its structural roles in the cytoplasm; however, mechanisms for the nuclear structure organization still need to be elucidated. Actin and actin-binding proteins have been shown to participate in such processes, either by a different form of actin polymerization or creating a link with different partners

binding. The fact that 2G2 antibodies, raised against actin–cofilin filaments, stain specifically nuclear actin (Gonsior et al. 1999) speaks in favor of such a model. Alternatively, actin and Arps could be used as bridges between different sets of chromatin remodelers. This would create a link for specific chromatin modifiers with ABPs to a network that can be used in several processes in the nucleus, where different modules can be exchanged depending on the function needed (Hogan and Varga-Weisz 2007). Nevertheless, it remains unclear how myosins could bind to such structures.

Babakov and co-workers (2004) found that α-actinin-4 co-localizes to the nucleus with p65/RelA subunit of NF-kappa B during cell activation by epidermal growth factor (EGF). Cell treatment with EGF leads to translocation of the proteins to membrane ruffles, and eventually to migration into the nucleus. Chakraborty and colleagues (2006) identified α-actinin-1 and -4 as class IIa HDAC-interacting proteins and isolated a novel splice variant of α-actinin-4 that is predominantly localized in the nucleus of HeLa cells.

FliI is a nuclear receptor coactivator, it contains gelsolin-like domain that can bind to actin. The same domain is important for the coactivator function. Co-immunoprecipitation experiments revealed FliI binding to BAF53 via the first of the two gelsolin-like motifs. A point mutation in the actin-binding motif of FliI reduced binding to BAF53 and substantially diminished the co-activator activity of FliI. Yeast two-hybrid screening assays demonstrated FliI binding to histone methyltransferase CARM1, which is a part of p160 coactivator complex (Lee et al. 2004). Based on these findings one can speculate that FliI may ensure the association of SWI/SNF complex to p160 to maintain ATP-dependent chromosome remodeling activity along with histone acetylation by histone acetyltransferase p300 and/or CBP and methylation by histone methyltransferase CARM1 and/or PRMT1.

In vitro studies of the binding properties of SWI/SNF-like BAF chromatin-remodeling complex to actin filaments revealed an interesting role of phosphatidylinositol 4,5-biphosphate (PIP2) (Rando et al. 2002). Isolated BAF complexes were able to associate with actin pointed ends and branch points upon binding to PIP2 micelles. The authors suggested a model where PIP2 binding relieved capping of actin associated with the BAF complex and therefore allowed actin filament binding. They provide some evidence that Brg1, which is also a component of BAF remodeling complex, could be this capping component. However, a role of another actin-capping protein cannot be excluded, and it was hypothesized that this protein could be CapG (Gettemans et al. 2005). CapG is a gelsolin family protein that has both nuclear and cytoplasmic localization (Onoda et al. 1993; Prendergast and Ziff 1991). One recent study shows CapG also in the nucleolus. This study indicates that transport of CapG to the nucleolus is an ATP-dependent process, which requires active RNA polymerase I transcription and translocation of Ran GTPase to the nucleolus along with CapG (Hubert et al. 2008).

All this data shows the relevance of ABPs in chromatin remodeling where they are required for several process as depicted in Fig. 1. However, recent transcriptional experiments using naked DNA templates have shown that actin and ABPs play additional roles in the control of gene expression, as outlined below.

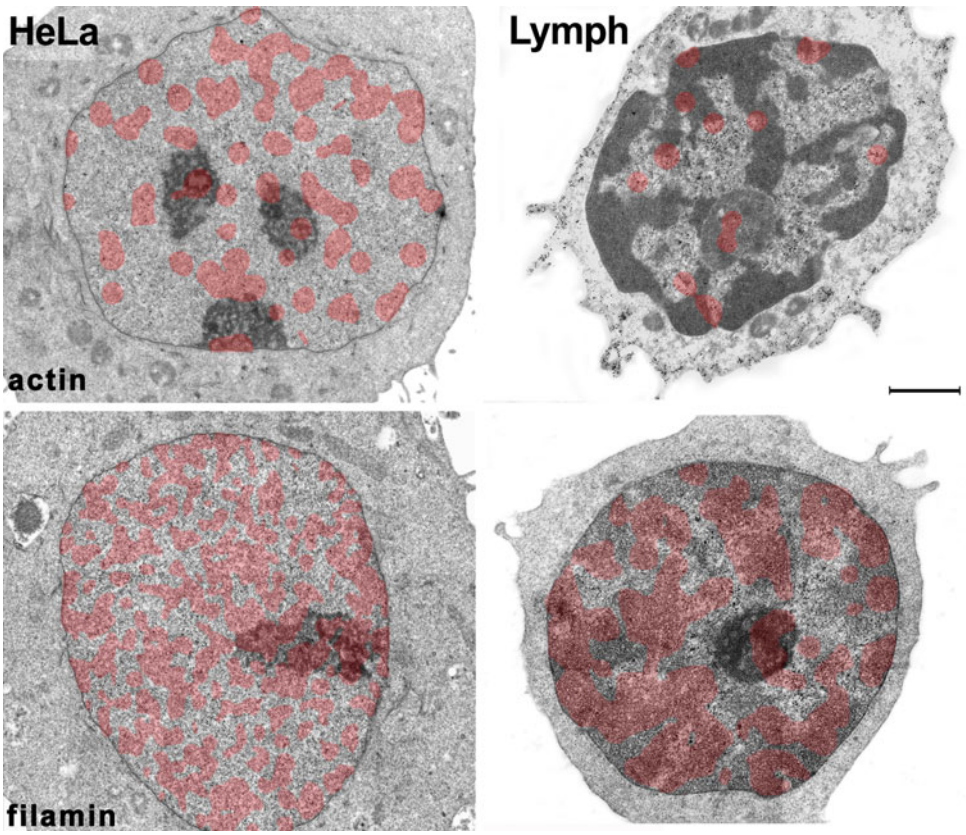

Fig. 3 The distribution of actin and filamin in the nuclei of transcriptionally active HeLa cells and resting human lymphocytes is compartment-specific. Actin labeling in HeLa cells is localized preferentially in decondensed chromatin and in the nucleolus. In resting human lymphocytes, clusters of actin labeling are located mainly in decondensed chromatin at the border of heterochromatin blocks. Filamin labeling in HeLa cells is intense and appears as a dense mesh throughout the whole nucleus, including nucleoli. In human lymphocytes, filamin clusters fuse together to form large labeled areas that occupy a significant proportion of condensed chromatin and perichromatin area but are mainly excluded from interchromatin granules. Ultrathin sections of cells were gold-immunolabeled with antibodies to actin and filamin, and the label density vas evaluated using our self-developed algorithms (Schöfer et al. 2004; http://nucleus.img.cas.cz/gold). The areas of increased labeling density in the nucleus are highlighted with *red* color. Labeling in cytoplasm is not depicted. *Bar* 1 μm. Reproduced from Dingova et al. 2009 with permission

ABPs in DNA transcription

Transcription initiation by all three polymerases was originally investigated at the level of transcriptional machinery recruitment. It became clear as early as in 70s, that polymerases alone were insufficient for this process. Roeder and colleagues were among the first to isolate a set of additional proteins essential for transcription—general transcription factors (GTFs). Their findings showed that GTFs are needed to sustain a low level of in vitro transcription on naked DNA with the addition of RNA polymerases (for a review, see Roeder 2005). Nevertheless, other additional factors are required for activating high-level transcription. Therefore, the hypothesis emerged for transcriptional regulation, in which regulation takes place not only at the promoter level but also at distant control elements, or enhancers. The activators bound to the enhancers were proposed to facilitate promoter initiation recruitment through coactivator mediation of activation signals and DNA looping to allow interaction of proteins bound to distal sites with the GTFs on the promoter. In some cases, it has been suggested that RNA pol II might relocate directly from the enhancer to the promoter during the activation process (Tchurikov et al. 2009). Tracking and looping requires a mechanical force in which actin-interacting molecules together with molecular motors like NM1 might be up to the task. Work by Yoo and colleagues (2007) showed that the Arp2/3 complex physically associates with RNA pol II and is involved in RNA pol II-dependent transcriptional regulation both in vivo and in vitro, as demonstrated by siRNA knockdown of Arp2 and Arp3. A further body of work suggests a bridge for Arp2/3 complex with Neuronal Wiskott–Aldrich syndrome protein (N-WASP) and PSF–NonO complex together with RNA pol II (Wu et al. 2006). It is conceivable that these proteins could properly position RNA pol II on the promoter. Some

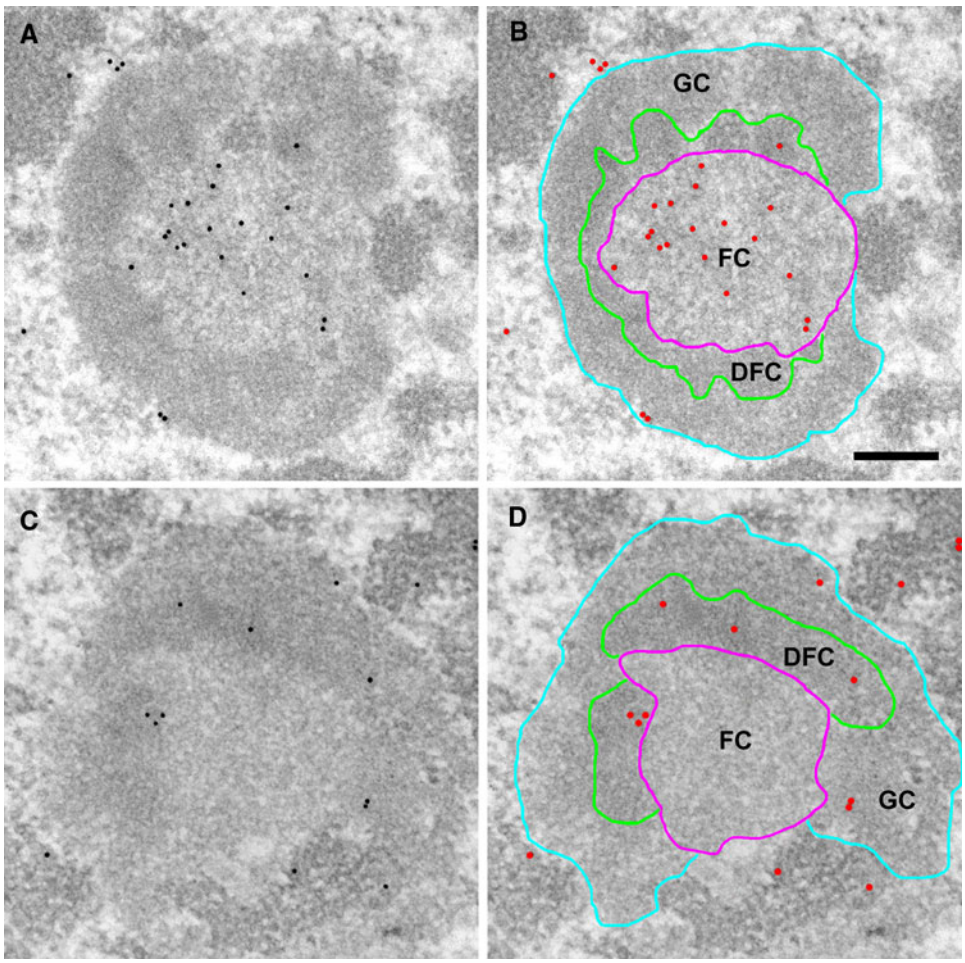

Fig. 4 Actin and NMI are localized in different nucleolar compartment in transcriptionally inactive human lymphocytes. **a** An electron micrograph of resting human lymphocyte nucleolus labeled with anti-actin antibody (12-nm gold particles). **b** The same image as **a**, but the nucleolar compartments are *outlined*. Fibrillar centers (FC), DFC and granular component (GC) are *marked*. Gold particles are highlighted in *red* color. FC is highly positive for actin. **c** An electron micrograph of resting human lymphocyte nucleolus labeled with anti-NMI antibody (12-nm gold particles). **d** The same image as **c**, the nucleolar components are *outlined* and gold particles are highlighted as in **b**. NMI is localized predominantly in DFC. *Bar* 200 nm. Reproduced from Kysela et al. 2005 with permission

experiments have emerged to sustain this hypothesis. It is known that Myo1c/NM1 can bind to actin via its head domain to exert physical tension or movement of their cargo and it was noted early on that NM1 can be found within sites of transcription activity of RNA polymerase I and II. Indeed, Hofmann and co-workers (2006) showed that NM1 colocalizes and copurifies with RNA pol II. Antibodies to NM1 inhibit Pol I in in vivo and in vitro transcription assays, while addition of NM1 activates RNA pol I transcription in vitro in a dose-dependent manner (Philimonenko et al. 2004). Microinjections of anti-NM1 antibodies reduce RNA pol II transcription in vivo. Interestingly, antibodies to NM1 added to reconstituted RNA pol II transcription system do not inhibit formation of the RNA pol II pre-initiation complex but block the formation of the first phosphodiester bond during transcription initiation (Hofmann et al. 2006). On the other hand, the experiments with RNA Pol I transcription in nuclear extracts indicate the involvement of NM1 in the later steps of transcription, such as promoter escape or elongation (Percipalle et al. 2006). Since NM1 can directly interact with actin and N-WASP-PSF-NonO-RNA Pol II, this may help the process of translocation from promoter regions to transcription sites within the nucleus, as well as for further fine positioning. However, the mechanism of how actin and NM1 may function within these processes has not yet been thoroughly tested.

Myo6 is another actin molecular motor recently found in the cell nucleus and it seems to have very similar properties to those of NM1. It colocalizes with RNA pol II and newly transcribed mRNA in a transcription-dependent manner, co-immunoprecipitates with RNA pol II complex, it is present at the promoter and coding region of active genes; knockdown of Myo6 inhibits transcription of these genes, and antibodies to Myo6 reduce RNA pol II transcription in vitro (Vreugde et al. 2006). Myo6 can also interact with RNA pol II, apparently dimerizing upon cargo binding and in this manner gaining processivity, i.e. the ability to "walk" many steps along a single actin filament before dissociating. Myo6 is therefore especially interesting because of its ability to exert force toward the minus end of the actin filament and it can play further roles in which a molecular motor is required (for review see Sweeney and Houdusse 2007).

Other mechanisms in which ABPs affect transcription involve interactions with additional activators or coactivators. In particular, the members of the nuclear hormone receptor family in which ABPs can activate or repress their function are an excellent example. Ozanne and co-workers (2000) identified filamin as an ABP that interacts with the androgen receptor affecting its nuclear translocation. Loy and colleagues (2003) showed that C-terminus of the filamin A (FLNa) inhibits transcription of the androgen receptor by modulating its activity since it competes with its coactivator TIF2. FLNa also interacts with regulatory proteins such as the tumor suppressor protein, BRCA2 (Yuan and Shen 2001) and protein FOXC1, which is a component of a larger complex that regulates the initiation of transcription. Moreover, FLNa is required for nuclear localization of transcription factor PBX1 (Berry et al. 2005). Filamin also plays an important role in the signaling mediated by Smad, protecting Smad proteins from ubiquitin-mediated degradation by masking an ubiquitin ligase-binding site of Smads (Sasaki et al. 2001). Supervillin, another member of villin/gelsolin family, is a nuclear/cytoplasmic actin-bundling protein and it is the primary coactivator of androgen receptor, as it enhances the activity of peroxisome proliferator-activated receptor-γ (PPAR-γ), glucocorticoid receptor and estrogen receptor (Archer et al. 2004; Ting et al. 2002). This stimulation differs from other nuclear receptor coactivator stimulations. While other coactivators increase the activation between N-terminal and C-terminal halves of the receptor, supervillin slightly suppresses it. A supervillin fragment (831–1281) was also found to colocalize with the androgen receptor in the nucleus when 5 alpha-dihydrotestosterone was added (Ting et al. 2002; Yu et al. 1990). The Ca^{2+}-calmodulin-dependent protein kinase type II binds to FliI and influences β-catenin dependent gene expression (Seward et al. 2008). FliI also regulates the development of *Caenorhabditis elegans* affecting anterior-posterior polarity and asymmetric cell division (Deng et al. 2007). FliI was shown to localize in the nucleus in the fibroblasts, where it translocates upon serum stimulation into the periphery of the cell from the nuclear/perinuclear region. FliI not only binds to actin but also binds to ARP4 as well as to p160 coactivators (Lee et al. 2004). Coimmunoprecipitation studies show that FliI associates with nuclear receptors such as thyroid hormone receptor and estrogen receptor, and siRNA silencing of FliI affects hormone-stimulated reporter gene expression (Lee et al. 2004). Furthermore, a G-ABP MAL is a coactivator of serum response factor. This is a transcription factor that regulates many mitogen-responsive and muscle-specific genes (Philippar et al. 2004). MAL binding to nuclear G-actin makes it unable to bind to serum response element and trigger transcription. MAL is then exported from the nucleus to the cytoplasm in complex with G-actin and Crm1 exporter. When actin is polymerized, the pool of free G-actin is reduced and the cofactor is released (Vartiainen et al. 2007). Free MAL can complex with serum response factor, which is able to bind serum response sequence and trigger transcription (Miralles et al. 2003).

The third mechanism in which ABPs affect transcription by RNA pol II is protein phosphorylation. It is known that phosphorylation of RB by cyclin-dependent kinases at the G1/S boundary leads to RB release and therefore activation of the ABP c-Abl tyrosine kinase. Activated nuclear c-Abl was shown to phosphorylate the C-terminal domain of RNA pol II to modulate transcription (Baskaran et al. 1993).

In the case of RNA pol I transcription, NM1 was observed in the dense fibrillar compartment, where transcription of ribosomal genes takes place. Here, it colocalizes with actin (Fomproix and Percipalle 2004; Nowak et al. 1997). Moreover, NM1 is indeed required for RNA Pol I transcription and it is associated with RNA Pol I through the basal transcription factor TIF-IA, a key regulator of Pol I transcription (Philimonenko et al. 2004).

In addition to the chromatin-remodeling process, these mechanisms for RNA pol I and II transcription show how ABPs are involved in several key steps for transcription control. ABPs together with actin and Arps may help to create a very dynamic scaffold in which nuclear processes can take place. As depicted in Fig. 1, ABPs are heavily involved at different stages of gene regulation. The elucidation of how the three-dimensional structures including actin, ABPs, and associated enzymes participate in these processed will be part of future research needed to truly understand gene activation in eucaryotic cells.

ABPs in DNA replication and repair

The most established role for ABPs in DNA replication and repair involves chromatin remodeling. The process of nucleosomes removal and their reincorporation are crucial steps both in DNA replication and repair, for which chromatin remodelers with bound actin and histone-modifying enzymes are needed. INO80 chromatin-remodeling complex plays a role in DSB DNA repair through recognition and eviction of phosphorylated form of histone H2A (in mammals called γH2AX) from DSB site to facilitate ssDNA formation and subsequent repair events. The Arp4, Arp5, Arp8 and actin form part of the INO80 complex and are essential for nucleosome-remodeling activity in vitro. It has been also shown that *arp5* and *arp8* mutants are hypersensitive to DNA damage agents. For Arp4, a role in recruitment of the complex to DSBs through interaction with γH2AX has been proposed. Furthermore, INO80 was shown to promote recovery of stalled replication forks in *S.*

cerevisiae. INO80 complex is associated with stalled replication forks genome-wide in yeast, and strains lacking INO80 show significant defects in the resumption of DNA replication. The mechanism of INO80 function in resumption of replication at stalled forks appears to be different from its activity in DSB repair. The recruitment of Arp5 to sites of initiation of DNA replication was independent of γH2A. Consistently, deletion of nhp10, which is responsible for the recruitment of INO80 complex to DSBs, did not have the same effect as *ino80* or *arp5* mutants on the recovery from replication arrest (Shimada et al. 2008).

Another chromatin remodeler, SNF2h, plays a role in DNA replication. It binds the WICH complex through WSTF to proliferating-cell nuclear antigen (PCNA) on replicated chromatin and prevents premature formation of heterochromatin. This therefore allows the re-binding of factors that transmit the epigenetic state to the newly synthesized DNA (Poot et al. 2005). NM1 was shown to be associated with a fraction of WICH involved in RNA Pol I transcription (Percipalle et al. 2006). It might be therefore interesting to investigate whether NM1 participates also in replication of heterochromatin.

Other functions of ABPs during DNA replication/repair could be recruitment of factors and scaffolding of functional complexes on filamentous actin. McMahon and colleagues (1999, 2001) identified spectrin as a direct binding partner of the Fanconi anemia protein FANCA. Sridharan and co-workers (2003) demonstrated that spectrin relocalizes to the same nuclear foci along with DNA-repair proteins, FANCA and XPF (nucleotide excision repair protein) after damage with a DNA cross-linking agent. This study suggested that nuclear spectrin may have an important function of in the nucleus providing a scaffold and aiding the recruitment of repair proteins to sites of the DNA damage. Myo16b displays overlapping localization with PCNA and cyclin in neuronal nuclei and tends to associate with nuclear compartment containing profilin and polymerized actin. It would be tempting to assume that Myo16b works as a motor to direct the movement of protein complexes in the nucleus during S phase. However, an analysis of the sequence of Myo16b ATP-binding pocket shows an amino acid replacement that significantly reduces actin-dependent ATPase activity. Thus, a scaffolding function of Myo16b is more likely than a motor function. Moreover, overexpression of Myo16b delays the progression through S-phase and decreases BrdU incorporation. In cells that incorporate BrdU, Myo16b does not colocalize with nascent DNA (Cameron et al. 2007). So, Myo16b is obviously involved in regulation of replication and S phase progression, but the precise mechanism should be elucidated. Furthermore, elevated Myo6 expression was induced by p53 during DNA damage. Upon p53 activation, Myo6 translocates from endocytic vesicles, membrane ruffles and cytoplasm to the Golgi complex, perinuclear membranes, and to the nucleus where it may be involved in DNA repair. RNAi knockdown of Myo6 leads to attenuated p53 activation and impaired Golgi integrity (Jung et al. 2006). Another example is thymosin beta$_4$, which is transported to nucleus in complex with mismatch DNA repair enzyme hMLH1, interacts with actin and may help to direct the enzyme to other steps of this process (Brieger et al. 2007).

ABPs involved in nucleocytoplasmic transport

The first evidence of actin involvement in nucleocytoplasmic transport was obtained as early as 1986 from isolated rat liver nuclei (Schindler and Jiang 1986). Antibodies to actin or myosin significantly reduced the flux rate of 64-kDa fluorescent dextran through the nuclear pores as measured by FRAP. Moreover, while addition of ATP greatly enhanced the flux rate in control nuclei, the addition of actin-affecting drugs such as cytochalasin D or phalloidin inhibited this stimulation of transport. Although these results may be criticized for use of isolated nuclei, more recent work confirms the role of actin in nucleocytoplasmic transport in intact cells. Hofmann and colleagues (2001) demonstrated that intranuclear microinjection of 2G2 antibodies raised against actin–profilin complex (Gonsior et al. 1999) blocked the nuclear Rev- and TAP-mediated export of viral RNAs as well as export of protein kinase A inhibitor both in Xenopus oocytes and in somatic cells. This export was also blocked by latrunculin B, which binds to G-actin but not by swinholide A that stabilizes actin dimers. By using 2G2 antibody, it was also possible to visualize actin present in the gelsolin-resistant fibers emanating from the nuclear envelope and associated with nuclear pore complexes (Hofmann et al. 2001). Another independent study also demonstrated a network of pore-linked filaments containing actin and an ABP 4.1 in Xenopus oocyte nuclei (Kiseleva et al. 2004). Interestingly, heterogeneous nuclear ribonucleoproteins (hnRNPs) appear to bind actin (Kukalev et al. 2005; Percipalle et al. 2002, 2003), some of them being able to shuttle between the nucleus and cytoplasm. This opens another interesting possibility that such actin fibers may in general help to dock the protein–RNA complexes at the pores via an ABP present in the complex and thus facilitating their export to cytoplasm. This model, at least for the case of preribosomal subunits protein–RNA complexes, was supported by very recent findings of Obrdlik and colleagues (2010). They showed that NM1 is present in the complex with mature rRNA transcripts and immunolocalized it at the top of nuclear pore basket in Xenopus oocyte nuclei.

Actin itself is exported from the nucleus by a nuclear export receptor Exportin 6 in complex with an ABP profilin (Stuven et al. 2003). Profilins are small proteins of molecular weight 18–14 kDa. They pass through the nuclear pores by simple diffusion and are quickly replenished in the nucleus. The dimer actin–profilin can couple profilin-binding proteins to Exportin 6 and they can be subsequently exported into the cytoplasm. Stuven et al (2003) have also found other proteins that bind profilin and actin to be present in a complex with Exportin 6. The survival motor neuron (SMN) protein has been shown to bind profilins I and II via the polyproline-rich motif (Giesemann et al. 1999). SMN is ubiquitously expressed protein that functions in assembly and transport of diverse ribonucleoprotein complexes (Kolb et al. 2007). Therefore, the Exportin 6-actin–profilin pathway may also participate in the export of SMN protein complex to the cytoplasm. Profilin also appears to play a significant role in nuclear export in Drosophila. The mutants with down-regulated profilin show mislocalization of RanGAP, protein that catalyses the hydrolysis of GTP in Ran, and have defects in nuclear export. Drosophila mutants that have down-regulated NTF2, a protein that imports small GTPase Ran into the nucleus, exhibits few survivals with small or no eyes. This phenotype was successfully rescued by crossing with a stream that has down-regulated profilin (Minakhina et al. 2005).

A CRM1-dependent nuclear export pathway is also involved in the regulation of the nuclear pool of actin, although actin does not bind CRM1 directly (Stuven et al. 2003). An earlier study by Wada et al. (1998) showed that after long exposure of cells to a CRM1 inhibitor leptomycin B, nuclear rod-like structures containing actin could be observed. The Exportin 6 pathway was not able to reduce the levels of nuclear actin so another mechanism to export actin to the cytoplasm must exist and is apparently dependent on CRM1. Thus, the plausible explanation for the nuclear rod effect is that they appear after nuclear sequestering of actin via blocking the nuclear export of another nuclear ABP that mediates binding of actin to CRM1 complex.

Due to its relatively low molecular weight of 43 kDa, monomeric actin may be able to pass the nuclear pore by diffusion. However, an active mechanism must exist that transports actin into the nucleus since actin fused to GFP is also able to localize into the nucleus (McDonald et al. 2006). One candidate for facilitating nuclear import of actin is cofilin. Actin and cofilin are observed together upon nuclear accumulation, and a function for cofilin was suggested to chaperone actin into the nucleus. Anti-cofilin antibody blocked nuclear entry of actin in permeabilized cells upon treatment with latrunculin B and ATP depletion (Pendleton et al. 2003). Nuclear translocation of cofilin is possibly triggered by dephosphorylation, as shown in fibroblasts (Nebl et al. 1996; Ohta et al. 1989) and in T cells (Samstag et al. 1994), while other data do not confirm this (Abe et al. 1993; Saito et al. 1994).

Another candidate for involvement in nucleocytoplasmic transport is paxillin. Paxillin shuttles between focal adhesions and the nucleus, as demonstrated by the experiments showing nuclear accumulation of paxillin after mutation/deletion of its NES domain or after inhibition of CRM1-dependent nuclear export by leptomycin B (Dong et al. 2009; Woods et al. 2002). A function of paxillin was proposed to assist in the translocation from the nucleus to focal adhesions for such proteins as Abl and STAT3 (Lewis and Schwartz 1998; Silver et al. 2004).

Taken together, these observations have shown that ABPs together with actin are significantly involved in the nuclear transport, as depicted in Fig. 2. However, the mechanisms in which they work during this process still remain mostly unresolved.

ABPs involved in nuclear architecture, dynamics and sensing outside

Structural integrity and spatial compartmentalization of the cell nucleus is important for its proper functioning. The best characterized structural component of the cell nucleus is nuclear lamina, composed of lamins belonging to the family of intermediate filament proteins. Furthermore, A-type lamin proteins are not only located at the nuclear periphery, but also are part of the internal nucleoskeleton/nuclear matrix. They are essential for nuclear structural integrity, DNA replication, transcription, splicing, cell signaling, DNA repair and cellular proliferation (Andres and Gonzalez 2009; Dechat et al. 2009; Gonzalez-Suarez et al. 2009; Vlcek and Foisner 2007). An interaction of the carboxy-terminal domain of lamin A with actin has been demonstrated in vitro, thus rendering lamin A a "non-classical" ABP (Sasseville and Langelier 1998). A-type lamins together with nesprins and SUN domain proteins participate in the formation of the linker of cytoskeleton and nucleoskeleton (LINC) complex. Nesprins are rod-like nuclear membrane proteins, which interact with F-actin and can mediate SUN protein binding. Nesprin 1 and 2 on the outer nuclear membrane connect cytoplasmic actin microfilaments to SuN1 and SuN2 on the inner nuclear membrane; SuN1, in turn, binds to lamin A on the nuclear scaffold (Crisp et al. 2006; Ostlund et al. 2009). Another protein belonging to the nesprin family, enaptin, was immunolocalized to actin stress fibers, the nuclear membrane, and interestingly, the nucleoplasm of COS7 cells thus being a part of the nuclear structural assembly (Padmakumar et al. 2004). LINC complex is important for nuclear

positioning and movement within the cell. It can also play a role in the transmission of mechanical signals from the cytoplasm to the nucleus. Pulling on integrins in cultured cells induces realignment of individual actin stress fibers and nucleoli, and alters their positions and orientation along the newly applied tension lines (Hu et al. 2005). It is not clear what is transferring the tension inside the nuclei; however, actin-based structures could well play this role.

Another protein that significantly contributes to the nuclear structure is emerin. Emerin is an inner nuclear membrane protein that is expressed in all differentiated cell types. It interacts with chromatin structure regulator barrier-to-auto integration factor, presumably via the LEM domain (Bengtsson and Wilson 2004). The lamin–emerin interaction has been implicated in maintaining the structural integrity of the nucleus and in efficient progression of the cell through the cell cycle. Emerin interacts with transcription factors (GCL, Btf, Lmo), splicing regulators (YT521-B), nuclear membrane protein nesprin-1α, actin and NM1 (Holaska 2008). The first evidence that emerin could bind α and β actin was provided by (Fairley et al. 1999). Holaska and collaborators (2007) showed later that emerin binds F-actin at the pointed end and identified NM1 as a direct ATP-independent binding partner of emerin, also by affinity purification of emerin-containing complexes from HeLa nuclear extracts. They found that NM1 is present along with actin, αII-spectrin and lamins in one protein complex that could participate in structural maintenance of the nuclear membrane.

There are two recent works that directly demonstrate the role of actin and ABPs in structural properties of nuclei. Bohnsack and co-workers (2006) found out that exportin 6, which is responsible for the nuclear export of actin, is not expressed in Xenopus oocytes before the onset of meiotic maturation. Furthermore, after microinjection of exogenous exportin 6 into oocyte nuclei, the intranuclear actin network disappeared, and at the same time the nuclei became extremely fragile. This effect was highly specific for exportin 6 injections and not observed upon control microinjections. These results point to the necessity of intranuclear actin network for the stabilization of giant oocyte nuclei. Indeed, application of actin-depolymerizing drug cytochalasin B also made the nuclei very fragile, while co-injection of F-actin-stabilizing phalloidin together with exportin 6 prevented nuclear fragility. Krauss and co-workers (2002, 2003) used Xenopus cell-free system of nuclear assembly to demonstrate that actin, ABP 4.1 and their functional interaction are required for proper formation of reconstituted nuclei and their replication competence. Protein 4.1 is an actin-scaffolding protein that can also bind spectrin (Correas et al. 2001). Interestingly, functional aminoacid sequences in the 4.1 domains responsible for binding to spectrin and to NuMa were also required for assembly of functional nuclei (Krauss et al. 2002).

This functional data is supported by ultrastructural work of several groups. Intranuclear actin-containing filaments were shown by electron microscopy in amphibian oocytes (Hofmann et al. 2001; Kiseleva et al. 2004). Such filaments emanated from the nuclear envelope and were often associated with nuclear pore complexes. Interestingly, filaments containing actin together with protein 4.1 formed a network that attached to Cajal bodies and other subnuclear organelles in Xenopus oocytes (Kiseleva et al. 2004; Pederson and Aebi 2002). Actin-based intranuclear structures might thus contribute to nuclear compartmentalization. Wang and co-workers (2006) reported that actin polymers participate in the reorganization of interchromatin granules clusters upon inhibition of transcription and form a special compartment, where transcription begins after release from inhibition. Preferential distribution of various ABPs, such as α-actinin, filamin, paxillin, spectrin and tropomyosin in specific nuclear compartments was shown ultrastructurally (Dingova et al. 2009), see an example in Fig. 3. Interestingly, the intranuclear distribution of actin and NMI was different in resting and activated human lymphocytes (Kysela et al. 2005). Figure 4 demonstrates that while actin and NMI are involved in rDNA transcription, in resting human lymphocytes with very low levels of transcription these two proteins localize in different nucleolar compartments. Actin and ABPs can be involved also in higher-order nuclear compartmentalization, such as organization and dynamics of chromosomal territories. Mehta and colleagues (2010) found that chromosome territories relocated rapidly within the cell nucleus when serum was removed from the culture medium. This relocation was energy-dependent and inhibited by drugs affecting polymeric actin or by NMI knock-down by RNA interference.

Other ABPs in the nucleus can contribute to the structural properties by regulating the polymerization state and dynamics of nuclear actin. Cyclase-associated protein 2 (CAP2) enhances actin filaments turnover. It is strongly enriched in the nuclear membrane fraction as well as in the nuclei. CAP2 is tightly bound and is released from the nucleus primarily by adding 2 M NaCl, which indicates that the detergent-resistant CAP2 is immobilized by attachment to non-chromatin structures (Peche et al. 2007). Furthermore, plastins may act in the nucleus as actin cross-linking proteins, which are generally thought to generate force by organizing actin filaments into bundles (Loomis et al. 2003; Vignjevic et al. 2003). Myopodin is also an actin-bundling protein, which is localized in the nuclei of differentiating myobasts (Weins et al. 2001). Formins also nucleate and elicit rapid processive assembly of filaments from profilin–actin, remaining bound to the growing barbed end (Chan and Leder 1996). Furthermore, in C2C12

cells, the Bpag1 localizes predominantly to the nucleus, its actin-binding domain interacts with the actin cytoskeleton and with the plakin domain region as it translocates the proteins to the nucleus. These results indicate that it is necessary for regulating the localization and function of plakin proteins required for structural work (Young et al. 2003).

Conclusions

Taken together, from all these observations, one can see the vast and complex scenario in which actin, ABPs and Arps are involved in a multitude of nuclear functions. Various functions are schematically summarized Figs. 1, 2. Interestingly, their actions extend from the nucleus to the cell surface as they seem to be also a part of the integral signaling pathways about events occurring on the cell surface, and our limited knowledge points now especially to the focal adhesions. Obviously, the still very fragmented data on nuclear functions of these proteins need to be seen not only as nuclear, but they need to be understood in the cellular aspect. The crucial questions about nuclear actin structure and about molecular properties of various sophisticated complexes that include varieties of actin, ABPs and Arps and their dynamics are still ahead. We can therefore foresee that there is a long way to understand the complexity of nuclear actions of actin, ABPs and Arps in the structural and functional networks in the entire cell.

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Specific Nuclear Localizing Sequence Directs Two Myosin Isoforms to the Cell Nucleus in Calmodulin-Sensitive Manner

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Abstract

Background: Nuclear myosin I (NM1) was the first molecular motor identified in the cell nucleus. Together with nuclear actin, they participate in crucial nuclear events such as transcription, chromatin movements, and chromatin remodeling. NM1 is an isoform of myosin 1c (Myo1c) that was identified earlier and is known to act in the cytoplasm. NM1 differs from the ''cytoplasmic'' myosin 1c only by additional 16 amino acids at the N-terminus of the molecule. This amino acid stretch was therefore suggested to direct NM1 into the nucleus.

Methodology/Principal Findings: We investigated the mechanism of nuclear import of NM1 in detail. Using over-expressed GFP chimeras encoding for truncated NM1 mutants, we identified a specific sequence that is necessary for its import to the nucleus. This novel nuclear localization sequence is placed within calmodulin-binding motif of NM1, thus it is present also in the Myo1c. We confirmed the presence of both isoforms in the nucleus by transfection of tagged NM1 and Myo1c constructs into cultured cells, and also by showing the presence of the endogenous Myo1c in purified nuclei of cells derived from knock-out mice lacking NM1. Using pull-down and co-immunoprecipitation assays we identified importin beta, importin 5 and importin 7 as nuclear transport receptors that bind NM1. Since the NLS sequence of NM1 lies within the region that also binds calmodulin we tested the influence of calmodulin on the localization of NM1. The presence of elevated levels of calmodulin interfered with nuclear localization of tagged NM1.

Conclusions/Significance: We have shown that the novel specific NLS brings to the cell nucleus not only the ''nuclear'' isoform of myosin I (NM1 protein) but also its ''cytoplasmic'' isoform (Myo1c protein). This opens a new field for exploring functions of this molecular motor in nuclear processes, and for exploring the signals between cytoplasm and the nucleus.

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Introduction

Nuclear myosin I (NM1) was the first unconventional myosin motor detected in the cell nucleus [1]. NM1 is an isoform of earlier identified cytoplasmic myosin Ic (Myo1c) containing additional 16 amino acids at the N-terminus. The mRNA of NM1 is differently spliced yielding 5′ introduction of exon containing alternative start of translation [2]. Importantly, the ubiquitous expression and nuclear localization of NM1 in mouse organs along with high degree of conservation of the N-terminal sequence across species has been confirmed [3,4].

This corresponds to its important functions. In the nucleus, there is ample evidence for functional involvement of NM1 in transcription by RNA polymerase I and II (Pol I and Pol II). NM1 co-localizes with both polymerases at the sites of transcription [2,5] and physically associates with both Pol I and Pol II complexes [6,7]. In-vivo rate of transcription is negatively affected by NM1 overexpression, and inhibited by NM1 knock-down and nuclear microinjections of anti-NM1 antibodies [7]. In an in-vitro transcription system, anti-NM1 antibodies inhibit transcription by both polymerases in a dose-dependent manner, whereas adding purified NM1 increases transcription [2,6,8]. Transcription initiation assays have revealed that NM1 exerts its function in early steps of Pol I and II transcription after the formation of pre-initiation complexes [6,7]. Indeed, NM1 interacts with Pol I transcription factor TIF-IA, which is present only in initiation-competent fraction of Pol I complexes [9], and actin that is associated with RNA polymerase I independently of active transcription [7]. According to Grummt [10], the binding of NM1 to Pol I via actin may help to initiate transcription by

recruiting TIF-IA to pre-initiation complex. This model is further supported by the fact that functional motor domain is needed for interaction of NM1 and Pol I [11]. In addition to transcription initiation, NM1 is also involved in Pol I transcription elongation since it associates with the chromatin remodeling complex WSTF-SNF2h and might therefore recruit this complex to the actively transcribing genes [12].

Interestingly, nascent ribosomal particles seem to be accompanied by NM1 during transport from nucleolus toward nuclear pores [13] and blocking of NM1 or actin by antibodies results in nuclear retention of small ribosomal subunits [14,15].

A role of acto-myosin motor in repositioning of chromosomes is emerging [16,17]. In pioneering work, Chuang and co-workers [18] showed that labeled artificial gene loci move, upon activation, toward the center of nucleus and that overexpression of mutated NM1 that lacks motor activity inhibits this effect. However, the exact mechanism behind these translocation phenomena is not clear.

Using specific antibodies generated against its N-terminal epitope, NM1 can be detected predominantly in the nucleus, nucleolus and at the plasma membrane of interphase cells [1,5,19]. NM1 is a short-tailed class I myosin that binds directly to actin via its head domain and the headgroups of acidic phospholipids via putative PH domain within positively charged tail [20]. Neck domain, located between head and tail, contains three IQ motifs that bind calmodulin [1]. To date, there are no data about biochemical characteristics of this protein. Because NM1 is almost identical to Myo1c, one can expect that its basic function is to maintain tensions as proposed for Myo1c [21] however, the exact function of the N-terminal extension in NM1 molecule that makes the only known difference form Myo1c is uncertain. The observation that NM1 is localized mainly in the nucleus and Myo1c at the plasma membrane has led to the opinion that the N-terminus could function as a nuclear targeting or nuclear sequestering sequence [2].

In this paper we identify the domains that direct the nuclear translocation of NM1 and decipher the mechanism of intracellular trafficking of NM1. We demonstrate that the N-terminal extension of NM1does not act as a nuclear localization sequence (NLS); instead, we identified a novel NLS within the the neck region of NM1 as crucial for nuclear import. In search for the possible import receptors of NM1 we found importin 5, importin-β1, importin 7 and Heat shock protein 90 (HSP90) to associate with truncated constructs as well as with the endogenous NM1. Since the identified NLS sequence is also present in the Myo1c protein we also investigated the localization of Myo1c. Using various experiments including the NM1 knockout mice derived cells we discovered the "cytoplasmic" Myo1c was also present in nuclei. This adds the traditional "cytoplasmic" Myo1c to the few molecular motors of the nucleus with potentially important functions in nuclear metabolism.

Results

NM1 is transported to the nucleus after mitosis

To study the dynamics of NM1 compartmentalization during cell cycle we followed the localization pattern of the endogenous NM1 during and after mitosis. Immunofluorescent labeling of NM1 in unsynchronized U2OS (Fig. 1A) and in NIH 3T3 (Fig. 1B) cells synchronized by mitotic shake off has shown that NM1 did not stay bound to chromatin during the mitosis and that its majority was released into the cytoplasm after the nuclear envelope breakdown in prophase (Fig. 1B). Soon after the reconstitution of nuclear envelope in early G1, most of NM1 was in the cytoplasm as shown in Fig. 1A and 1B (Early G1). In unsynchronized population of cells, this pattern was very rarely observed, and the vast majority of cells had clearly nuclear staining of NM1 (Fig. 1A, Interphase). This demonstrates that nuclear import of endogenous NM1 is accomplished in G1 phase.

To begin identifying import signals in NM1, we first tested the localization of full length NM1 constructs fused to different tags. Untagged overexpressed mouse and human NM1 localized predominantly in the nucleus in 80% of cells, and V5/His-tagged NM1 was predominantly nuclear in 50%, whereas EGFP-tagged or FLAG-tagged NM1 was predominantly nuclear in less than 20% of cells (data not shown). Further studies used the V5/His-tag because it interfered with nuclear import the least.

To visualize the timing of V5/His-tagged NM1 (NM1-V5/His) transport into the nucleus after mitosis, we transfected U2OS cells and the next day added either nocodazole (depolymerizes microtubules) or aphidicolin (DNA polymerase inhibitor) for 16 hours to accumulate cells in M-phase or S-phase respectively, then washed out the inhibitor and used indirect immunofluorescence to localize NM1-V5/His at different times after release from the block (Fig. 1C,D). Nocodazole-treated (metaphase-enriched) cells continued with mitosis after washout. The lowest nuclear levels of tagged NM1 were seen at 2 and 4 hours after release, but increased gradually at 6–10 hours after release (Fig. 1C). After release from aphidicolin, cells maintained high nuclear levels of NM1-V5/His for ~11 hours, consistent with the expected time needed to complete S-phase and enter G2 (Fig. 1D). The lowest levels of nuclear NM1-V5/His were detected 17 hours after release from aphidicolin (Fig. 1D), when many cells were in mitosis or early G1. Together these results suggested both endogenous and tagged NM1 are released from the nucleus during mitosis. Endogenous NM1 is transported into renewed nuclei shortly after the nuclear envelope reconstitution in the early G1, while nuclear import of the ectopically expressed NM1 with a tag is slower.

First two IQ domains are needed for nuclear transport of NM1

Because the N-terminal part of NM1 was suggested to be crucial for nuclear localization [2], we prepared various deletion and truncation mutants of the NM1 in fusion with V5/His at its C-terminus. We compared their localization in U2OS cells with the full length NM1-V5 which was detected in the cytoplasm and faintly in the nucleus (Fig. 2A, anti-V5). Surprisingly, the deletion of the neck and the tail domain led to the cytoplasmic retention of the mutant (Fig. 2B). This suggested that the NLS sequence is located within the neck or in the tail domains. After deletion of the head domain, we observed enhanced nuclear signal with short C-terminal V5/His (not shown) as well as with the bulky N-terminal EGFP tag (Fig. 2C). This suggested that the EGFP-fused myosin neck-tail fragment is imported efficiently. Further deletion of half of the tail disrupted the plasma membrane association of the protein but not its nuclear translocation (Fig. 2D). The tail together with the third IQ domain of the neck stayed out of the nucleus and associated with plasma membrane (Fig. 2E) while the construct with first two IQ domains was located exclusively to the nucleus and nucleoli (Fig. 2F). This localized a putative nuclear localizing sequence within the first two IQ domains of NM1 neck residues 712–770.

The second IQ domain contains a novel NLS sequence

To pinpoint the exact part of the neck needed for nuclear translocation, we prepared a set of fusion constructs containing GFP and the cytosolic pyruvate kinase (PK) enzyme [22]. We used PK to enlarge the proteins so that they would not diffuse passively

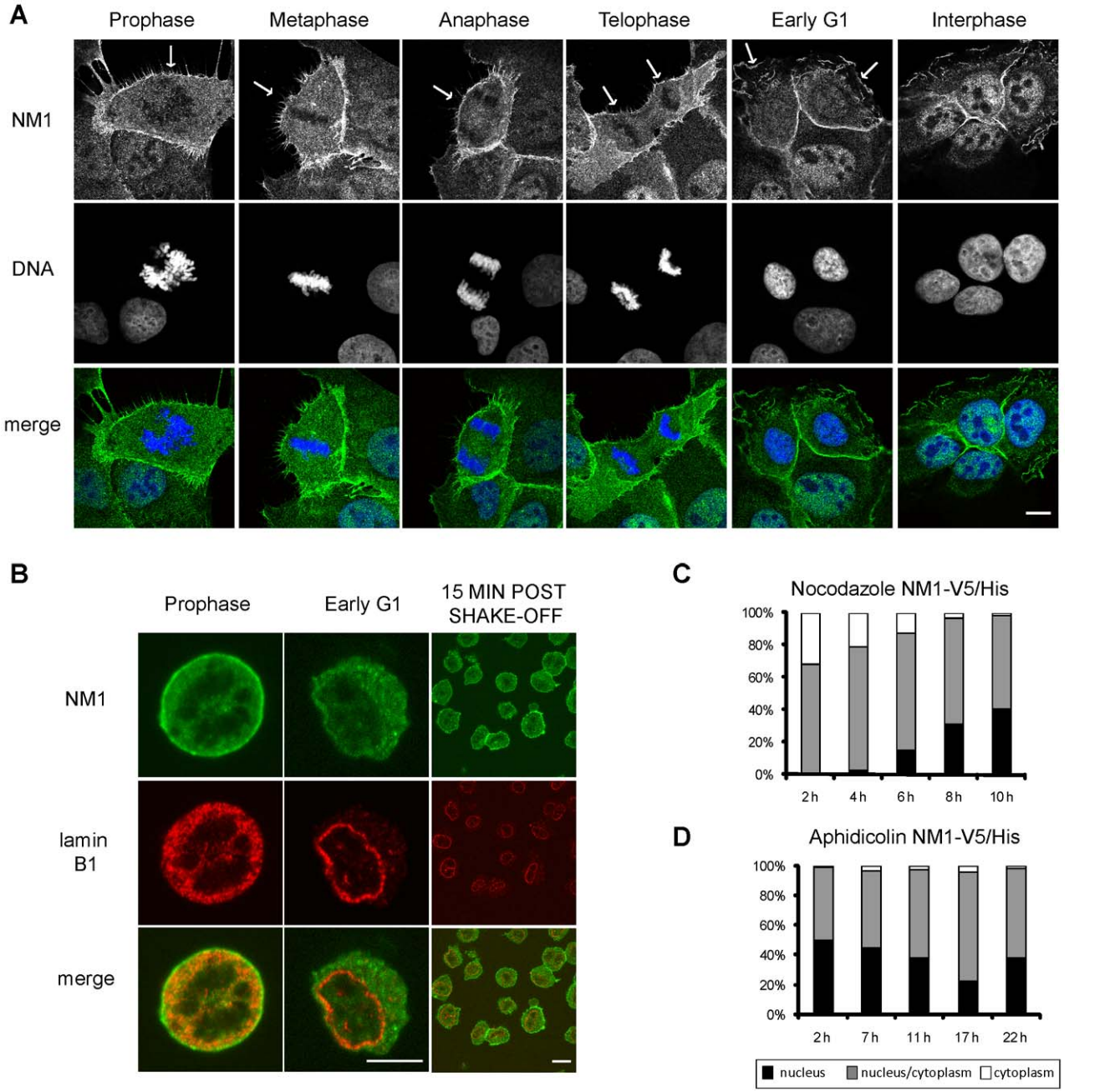


Figure 1. Localization NM1 during mitosis. (**A**) Unsynchronized U2OS cells were fixed and labeled with antibody to NM1. Localization of NM1 is shown at various stages of mitosis. DNA was visualized by DAPI. (**B**) Mitotic NIH 3T3 cell were seeded onto the poly-L-lysine coated coverslips, fixed and labeled with antibodies to NM1 and Lamin B1. Cells fixed immediately after seeding (**Prophase**) and 15 min after seeding (**Early G1, 15 min post shake-off**). Nuclear lamina was reconstituted in Early G1 as visualized by Lamin B1 labeling. All immunofluorescence pictures were obtained using confocal microscope, single confocal sections are shown. Scale bar: 10 μm. U2OS cells were transiently transfected with NM1-V5/His. 24 hours after transfection cells were treated with nocodazole (**C**) or aphidicolin (**D**), to stall the cells either in G2/M or in G1/S phase of cell cycle. After the release from the block cells were cultivated for another 24 hours. Samples were taken in indicated time points. Cells were labeled with antibody to V5 tag, patterns counted and divided into three groups according to the localization of fluorescent proteins. More than 100 cells were counted in each time point, experiment was repeated twice with similar result.
doi:10.1371/journal.pone.0030529.g001

to the nucleus as GFP alone would [23]. The 87 kDa GFP-PK fusion construct was located solely to the cytoplasm (Fig. 2G). When the sequence of the first two IQ domains was added to GFP-PK strong nuclear and nucleolar signal was observed (Fig. 2H). Next, we examined the capability of each IQ domain to drive the nuclear transport (Fig. 2I, J). Nuclear accumulation was specifically driven by the second IQ motif (Fig. 2J), not the first IQ motif (Fig. 2I). The IQ2 motif and its c-terminal flanking sequence contains two clusters of basic amino acids. Next, we preserved only the basic amino acid clusters with the intermitting non-polar amino acids, resulting in 13 amino acid peptide, ^{754}GRRKAAKRKWAAQ^{766}. This sequence was sufficient for

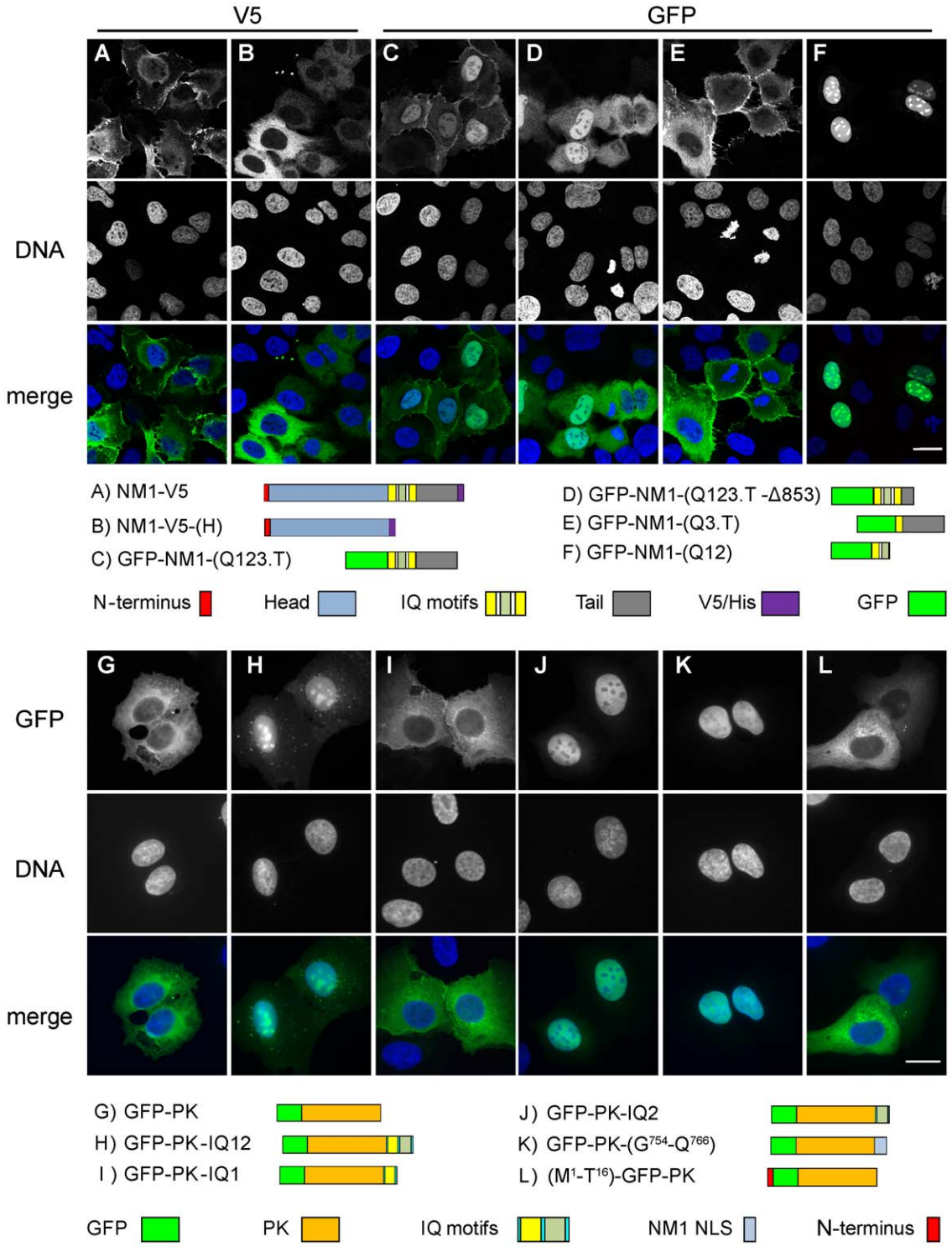


Figure 2. Neck domain of NM1 contains the NLS. U2OS cell transfected with a panel of truncation constructs of full length NM1 (**A–F**) and IQ domains fused to GFP-PK (**G–L**). Cells were fixed 48 hours post transfection. Below the pictures are schematic representations of the truncations affecting various NM1 domains as well as the GFP-PK phusions. Pictures (**A–F**) were acquired using confocal microscope, single confocal planes are shown. Pictures (**G–L**) were photographed using wide-field fluorescent microscope. Scale bar: 10 μm.
doi:10.1371/journal.pone.0030529.g002

nuclear translocation (Fig. 2K). On the other hand, the N-terminal 16 amino acids from NM1, fused to the N-terminus of the GFP-PK construct, did not localize to the nucleus at all (Fig. 2L). To rule out the possibility that it serves as a nuclear retention signal, we fused the N-terminal sequence to EGFP that diffuses freely into nucleus. We did not observe nuclear enrichment of the signal that would be caused by an interaction of the protein inside the nucleus (not shown). In contrast to the full length NM1 (Fig. 3A), a C-

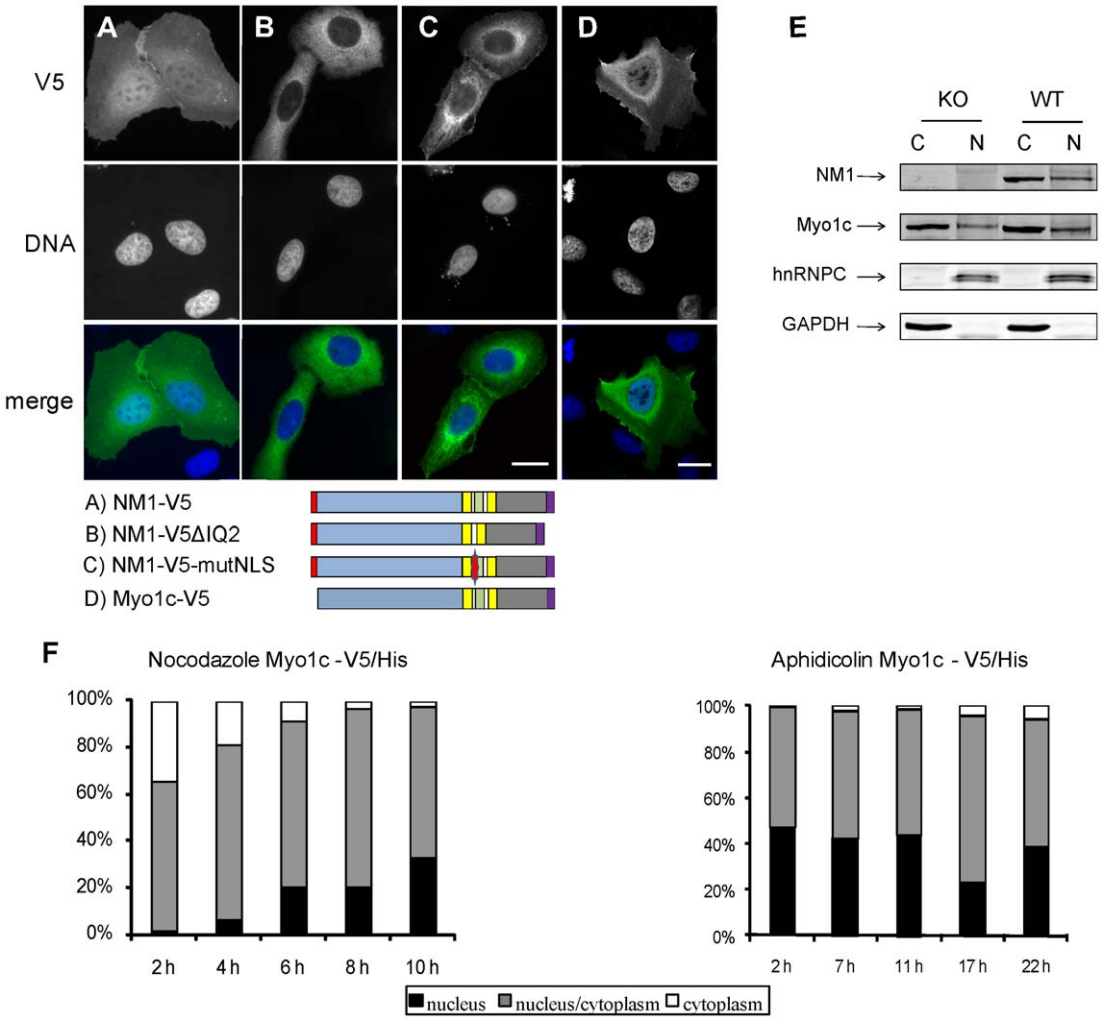


Figure 3. Mutation of basic residues in the neck of NM1/Myo1c abolishes its nuclear import. U2OS cells were transfected with full length NM1-V5/His (**A**), NM1-V5/His lacking the second IQ motif (**B**), and NM1-V5/His with point mutation of basic amino acids within the NLS into alanines (**C**). Below the pictures are schematic representations of constructs used. Color coding is the same as in **Fig. 2**. Cells were fixed 48 hours post transfection and labeled with anti-V5 antibody, pictures were obtained using wide-field microscope, scale bar: 10 μm (**D**) U2OS cells transiently transfected with Myo1c-V5/His show nuclear localization of the protein Picture is a single confocal plane, obtained by confocal microscope. Scale bar: 10 μm. (**E**) Nuclear and cytosolic extracts were prepared from liver of either wild type (WT) or NM1 knock-out (KO) mice. Equal amount of protein was resolved using SDS-PAGE and electro-transferred to nitrocellulose. Membrane was probed with anti-NM1, anti-Myo1c, anti hnRNP C1/C2 and GAPDH antibody. Signal was detected using LI-COR Odyssey infrared imaging system. (**F**) U2OS cells were transiently transfected with Myo1c-V5/His. 24 hours after transfection cells were treated with nocodazole or aphidicolin to stall the cells either in G2/M or in G1/S phase of cell cycle. After the release from the block cells were cultivated for another 24 hours. Samples were taken in indicated timepoints. Cells were labeled with antibody to V5 tag, patterns counted and divided into three groups according to the localization of fluorescent proteins. More than 100 cells were counted in each timepoint, expreriment was repeated twice with similar result.
doi:10.1371/journal.pone.0030529.g003

terminally fused NM1 construct lacking the residues 739–762 accumulated in the cytoplasm of U2OS cells (Fig. 3B), supporting an important role for the second IQ. No single K/R-to-A substitution in residues 754–766 was able to disrupt the NLS activity (data not shown). However mutating all 6 basic residues to Ala completely abolished nuclear import (Fig. 3C; NM1-V5-mutNLS). NLS Database [24] and literature searches revealed no known NLS homologous to that of NM1. We therefore conclude that NM1 and cytoplasmic Myo1c share a novel type of NLS.

Myo1c is able to translocate into the nucleus

N-terminus of NM1 alone did not possess a nuclear localization potential and the NLS was located in region shared by both the NM1 and Myo1c. We therefore inspected the localization of Myo1c under overexpressed condition. V5/His tagged Myo1c was localized to the nuclei of transfected U2OS cells (Fig. 3D). The nuclear localization of Myo1c-V5/His in nocodazole- or aphidicolin-treated cells was also cell cycle dependent, with profiles (Fig. 3F) similar to that of cells that overexpressed NM1 (Fig. 1F).

To test the influence of the N-terminal 16 amino acids on NM1 functions we prepared knock-out mice lacking the exon-1 that contains the NM1 start codon (Venit et al, in preparation). Resulting mRNA contains only the downstream start of translation which gives rise to Myo1c protein. We used purified liver nuclei from NM1 knock-out mice to confirm that the N-terminus is not required for nuclear transport of endogenous NM1. Fig. 3E shows the presence of Myo1c in the purified liver nuclei from NM1 knock-out mice visualized by antibody to the C-terminus of NM1/

Myo1c. Weak signal of GAPDH indicates negligible contamination of nuclei with cytosol, while signal of hnRNP C1/C2 shows marked enrichment of nuclear proteins compared to cytosolic liver extract. This shows that both NM1 and Myo1c have the ability to enter the nucleus. Further, we have observed the co-localization of NM1 and myo1c in a single cell. However, we were not able to co-localize NM1 and myo1c in nuclei of untransfected cells using the polyclonal antibody directed to the tail region of NM1/Myo1c (R2652) [25] since it failed to label endogenous epitopes within nuclear environment. Therefore, U2OS cells were cotransfected with FLAG tagged NM1 and V5/His tagged Myo1c. Fig. 4A shows a cell expressing both proteins. Myo1c and NM1 co-localized at the plasma membrane (arrows) and in the nucleus. Interestingly, R2652 antibody revealed the signal in nucleus and nucleolus when myo1c was overexpressed Fig. 4B.

Importins bind the NM1 neck region

The transport of nuclear proteins through nuclear pores is often facilitated by importins that recognize their NLS in cytoplasm [24]. To discriminate between cytoplasmic and nucleus/plasma membrane-associated myosin, cells were extracted with buffers containing digitonin that is known to extract cytosolic myosin 1c [26]. We sought to identify the transport receptors that bind NM1/Myo1c NLS. Using pull down assay with recombinant IQ12 as the bait we identified importin 5 (IPO5) and Heat shock protein 90 beta (HSP90) as the proteins that associate with IQ12 in the cytoplasm of HeLa cells (Fig. 5A). To verify the obtained result by another method, we looked for interacting partners of GFP-NM1-(Q123.T) in HEK-293T cells. Mass spectrometry analysis of bands that co-purify specifically with GFP-NM1-(Q123.T) but not with the control Str-GFP contruct, revealed importin 5, importin 7 (IPO7), importin-β1 (KPNB1) and HSP90 beta (Fig. 5B). Additional bands that were present on the gel were not identified. To verify that the importin 5, importin 7 and importin-β1, which were found to bind the truncated constructs, recognize also the endogenous protein, we performed co-immunoprecipitation with a polyclonal antibody directed to N-terminus of NM1. Since most of endogenous NM1 molecules potentially accessible to importins are located in cytosol of the G1 cells (Fig. 1A), we synchronized the HeLa cells with nocodazole and harvested them 3 hours after nocodazole wash-out. As shown by western blot (Fig. 5C), endogenous NM1 specifically binds to importin 5 (IPO5), importin 7 (IPO7), and importin-β1 (KPNB1) in digitonin extracts of the G1 cells.

Next, to confirm that importin 5 binds specifically to NM1 NLS via the interaction with positively charged amino acids, we compared the proteins that co-purify with headless NM1 with wild type NLS (GFP-NM1-(Q123.T) ^{NLSwt}) and headless NM1 with all basic residues in the NLS mutated to alanines (GFP-NM1-(Q123.T) ^{NLSmut}) from electroporated HEK293T cells. Fig. 5D shows that importin 5 interacts only with GFP-NM1-(Q123.T) ^{NLSwt} and that this interaction occurs in digitonin extract in contrast to triton X-100 that liberates the plasma membrane bound myosin [26]. Taken together, the aforementioned data show that the importin 5, importin 7 and importin-β1 bind the newly identified NLS.

NM1 nuclear import does not follow the canonical nuclear import pathway

The direction of canonical nuclear import pathway is controlled by the small GTPase Ran. High levels of GTP-loaded Ran in the nucleoplasm cause the dissociation of importin-cargo complex upon translocation through the nuclear pore complex [27]. We probed the stability of the NM1-importin complexes in the presence of RanGTP in order to test whether the nuclear import of NM1 follows the canonical nuclear import pathway.

Complexes containing Str-GFP-NM1-(Q123.T) and associated importins were purified from electroporated HEK293T cells using streptactin affinity column and incubated with recombinant Q69L mutant of Ran, preloaded with GTP. This mutant is not able to hydrolyze GTP [28] and should cause elution of importins from the Str-GFP-NM1-(Q123.T) column The activity of Q69L mutant of Ran was confirmed by its ability to dissociate importin β1 from its well known cargo, SV40 NLS (Fig. 5E). In contrast to SV40 NLS, the GFP-NM1-(Q123.T) remained associated with importin 5 even in the presence of RanGTP Q69L. As shown by western blot (Fig. 5E), the complex of GFP-NM1-(Q123.T) and importin 5 co-eluted from the column by the addition of biotin that disrupts the binding of Str-GFP-NM1-(Q123.T) to the streptactin resin. Taken together, these data suggested that the NM1 nuclear import does not follow the canonical nuclear import pathway regulated by GTPase Ran.

Overexpression of calmodulin negatively influences NM1 nuclear import

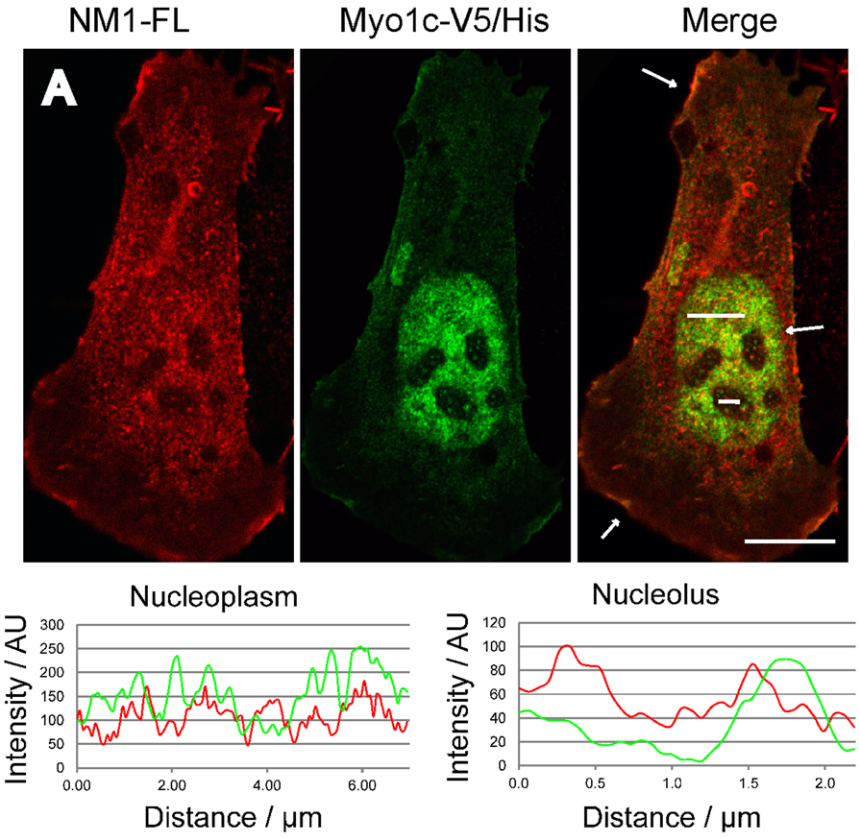
Neck region of NM1 is characterized by the presence of IQ motifs that bind calmodulin in Ca^{2+}-dependent manner [29]. As NLS sequence of NM1 is present within one of these IQ motifs, we tested the influence of increased calmodulin levels on the NM1 localization. When GFP-PK-IQ12 was co-expressed with calmodulin in U2OS cells, elevated levels of calmodulin blocked the nuclear import of the IQ12 construct (Fig. 6B). Calmodulin, on the other hand, did not block the import of GFP-PK-IQ2 (Fig. 6C). Importantly, calmodulin did not inhibit the nuclear import of the GFP-PK-SV40 NLS construct, suggesting that the observed effect is not a general inhibition of nuclear import pathways (Fig. 6A). To compare the amount of calmodulin associated with GFP-PK-IQ2 and GFP-PK-IQ12 we immunoprecipitated the proteins from extracts of electroporated HEK293T cells using GFP-trap magnetic beads. As shown in Fig. 6D calmodulin associated with GFP-PK construct only when both IQ domains were present (GFP-PK-IQ12). In conclusion calmodulin binding to IQ12 appears to regulate nuclear import of NM1.

Discussion

Nuclear myosin 1 is ubiquitously expressed protein that localizes to the nuclei of all cell types tested so far with the exception of cells in germinal stage of spermiogenesis [3]. Our previous work described the dynamics of intranuclear relocalization of NM1 [8,19] and involvement of NM1 in important nuclear processes – namely gene transcription. In this paper, we further contribute to the knowledge of NM1 cellular trafficking by describing the dynamics of its nuclear import and identification of the sequence that is necessary for the nuclear entry of NM1.

NM1 contains NLS within the IQ domain

We used tagged constructs in search for the NLS of NM1. By deletions and truncations of the full length protein, we narrowed down the region of NM1 required for its nuclear import to a short sequence within the second IQ motif of the neck domain. The sequence contains clusters of basic amino acids intermingled with non-polar amino acids and mutation of the basic residues into alanines blocked the nuclear import of NM1. The NLS does not resemble to any of the NLSs already described in the literature and, thus, it might be expected to have some unique properties. Similarly to NM1, also the neck of other unconventional myosin, myosin Vb, contains IQ sequence, that was shown to be


NM1-FL
Myo1c-V5/His
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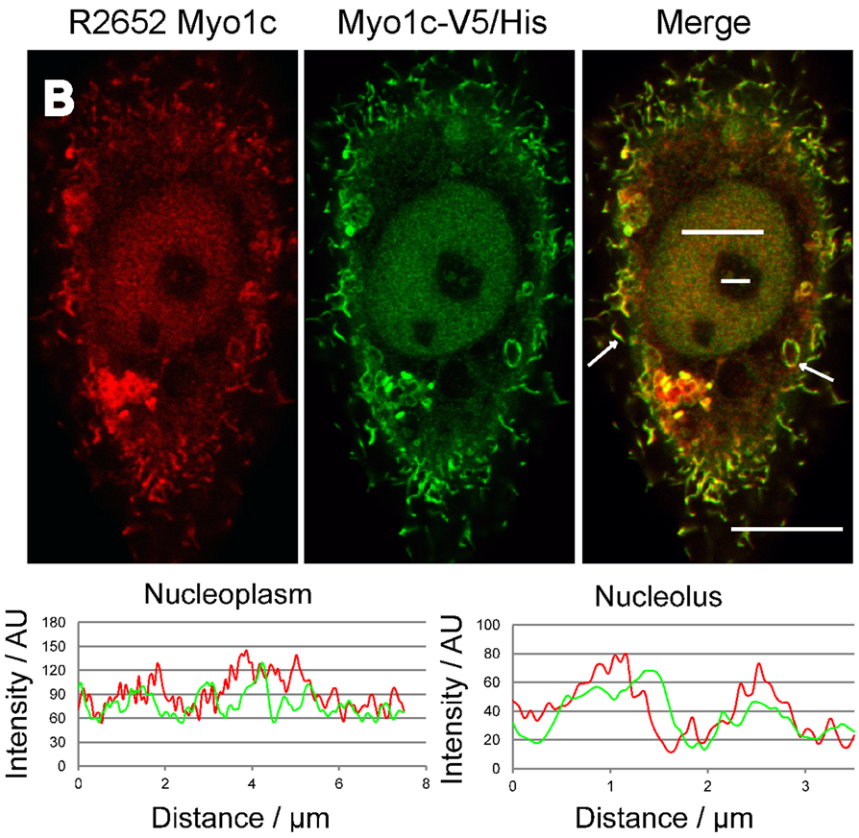
R2652 Myo1c
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Figure 4. Co-localization of overexpressed NM1 and Myo1c. U2OS cells were co-transfected with FLAG-tagged NM1 (**NM1-FL**) and V5/His tagged Myo1c (**Myo1c-V5/His**). Cell showing nuclear localization of NM1 and Myo1c was photographed using confocal microscope (**A**). U2OS were transfected with Myo1c-V5/His. 48 h post transfection cells were fixed, and labeled with polyclonal antibody (R2652) directed toward the tail region of NM1/Myo1c and with monoclonal antibody against V5 (**B**). Intensity profiles along the regions of interest in the nucleus and nucleolus are shown under the pictures. White arrows are pointing to regions at the plasma membrane where both proteins are enriched. Scale Bar: 10 μm.
doi:10.1371/journal.pone.0030529.g004

responsible for nuclear and nucleolar localization of this myosin. Furthermore, it also mediates interaction with RNA pol I [30]. An IQ motif of another actin and PIP2 binding protein, the neural Wiskot-Aldrich syndrome protein (N-WASP), serves as an NLS [31]. In conclusion, the ability to drive nuclear import appears to be common to various IQ motifs.

Role of IQ2 in plasma membrane localization of NM1/Myo1c

NM1/Myo1c neck-tail domain (NM1-(Q123.T)), was shown previously to associate with the plasma membrane through interaction with PIP2. This interaction was assigned to the putative PIP2-specific PH domain in the tail region of myo1c [20]

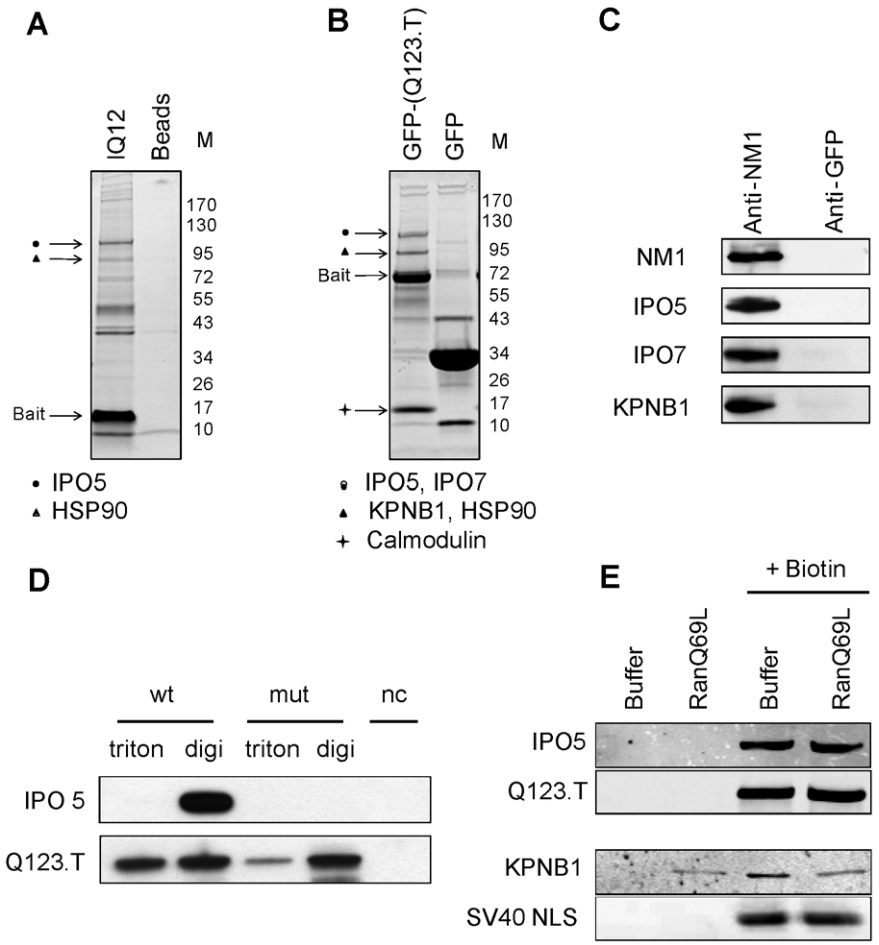

Figure 5. Identification of NM1 interacting proteins in the cytosol. Digitonin extract from suspension HeLa cells was incubated with recombinant Str-IQ12-His peptide containing N-terminal OneStrep tag (IQ12) and Streptactin beads as a control for background binding. Bound proteins were resolved on 4–20% SDS-PAGE gel and stained with SimplyBlue. Mass spectrometric analysis of the protein bands that co-purified with bait (arrows) identified importin 5 and heat shock protein 90 beta (HSP90) (**A**). SimplyBlue stained 4–20% SDS-PAGE gel with proteins that interacted with Str-GFP-NM1-(Q123.T) and Str-GFP as a control in digitonin extract of HEK293T cells. The arrows show positions of bands that contained proteins identified using mass spectrometry as importin 5, importin 7, importin-β1, HSP90 beta and calmodulin (**B**). Proteins that co-immunoprecipitate with antibody to endogenous NM1 from HeLa extracts were resolved using SDS-PAGE and tranferred onto nitrocelulose membrane. Membrane was probed with with anti-NM1, anti-importin 5 (IPO5), anti-importin 7 (IPO7), anti-importin-β1 (KPNB1). Rabbit polyclonal antibody against GFP was used as a control for backgroung binding (**C**). N-terminally Strep tagged GFP-NM1-(Q123.T) ^{NLSwt} (wt), GFP-NM1-(Q123.T) ^{NLSmut} (mut) and GFP as negative control (nc) were expressed in HEK293T cells. Cells were extracted with buffer containing digitonin (digi) to obtain soluble cytosol; pellet was re-extracted with the same buffer containing 1% Triton X-100 (triton). Bound proteins were resolved on SDS-PAGE, transferred to nitrocelulose. Membrane was incubated with antibody to importin 5 ans GFP (**D**). Beads containing Str-GFP-NM1-(Q123.T) and Str-GFP-SV40 NLS and associated proteins were eluted first with buffer containing GTP-loaded RanQ69L or buffer alone and then with biotin containig buffer that liberated Strep-tagged bait proteins from the column. Proteins eluted from the beads were resolved on SDS-PAGE and transferred to nitrocelulose membrane. GFP, importin 5 and importin-β1 signals were detected using specific antibodies (**E**). Signal from secondary antibodies was detected using LI-COR Odyssey infrared imaging system.
doi:10.1371/journal.pone.0030529.g005

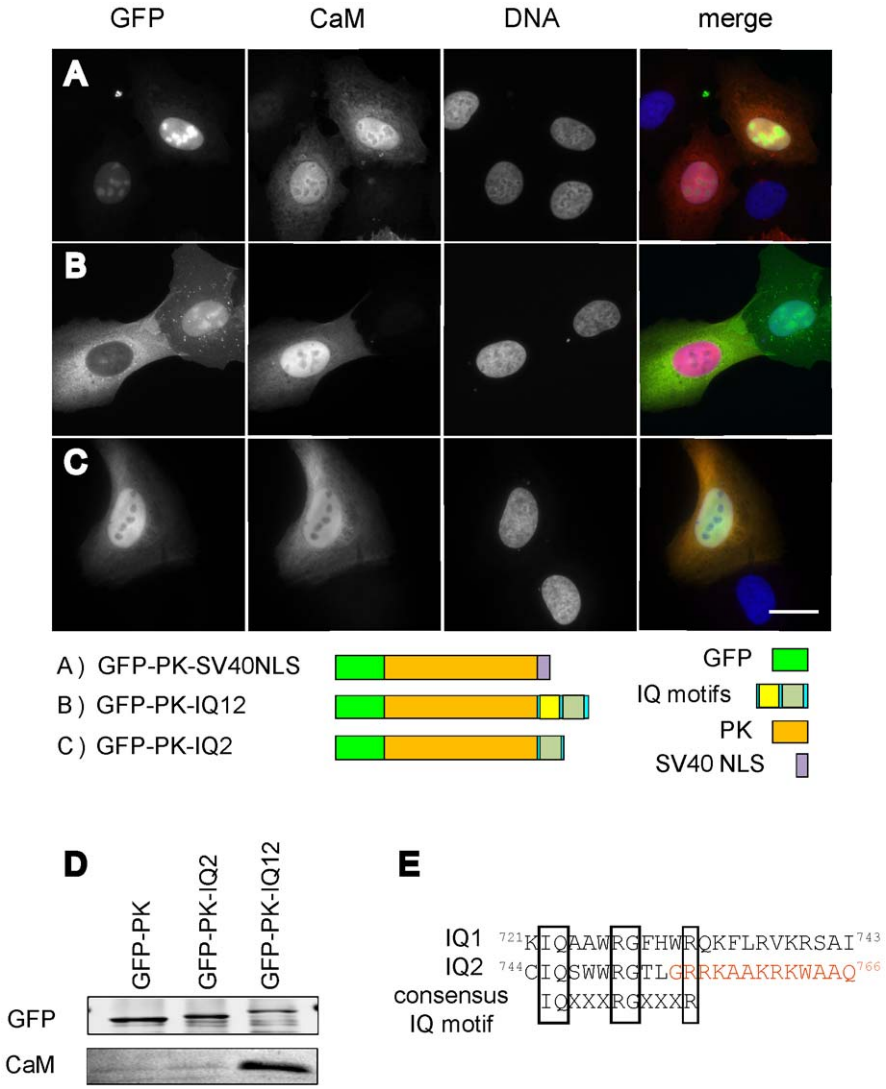

Figure 6. Overexpression of calmodulin influences the nuclear import of NM1. U2OS cells were co-transfected with GFP-PK constructs containing IQ domains, and calmodulin. Calmodulin was visualized using specific antibody (**A**,**B**,**C**). Scale bar 10 μm. HEK293T cells electroporated with the same constructs as in (**A**,**B**,**C**). Whole cell extracts were subjected to immunoprecipitation with anti-GFP nanobody. Bound proteins were resolved on SDS-PAGE and transferred to nitrocelulose. GFP and CaM were visualized using specific antibodies (**D**). (**E**) Comparison of IQ1 and IQ2 sequences. The consensus IQ motif is shown below. The NM1 NLS sequence is highlighted in red.
doi:10.1371/journal.pone.0030529.g006

and/or another less specific to the neck region [32]. Interestingly, NM1 mutants which either lacked the second IQ motif or the basic residues within the second IQ sequence were misslocalized from the plasma membrane to the cytosol (Fig. 3B and 3C). These data are in agreement with previously published work that identified the IQ2 as additional plasma membrane binding site [32].

Mechanism of NM1 nuclear import

The basic mechanism of NM1 nuclear import appears to involve karyopherins, because importin 5, importin 7 and importin-β1 were found to be associated with both overexpressed and endogenous NM1. The interaction of importin 5 with NM1 NLS seems to be specific since NM1 mutant lacking the basic amino acids in NLS did not bind this karyopherin.

Canonical importin-mediated nuclear entry is controlled by the nuclear RanGTP which, upon binding to importins, releases their cargos. Surprisingly, the complex of NM1 with importin 5 is stable in the presence of RanGTP (Fig. 5E) and its nuclear import is rather dependent on the levels of calmodulin (Fig. 6B). This suggests that nuclear import of NM1 is mediated by a non-canonical pathway. Indeed, such a calmodulin-dependent and Ran-independent nuclear import pathway has been shown to regulate the nucleocytoplasmic localization of several transcription factors (SOX9, SRY, c-Rel). The N-terminal domain of SRY and SOX9 contains a calmodulin-binding domain followed by an NLS [33]. It was shown that calmodulin binding stimulates the nuclear entry of SRY and SOX9 [34,35]. On the other hand the NF-κB/Rel family protein c-Rel binds Ca^{2+}-calmodulin via sequence near the NLS and this binding blocks its nuclear accumulation [36]. The NLS of NM1 resides in close vicinity of the second IQ motif. We showed that IQ2 alone is able to drive the translocation of heterologous construct GFP-PK to the nucleus (Fig. 2J). Interestingly, IQ1 seems to play a key role in regulation of NM1 nuclear

import (Fig. 6B). In presence of elevated levels of calmodulin, IQ1 mediated the inhibition of nuclear import (Fig. 6B) and it also substantially increased the binding of CaM the IQ2 (Fig. 6D).

On the other hand, the crystallographic [37] and biochemical studies have shown that calmodulin binds the IQ motifs of unconventional myosins in Ca^{2+}-free state and that elevated Ca^{2+} dissociates calmodulin from the neck of Myo1c [29]. More recent study showed that in the absence of calcium, Ca^{2+}-free calmodulin (apo-CaM) was bound to the IQ1 with highest affinity whereas in the presence of calcium IQ1 dissociated Ca^{2+}-CaM most rapidly. Ca^{2+} - induced dissociation of calmodulin molecules from the neck increases ATPase rate and inhibits the motility of Myo1c [38]. We propose a scenario in which the calmodulin occupies the IQ12 at low Ca^{2+} cellular levels preventing the importin from binding to NM1. The Ca^{2+} oscilations which occur during G1 phase of cell cycle [39] or which follow the signal transduction events [40] might cause the calmodulins to dissociate from the NM1 neck. As a result, the CaM-free IQ2 will subsequently bind to the import receptor that transports the NM1 to the nucleus. Taken together, at the cellular level, both the motor function of NM1/Myo1c and its localization appear to be dynamically regulated by transient transient changes in Ca^{2+} concentration. Obviously, further experiments are needed to reveal the details of the mechanism.

Nuclear localization of NM1/Myo1c

The fact that both Myo1c and NM1 contain the same NLS sequence points to the question whether also Myo1c would be present in the nucleus along with NM1. NM1 was first detected in the nucleus in 1997 [1] using the antibody specific to N-terminus of NM1. Myo1c has never been reported in the nucleus since the antibody directed to the C-terminus [41] does not label the nuclei of cells. Intriguingly, upon transfection with either NM1 or Myo1c, the nuclear signal could be readily detected also by this antibody (Fig. 4B). One plausible explanation of this discrepancy would be that the epitope is probably masked by nuclear binding partners, posttranslational modifications or adopts a different conformation. However, the steady-steate cytosol to nucleus distribution of endogenous NM1 and Myo1c in mouse liver is approximately 70% (cytosol) to 30% (nucleus) Fig. 3E. Therefore another possible intepretation is that the level of endogenous nuclear myosins at steady state is is below the detection limit of the antibody directed to the C-terminus.

In conclusion, our work revealed a novel NLS sequence responsible for nuclear translocation of NM1. This sequence acts as an efficient NLS when fused to different otherwise cytosolic proteins, while the AA 1–16 lacks this capacity. This means that NM1 does not need N-terminal sequence for nuclear import, and that both NM1 and Myo1c can function in the cell nucleus which is supported by detection of both isoforms in the cell nucleus. Finally, our work suggests a complex regulation of myosin 1c nuclear import, mediated by both CaM and importins. This data opens additional interesting questions: Do the two myosins serve the same functions in the nucleus and in the cytoplasm? Do these two myosins have the same molecular properties (e.g. binding to actin, nucleotide, mechanism of strain-dependent release of ADP) or do they somehow differ? Are they tuned to serve the same function at the level of cell, tissue and organism? Obviously, further investigation is needed to answer these questions.

Materials and Methods

Antibodies

In immunofluorescence and co-immunoprecipitation experiments we used affinity purified antibody directed to the NM1 N-terminus M3567 (Sigma), antibodies to lamin B (M-20, Santa Cruz Biotechnology), V5-tag (Serotec), V5-tag (V8137-Sigma), FLAG-tag (Stratagene) and to calmodulin (Upstate, cat. No. 05-173). Anti-importin 5 (sc-17802), importin beta (sc-1919) and importin 7 (sc-55235) were purchased from Santa Cruz Biotechnology. R2652 rabbit polyclonal antibody against the tail domain of Myo1c was kindly provided by Peter G. Gillespie, Oregon Hearing Research Center and Vollum Institute [41]; rabbit polyclonal anti-NM1 for western blots was kindly provided by Piergiorgio Percipalle [5], and EGFP antibody was purchased from Invitrogen (cat. No. A11122).

Cells and transfections

Cell lines cells were obtained from American Type Culture Collection. NIH/3T3 (ATCC No. CRL-1658), U2OS (ATCC No. HTB-96), HeLa (ATCC No. CCL-2) and HEK 293T/17 (ATCC No CRL-11268) were kept in DMEM supplemented with 10% fetal bovine serum (FBS) in 5% CO2/air, 37°C, in humidified atmosphere. HeLa S3 (ATCC No. CCL-2.2) were kept in S-MEM supplemented with 5% FBS and grown in spinner flasks. The U2OS cells were transfected with FUGENE 6 (Roche) according to the manufacturer's protocol, fixed after 48 h and either observed directly under the microscope or labeled with antibodies. HEK293T cells were electroporated using GenePulser (Biorad) electroporator as described [42]. The efficiency of electroporation was about 90%.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with freshly prepared 3% formaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, incubated with primary antibodies for 1 hour at room temperature. Primary antibodies were diluted in PBS containing 0.05% Tween-20 (PBST) to 16 μg/ml (NM1), 5 μg/ml (lamin B1), 5 μg/ml (calmodulin), 1 μg/ml (V5-tag), 5 μg/ml (Flag-tag). After washing in PBST, coverslips were incubated with FITC or Cy5-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch). Coverslips were mounted with Mowiol (Sigma) containing DABCO (Sigma) as an anti-fading agent and 0.1 μg/ml DAPI (Sigma), and observed under fluorescent or confocal microcopes (LEICA DM 6000, LEICA DMI 6000, LEICA TCS SP5 AOBS TANDEM). Brightness and contrast of captured digital images was adjusted with Photoshop software (Adobe).

Cell synchronization

U2OS cells were treated with nocodazole (80 ng/ml or 400 ng/ml) for 16 h. Mitotic cells were washed off the dish with medium, spun down and resuspended in fresh medium, and seeded on coverslips. Cells on coverslips were cultivated further in fresh medium and fixed 2,4,6,8, and 10 hours after the nocodazole block. Aphidicolin (1 μg/ml) was applied for 16 hours, cells were washed, cultivated in fresh medium, and then fixed 2, 7, 11, 17, and 22 hours post aphidicolin block. NIH 3T3 cells were synchronized by mitotic shake-off. Harvested cells were seeded on poly-lysine coated coverslips, and allowed to attach for 15 min. HeLa cells, used for co-immunoprecipitation of endogenous NM1, were incubated for 16 hours with nocodazole (400 ng/ml), washed in PBS, and then cultivated in complete medium for additional 3 h prior to the harvest.

Plasmid DNA preparation

NM1-GFP, GFP-NM1, Myo1c-GFP, NM1-V5, Myo1c-V5 were obtained by ligation of full length mouse NM1 (amino acids

1–1024) and Myo1c (aa 1–1028) [2] cDNA into pEGFP-C3, pEGFP-N3 (Clontech) and pcDNA3.1/V5-His (Invitrogen) vectors. Truncations containing head (H) neck with IQ domains (Q123) and tail (T) domains were generated using inverse PCR. NM1-V5-(H) (aa 1 to 716) was generated from NM1-V5.GFP-NM1-(Q123.T) (aa 712 to 1044),GFP-NM1-(Q3.T) (aa 763 to 1044), GFP-NM1-(Q123.T -Δ853) (aa 712 to 853), GFP-NM1-(Q12) (aa 712 to 770) were constructed from GFP-NM1 using standard cloning methods. For inspection of NLS-peptide localization, we produced a testing construct GFP-PK that contains in-frame fusion of EGFP and cytosolic enzyme pyruvate kinase (PK) [22], GFP-PK-IQ-12 was produced by ligation NM1-(Q12) sequence into GFP-PK vector. GFP-PK-IQ1 (aa 712 to 740), GFP-PK-IQ2 (aa 739 to 766) GFP-PK-NLS $^{(NM1)}$ (aa 754 to 766) were generated by PCR deletions from GFP-PK-IQ1,2. Nt-GFP-PK was produced by ligation of NM1 N-terminal sequence (aa 1–16) in front of EGFP in GFP-PK vector. Ligation of the OneStrep tag sequence (IBA) in front of EGFP in the pEGFP-C3 vector generated Str-GFP. Str-GFP-NM1-(Q123.T) was produced by ligating the Q123.T (aa 712 to 1044) sequence into Str-GFP vector. Calmodulin cDNA was prepared from HeLa total cell RNA using RT-PCR and cloned into pcDNA3.1 vector (Invitrogen). Bacterial expression vector pET-Str-His was generated by ligation of OneStrep sequence into the pET28b vector (Novagen). Str-IQ12-His was produced by an in-frame ligation of the PCR-amplified fragment of NM1-(Q12) (aa 712 to 770) between the OneStrep- and His-tag. Point mutations in the NLS sequence of NM1 were generated by the site directed mutagenesis protocol (Stratagene). Bacterial expression plasmid pQE-RanQ69L was kindly provided by Prof. Dirk Görlich. Detailed description of all cloning procedures is available upon request. Recombinant proteins were expressed in bacteria and purified using Ni-NTA agarose column as described [28,38].

Pull-down assays and immunoprecipitation

Digitonin extract from suspension HeLa cells, prepared as described [43], was diluted to 2 mg/ml of total protein in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, protease inhibitors). After dilution, purified bacterially expressed Str-IQ12-His was added to the lysate. After 3 hours of incubation, the extract was centrifuged to remove precipitated proteins and supernatant was further incubated for 1 hour with StrepTactin Beads (IBA) to capture the bait and associated proteins. Beads were briefly washed 3 times with 1 ml of the lysis buffer followed by brief wash with IBA wash buffer (100 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA) and captured proteins were eluted from beads with the wash buffer supplemented with 2 mM biotin. HEK 293T cells electroporated with Str-GFP-NM1-(Q123.T) or Str-GFP were collected by trypsinization into serum-containing medium. After centrifugation 300 g/3 minutes, the cells were washed twice with ice-cold PBS and extracted twice with lysis buffer containing 50 mM HEPES pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 1 mg/ml digitonin (Fluka), EDTA-free COMPLETE inhibitors (Roche). After 4 hours of incubation with StrepTactin resin, the captured protein complexes were washed briefly 3 times with 1 ml of the lysis buffer followed by wash with 1 ml of IBA wash buffer. Proteins were eluted from beads with 2 mM biotin added into the wash buffer. The experiments with RanQ69L mutant were performed as described above, with the exception that after the 3rd wash a half of the beads was incubated for 10 min with buffer containing recombinant RanQ69L and the other half was incubated only in buffer. Elution with Ran mutant was repeated twice and remaining proteins were eluted from beads with IBA elution buffer containing 2 mM biotin. Eluates were concentrated ultrafiltration (Ultracel 10K, Milipore) and resolved on 6–20% gradient polyacrylamide gel.

Endogenous NM1 was immunoprecipitated from adherent HeLa cells synchronized with nocodazole. Cells were extracted twice in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 1 mg/ml of digitonin and protease inhibitors), lysates were clarified by centrifugation (10 min, 16 000 g, 4°C), and incubated with beads containing either covalently bound antibody antibody to NM1 (Sigma, cat no M3567) or to EGFP (Exbio, Czech Republic, cat no 11-473-C100). After 3 washes in 1 ml of lysis buffer beads were washed in 1 ml of 50 mM amonium bicarbonate pH 7.5 to remove salts and detergent. Bound proteins were eluted twice with 500 µl of 500 mM amonium hydroxide. Eluates were evaporated using SpeedVac concentrator (Savant, Holbrook, NY, USA), dry pellets were resuspended in 20 ul of 1× SDS loading buffer, boiled and resolved on 6–20% gradient SDS PAGE. After transfer to nitrocelulose proteins were visualized using specific antibodies.

GFP-PK constructs in Fig. 6D were immnuoprecipitated from lysates of electroporated HEK23T cells as follows. Cells were harvested by trypsinization, washed in PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCL pH-7.5, 10 mM EGTA, 2 mM EDTA, 1% Triton X-100, protease inhibitors ROCHE). After clarification by centrifugation (10 min, 16 000 g, 4°C), supernatans were incubated with 20 µl of GFP-trap magnetic particles (ChromoTek GmbH, Germany). After 5 washes in 1 ml of lysis buffer particles were washed in 1 ml of 50 mM amonium bicarbonate pH 7.5 to remove salts and detergent. Bound proteins were eluted twice with 500 µl of 500 mM amonium hydroxide. Eluates were evaporated using SpeedVac concentrator (Savant, Holbrook, NY, USA), dry pellets were resuspended in 20 ul of 1× SDS loading buffer, boiled and resolved on 6–20% gradient SDS PAGE. After transfer to nitrocelulose proteins were visualized using specific antibodies.

Proteolytic digestion and sample preparation

Protein bands were cut from the gel, sliced into the small pieces, and decolorized in sonic bath at 60°C several times with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (ACN). After complete destaining, proteins were reduced by 50 mM TCEP in 0.1 M 4-ethylmorpholine acetate (pH 8.1) for 5 min at 80°C and alkylated using 50 mM iodoacetamide in 0.1 M 4-ethylmorpholine acetate (pH 8.1) for 30 min in dark at room temperature. Then, the gel was washed with water, shrunk by dehydration with ACN and reswollen in water. The rehydratation and dehydration of the gel was repeated twice. Next, the gel was reswollen in 0.05 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (ACN) and then the gel was partly dried using a SpeedVac concentrator (Savant, Holbrook, NY, USA). Finally, the gel was reconstituted with cleavage buffer containing 0.01% 2-mercaptoethanol, 0.05 M 4-ethylmorpholine acetate (pH 8.1), 10% ACN, and sequencing grade trypsin (Promega, 10 ng/µl). Digestion was carried out overnight at 37°C; the resulting peptides were extracted with 30% ACN/0.1% TFA and subjected to mass spectrometric analysis.

Mass spectrometric analysis

Mass spectra were acquired in the positive ion mode on a MALDI-FTMS APEX-Ultra (Bruker Daltonics, Bremen, Germany) equipped with 9.4 T superconducting magnet and SmartBeam laser. The acquisition mass range was 700–3500 m/

z and 512k data points were collected. A 280 V potential was applied on the MALDI plate. The cell was opened for 2500 ms, 4 experiments were collected for one spectrum where one experiment corresponds to 300 laser shots. The instrument was externally calibrated using PepMix II peptide standard (Bruker Daltonics, Bremen, Germany). It results in typical mass accuracy below 2 ppm. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.2% TFA was used as a MALDI matrix. A 1 µl of matrix solution was mixed with a 1 µl of the sample on the target and the droplet was allowed to dry at ambient temperature. After the analysis the spectra were apodized using square sin apodization with one zero fill. The interpretation of mass spectra was done using DataAnalysis version 3.4 and BioTools 3.2 software packages (Bruker Daltonics, Billerica, MA). Proteins were identified by peptide mass fingerprinting (PMF) using a search algorithm MASCOT (Matrix Science).

Generation of the NM1 knock-out mice

To generate NM1-KO mice, loxP-recombination sites were introduced into NM1 gene by homologous recombination in R1 embryonic stem cell line [44]. Cre-mediated recombination in germline cells, achieved by cross breeding with the meu-cre expressing mice [45] resulted in removal of the loxP-flanked exon-1 from the mouse NM1 genomic sequence (sequence from −165 to +116 base pairs from NM1 translation initiation site). In the mutant NM1 allele, only the start codon initiating the translation of Myo1c is present. As a result, only Myo1c protein is expressed in all tissues. Mice were genotyped using genomic PCR, and the absence of NM1 protein was confirmed by Western blotting (Venit et al., in preparation).

Isolation of nuclei from mouse liver

Nuclei from mouse liver were isolated as described [46]. Briefly, mice were killed by CO_2 and liver was homogenized in ice-cold buffer A (250 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES pH 8) in glass Dounce homogenizer. The homogenate was spun down (600 g/10 min), the supernatant was taken as the cytosolic fraction and the pellet was washed once in buffer A. The crude nuclear pellet was resuspended in buffer B (2.0 M sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES pH 8) and centrifuged 30 minutes/16000 g. Purified nuclei were resuspended in buffer Z (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS), heated to 90°C for 10 minutes, sonicated, and centrifuged again (16000 g/10 min). The amount of protein in the supernatant was measured using BCA (Pierce).

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Author Contributions

Conceived and designed the experiments: RD MK PH. Performed the experiments: RD SY MK PN JH TV. Analyzed the data: RD SY MK PN JH TV PH. Wrote the paper: RD PH.

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Phosphatidylinositol-4,5-bisphosphate serves as intranuclear anchor for nuclear myosin 1 and myosin 1C

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Abstract

Myosins are motor proteins which use ATP to carry cellular cargos along actin filaments. Nuclear myosin 1 (NM1) and myosin 1C (Myo1C) are two identical proteins except for 16 extra residues at the N-terminus of NM1. Known cargo molecules that bind to the tail domain of NM1 in the nucleus are DNA, RNA and emerin. Actin and phosphotidylinositol 4,5 bisphosphate (PIP2) are reported to bind to the tail domain of Myo1C in the cytoplasm. PIP2 is a minor membrane phospholipid which is also localized to intranuclear structures. Since membranous structures have not been seen inside of the nucleus, it was suggested that PIP2 is anchored within the nucleus by forming complexes with specific nuclear proteins. We explored therefore if PIP2 also interacts with NM1 and Myo1C in the cell nucleus. We show that both NM1 and Myo1C bind to PIP2 via their plecstrin homolohy (PH) domains also in the nucleus. Furthermore, this binding results in slower mobility of NM1 and Myo1C as shown by fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP) methods. PIP2 interaction with NM1 and Myo1C recruits lamin A and farnesylated proteins to the lipo-protein complex. Moreover, several lipid molecules such as ceramide phosphocholines (sphingomyelins), sulfoglycosphingolipids (sulfatides), 1-acyl,2-alkylglycerophosphoethanolamines, 1-acyl,2-alkylglycerols, diacylglycerophosphocholines, diacylglycerophosphoethanolamines, 1-alkyl,2-acylglycerophosphocholines, diacylglycerophosphates were also found to associate with nuclear PIP2. In addition, nuclear proteins involved in chromatin regulation, transcription, splicing, ribosome synthesis and genomic stability were also found to interact with NM1. These findings suggest that NM1 and Myo1C are tethered within the nucleus via PIP2 possibly nucleating lipo-protein complexes which function in various nuclear processes.

Introduction

NM1 was the first class I myosin motor detected in the cell nucleus (Nowak et al., 1997). Class I myosins have an N-terminal head domain responsible for binding to ATP and actin, a neck domain responsible for binding to calmodulin, and a tail domain responsible for binding to cellular cargos (Batters et al., 2004; Hokanson and Ostap, 2006; Houdusse et al., 2006; Sellers, 2000). NM1 binds directly to DNA (Hofmann et al., 2006), RNA (Obrdlik et al., 2010) and emerin (Holaska and Wilson, 2007) through its tail domain within the nucleus. Monomeric actin transport to the leading edge of the cell is facilitated by interaction with tail domain of Myo1c (Fan et al., 2012). Myo1c is also involved in the transportation of glucose transporter GLUT4 vesicles to the plasma membrane (Bose et al., 2004; Bose et al., 2002). Moreover, cargo-binding tail domain of Myo1c binds directly to PIP2 in cytoplasm, and this interaction tethers Myo1C to the plasma membrane (Hokanson and Ostap, 2006; Hokanson et al., 2006). Apart from the tail domain, IQ motifs in the neck domain are also involved in PIP2-binding due to the interaction with two myristoylated proteins (calcium binding protein 1 and calcium and integrin binding protein 1) in a calcium-dependent manner. This kind of interaction provides an additional attachment of Myo1C to plasma membrane (Cyr et al., 2002; Tang et al., 2007) and it is required for mechanical transduction and adaptation in inner ear cells (Gillespie and Cyr, 2004; Hirono et al., 2004).

Other members of the class 1 myosin family also interact with plasma membrane via their tail domains. Myo1A binds to phosphatidylserine and PIP2, and anchors actin filaments to plasma membrane in microvilli of small intestine (Hayden et al., 1990; Tyska et al., 2005). Myo1B localizes in actin-enriched membrane projections such as filopodia due to PIP2-binding (Komaba and Coluccio, 2010). Besides Class I myosin family, Myosin VI, which walks towards minus end

of actin filaments, binds to PIP2 in clathrin-coated structures (Spudich et al., 2007). Since the sequence of Myo1C and NM1 tails are identical, one could expect that NM1 is able to bind PIP2 as well.

NM1 is as a member of nucleoskeletal proteins which include especially lamins, actin, emerin, spectrin and titin (Castano et al., 2010; Simon and Wilson, 2011). NM1 is crucial for gene expression by modulating chromatin remodeling (Percipalle and Farrants, 2006; Percipalle et al., 2006) and for gene transcription (Fomproix and Percipalle, 2004; Hofmann et al., 2006; Philimonenko et al., 2004; Ye et al., 2008). Nuclear localization sequence (NLS) of NM1 resides in the second IQ motif, which is present in both Myo1C and NM1. Therefore both isoforms localize in the nucleus (Dzijak et al., 2012) and substitute each other in transcription by RNA polymerase I (Venit et al., 2013). Also PIP2 resides in the nucleus (Boronenkov et al., 1998; Mellman et al., 2008; Osborne et al., 2001) where it is involved in transcription by RNA polymerase I (Yildirim et al., 2013) and RNA polymerase II (Yu et al., 1998), splicing (Mellman et al., 2008), chromatin remodeling (Zhao et al., 1998) and actin polymerization (Rando et al., 2002). Class 1 myosins bind to PIP2 in the plasma membrane, but up to date no information is available on the nucleus where membranes are internally absent. Therefore, we investigated Myo1C and NM1 interaction with PIP2. Here we report that Myo1C and NM1 bind to PIP2 via their PH domains also in the cell nucleus. They make complexes with proteins such as lamin A and farnesylated proteins, which might function as anchorage sites for large macromolecular complexes during gene expression.

Results

Myo1C and NM1 are anchored in the nucleus via interactions with PIP2

To investigate whether Myo1C and NM1 are able to bind to PIP2 in the cell nucleus, we performed pull down experiments and direct binding assays. We found that Myo1C and NM1 make complex with PIP2, since Myo1C and NM1 were pulled down with PIP2-coupled agarose beads from nuclear extracts (Figure 1A-i).

Cell nucleus contains lipid microdomains enriched in sphingomyelin and cholesterol (Cascianelli et al., 2008; Scassellati et al., 2010), which are similar to microdomains in the plasma membrane (Edidin, 2003). These microdomains might target different protein complexes due to their highly saturated and tightly packed lipid composition. Proteins with lipid modifications such as farnesylation, palmitoylation and myristoylation can be recruited to these lipid domains (Zacharias et al., 2002). On the other hand, proteins lacking these modifications can be tethered to these microdomains via their lipid binding domains. PIP2 is present in these microdomains (Johnson and Rodgers, 2008) and it anchores Myo1C to these microdomains on the plasma membrane (Maravillas-Montero et al., 2011). To identify if NM1 and PIP2 are present together in intranuclear microdomains, we made a sucrose gradient ultracentrifugation from highly purified nuclear extracts. We found both NM1 and PIP2 in the light fraction (#1, detergent-insoluble microdomains) (Simons and Toomre, 2000) suggesting that NM1 and PIP2 are present in similar intranuclear microdomains (Figure 1A-ii). To test whether NM1 and PIP2 interact directly, we prepared a single point mutation (K908A) in the PH domain of NM1 that is critical for PIP2-binding in MyoIc (Hokanson and Ostap, 2006; Hokanson et al., 2006). We purified wild type and mutat (K908A) NMI and used then on PIP2 spotted membrane for direct binding assays. We

found that wild type NM1 binds PIP2 directly while mutant lacks the PIP2-binding (Figure 1A-iii).

Since PIP2 is relatively small molecule (~1.042 g/mol), Myo1C and NM1 binding to PIP2 should not interfere with the mobility of proteins unless PIP2-binding mediates the interactions with other molecules. To test this hypothesis, we measured the mobility of Myo1C and NM1 using FCS and FRAP methods. In comparison with non-mutated NM1, the mobility of the mutant (K908A, R919A) NM1 became 2 times faster (Figure 1B-i). Similarly, FCS results showed that NM1 mobility increased almost 2 times when PIP2-binding to NM1 was lost (Figure 1B-ii). These results confirm that NM1 binds to PIP2 in the cell nucleus. Since this interaction reduces NM1 mobility, NM1-PIP2 probably associate with larger protein-lipid complexes within the nucleus.

To see if PIP2 dephosphorylation effects NM1 mobility, we created a construct consisted of NLS-fused inositol 5-phosphatase. This enzyme cleaves 5-phosphate of PIP2 and converts it to PI4P in the nucleus. We co-transfected cells with NM1-EGFP and NLS-mRFP-inositol 5-phosphatase, and measured NM1 mobility in the nucleoplasm. When PIP2 was dephosphorylated by inositol 5-phosphatase, NM1 mobility increased 3 times, indicating again that NM1 mobility depends on PIP2 binding (Figure 1C).

The phospholipase C delta1 (PLCδ1) enzyme binds to PIP2 via its PH domain and cleaves PIP2 into inositol (1,4,5) triphosphate and DAG (Okada et al., 2002; Yamaga et al., 1999). A single point mutation of basic amino acid directly involved in interactions with 4- and/or 5-phosphoryl groups of phosphatidylinositol (R40A) results in loss of PIP2-binding and hydrolysis and therefore loss of membrane attachment (Yagisawa et al., 1998). To test if NM1 and PLCδ1PH

domain could compete for binding to PIP2, we mutated the PIP2-binding domain of PLCδ1PH (R40A), and co-transfected cells with constructs coding for NM1 and PLCδ1PH, or NM1 and PLCδ1PH-R40A. FRAP results showed that NM1 mobility increased ~ 2 times when PIP2 was bound by PLCδ1PH domain compared to the PLCδ1PH-R40A mutant (Figure 1D). Moreover, we show that also Myo1C bind to PIP2 in the nucleus, since Myo1C became 2 times faster due to abolishment of PIP2-binding (Figure 1E). In parallel, PIP2 dephosphorylation by inositol 5-phosphatase caused an increase in Myo1C mobility (Figure 1F).

All these data indicate that NM1 and Myo1C bind to PIP2 in the cell nucleus of living cells via their PH domain and this binding alters the mobility of NM1and Myo1C most likely due to their anchorage to larger molecules or complexes via PIP2.

NM1 mobility is dependent on transcription activity and actin polymerization

NM1, actin and PIP2 localize to transcriptionally active sites of nucleoli and make complexes with RNA polymerase I (Fomproix and Percipalle, 2004; Nowak et al., 1997; Philimonenko et al., 2010; Philimonenko et al., 2004: Yildirim et al. 2013). Because NM1, actin and PIP2 promote transcription of ribosomal genes (Philimonenko et al., 2004; Ye et al., 2008; Yildirim et al. 2013), we tested if NM1 mobility depends on active transcription and actin polymerization. Therefore, we inhibited transcription of all three RNA polymerases by high doses of actinomycin D (AMD) or we blocked only RNA polymerase I by low doses of AMD (Figure 2B). The mobility of non-mutated and mutated NM1 decreased ~ 1.5 times after inhibition of transcription by either all RNA polymerases (Figure 2A), or only RNA polymerase I (Figure 2B). Moreover, the mobility of both non-mutated and mutated NM1 decreased ~ 1.6 times after treatment of cells by latrunculin A, a toxin that disrupts actin polymerization (Figure 2C). On the other hand, the

mobility of RAB6 protein, a small GTP-binding protein involved in recycling of vesicles from endosomes to golgi (Short et al., 2002; Young et al., 2005), did not change significantly upon inhibition of RNA polymerases and inhibition of actin polymerization (Figure 2D). These results indicate that NM1 mobility in the nucleus is dependent on active transcription and actin polymerization.

PIP2-binding to Myo1C and NM1 facilitates formation of protein-lipid complex containing lamin A and farnesylated proteins

Because there are no visible membranous structures within the nucleus, it was suggested that proteins with hydrophobic pockets bind to PIP2 and protect it from the hydrophilic environment (for a review see, Irvine, 2003). Together with results of our mobility assays it suggests that PIP2 binding to NM1 and Myo1C might be responsible for recruiting of other proteins into this lipo-protein complex. To test this hypothesis, we used pull-down assays and immunofluorescence detection. We found that lamin A forms a complex with NM1 and Myo1C via interacting with PIP2 (Figure 3A). Since post-translational modifications such as farnesylation, palmitoylation and myristoylation can facilitate the interaction between proteins and lipids, we have also tested whether NM1/Myo1C-PIP2 complex contains farnesylated proteins. Indeed, farnesylated proteins were pulled down specifically with NM1 and Myo1C compared to their PIP2-binding mutants (Figure 3B).

To further study components of NM1/Myo1C PIP2 complex within the nucleus, we overexpressed NM1 and Myo1C in U2Os cells and investigated the localization of its binding partners (PIP2, lamin A and farnesylated proteins) by immunofluorescence. We found that NM1 colocalizes with PIP2 in nucleoplasm, especially in the nuclear speckles. Furthermore, NM1

colocalizes with lamin A in nucleoplasm and at nuclear lamina, and with farnesylated proteins in nucleoplasm, cytoplasm and at the plasma membrane (Figure 4A). Intensity profiles demonstrate the colocalization patterns with fluorescence maxima of both proteins clearly overlapping (Figure 4B). Moreover, nuclear colocalization plots show the pixels from both channels with yellow pixels indicating colocalization between investigated molecules (Figure 4C). Myo1C also colocalizes with PIP2, lamin A and farnesylated proteins in the cell nucleus (Figure 5A) as shown by intensity profiles (Figure 5B) and colocalization plots (Figure 5C).

NM1 and PIP2 form complexes with proteins and lipids in the cell nucleus

To further investigate proteins that interact with NM1, we carried out co-immunoprecipitation of NM1 from nuclear extracts using anti-NM1 antibody and preimmune sera as a control (Figure 6A). Co-immunoprecipitation results showed that NM1 binds to several nuclear proteins listed in Table 1. These results indicate that NM1 might be also involved in other important processes besides transcription and chromatin remodeling such as DNA damage, splicing and ribosome maturation.

We used also another approach to study PIP2-binding partners. We over-expressed PLCδ1PH domain and PIP2-binding mutant PLCδ1PH-R40A domain fused with nuclear localization sequence in U2Os cells. We prepared nuclear extracts from these cells and performed *in vivo* pull-down experiments (Figure 6B). We show that histone H2A and H2B bind to PLCδ1PH but not to PLCδ1PH-R40A domain, suggesting a possible involvement of PIP2 in chromatin remodeling. Moreover, a number of proteins involved in transcription make complex with PLCδ1PH domain but not PLCδ1PH-R40A domain, indicating PIP2 involvement in transcription (Yildirim et al. 2013; Yu et al., 1998). Similarly, we determined by mass spectroscopy that

ceramide phosphocholines (sphingomyelins), sulfoglycosphingolipids (sulfatides), 1-acyl,2-alkylglycerophosphoethanolamines, 1-acyl,2-alkylglycerols, diacylglycerophosphocholines, diacylglycerophosphoethanolamines, 1-alkyl,2-acylglycerophosphocholines, diacylglycerophosphates bind specifically to PLCδ1PH domain as compared to PLCδ1PH-R40A domain (Table 2).

Discussion

Myosins are motor proteins which hydrolyze ATP to convert chemical energy into mechanical force and transport cargo along actin filaments. Myo1C is known to be important for trafficking various cytoplasmic cargos such as GLUT4 transporter containing vesicles (Bose et al., 2004; Bose et al., 2002) and actin (Fan et al., 2012). It also interacts with the inositol domain of PIP2 (Hokanson and Ostap, 2006; Hokanson et al., 2006) in the cytoplasm. The interaction occurs through Myo1c PH domain in the tail domain, which is identical to the tail domain of NM1. However, it is not known if NM1 also can bind to PIP2 within the nucleus. As a nuclear molecular motor, NM1 is involved in long range chromosomal movements together with actin (Hu et al., 2008). Another study also showed the interaction of NM1 with emerin (Holaska and Wilson, 2007), which is a nuclear membrane protein interacting with lamins. Here, we investigated PIP2 interaction with Myo1C and NM1 and their binding partners in the cell nucleus.

Our pull-down and direct binding assays proved the interaction of PIP2 with both Myo1C and NM1 through their PH domain in the tail region. We also showed that Myo1C and NM1 binding to PIP2 decreases the mobility of these myosin isoforms in the nucleus. In accordance, depletion

of PIP2 by dephosphorylation via inositol 5-phosphatase causes an increase in the mobility of NM1 and Myo1C, indicating the ability of Myo1C and NM1 to bind to PIP2, but not to PI4P. We then tested if the PH domain of Myo1C and NM1 has higher affinity for binding to PIP2 than the PH domain of PLCδ1 enzyme which also interacts with PIP2 (Okada et al., 2002; Rebecchi et al., 1992; Yamaga et al., 1999). We found that binding of PLCδ1PH domain to PIP2 interferes with binding of the PH domain of NM1 to PIP2, indicating a higher affinity of PLCδ1PH domain for binding to PIP2 compared to PH domain of NM1.

Because PIP2 is a very small molecule, the decrease in the mobility of Myo1C and NM1 is most likely due to recruitment of other proteins and/or lipids into the complex via PIP2. To test this hypothesis, we performed pull-down assays using a tail domain of these myosin isoforms. The results showed the presence of lamin A in PIP2-myosin complex. It has been documented that lamin A binds to DNA directly or via binding to BAF and core histones (Taniura et al., 1995). Mutations in lamins alter positioning of chromosomes and disrupt epigenetic regulation, suggesting that lamin scaffolds might serve as an anchoring site for chromatin and regulate its status (Dechat et al., 2008; Simon and Wilson, 2011). We suggest that lamin A might provide a docking site for this complex in the nucleus. The question regarding the involvement of lamin A-NM1/Myo1C-PIP2 complex in the modulation of chromatin status still remains to be answered. Farnesylated proteins carry 15C fatty acid chain at the C-terminus, they can attract proteins with hydrophobic domains and form lipo-protein complexes (Zacharias et al., 2002). Since we found farnesylated proteins as a part of NM1/Myo1C-PIP2 complex in the nucleus, we propose that PIP2 binding to NM1/Myo1C recruits proteins with farnesyl groups due to interaction between fatty acid chains of PIP2 and farnesylated proteins.

Moreover, our co-immunoprecipitation experiments showed that NM1 binds to proteins involved in cytoskeleton arrangement, DNA damage, splicing, ribosome synthesis, and genomic stability, suggesting a diverse set of functions in which NM1 may be involved, possibly as a molecular motor. In addition, our pull-down experiments showed that core histones and other lipids (sphingomyelins, sulfatides, 1-acyl,2-alkylglycerophosphoethanolamines, 1-acyl,2-alkylglycerols, diacylglycerophosphocholines, diacylglycerophosphoethanolamines, 1-alkyl,2-acylglycerophosphocholines, diacylglycerophosphates) bind to PIP2, indicating that PIP2 can be anchored in nucleus via lipid-protein and lipid-lipid interactions.

NM1 is implicated in nuclear processes such as transcription (Philimonenko et al., 2004; Ye et al., 2008), chromatin remodelling (Fomproix and Percipalle, 2004; Percipalle et al., 2006), and transport of small ribosomal subunits (SSUs) from nucleolar periphery towards nuclear pores (Obrdlik et al., 2010). Ye et al., 2008 showed that polymerized actin and NM1 motor domain are required for the activation of RNA polymerase I transcription. Recently, we showed that PIP2 also forms complexes with RNA polymerase I and promotes transcription of ribosomal genes (Yildirim et al. 2013). PIP2 also displaces actin from the actin binding site of ATP-dependent chromatin remodeling protein BRG1 (Rando et al., 2002), analogously to uncapping of actin via PIP2 in cytoplasm (Yin and Janmey, 2003), and thus facilitates the synthesis of filamentous actin in the nucleus (Rando et al., 2002). PIP2 also promotes binding of SWI-SNF like BAF chromatin remodeling complex to nuclear matrix/chromatin in actin-dependent manner (Zhao et al., 1998). However, nothing is known about the functional interplay between actin and NM1-PIP2 complex in the nucleus. Therefore, we studied NM1-PIP2 complex mobility and its dependence on active transcription and actin polymerization. We found that inhibition of transcription and disruption of actin polymerization significantly decreases NM1 mobility in the cell nucleus. These data suggest

that NM1 interaction with polymerized actin stimulates ATPase activity of myosin, which is required for force generation to drive transcription machinery. However, further studies are needed to elucidate the significance of NM1-PIP2 interaction in gene transcription.

Taken together, our data demonstrate for the first time that in the cell nucleus, PIP2 interaction with NM1/Myo1C is required for association of these myosin isoforms with a subset of proteins and lipids. Our data also answer the question how PIP2 is retained within nucleus in a different form than membranous structures, since we showed the interaction between PIP2 and farnesylated proteins via their fatty acid chains. Based on these findings, we suggest that PIP2-linked lipo-protein complexes might form a structural framework that is required for actomyosin-dependent nuclear processes.

Materials and Methods:

Cells and transfections

U2OS osteosarcoma cells were kept in DMEM with 10 % fetal calf serum in 5 % CO_2/air, 37°C, humidified atmosphere. The cells were transfected with Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol, and observed under confocal microscope Leica TCS SP5. U2OS stable cell lines (PLCδ1PH and PLCδ1PH-R40A mutant fused with GFP-NLS-STR at the C-terminus) were selected in presence of 400 μg/ml geneticin (G418, Gibco BRL Life Technologies, Inc).

For pulling down binding partners of NM1/Myo1C-PIP2 complex, we created U2OS cells stably expressing NLS sequence of NM1/Myo1C (Dzijak et al., 2012), IQ3 and tail domains of NM1/Myo1C at the C terminus of STR-GFP construct. As control, we prepared PIP2 binding

mutant constructs expressed stably in the same cell line. Stable cell lines selected in presence of puromycin (2.5 μg/ml).

Constructs used in this study

Full length NM1/Myo1C cDNAs were ligated into EGFP-N3 vector or into EGFP-C3 (Clontech). Mutants were obtained by site-directed mutagenesis using following primers: K892A/K908A forward primer 5`- GCCGTGCCCGTGGTAGCATACGACCGTAAGGG-3′ and reverse primer 5`- CCCTTACGGTCGTATGCTACCACGGGCACGGC-3′; R903A/R919A forward primer 5`- CAAGCCTCGCCCCGCGCAGCTGCTGCTC-3′ and reverse primer 5`- GAGCAGCAGCTGCGCGGGGCGAGGCTTG-3′.

SV40 NLS sequence was prepared by PCR using following primers: forward primer 5` CTAGCGCCACCATGGCTCCAAAAAAGAAGAGAAAGGTAGATCCA-3′ and reverse primer 5` CCGGTGGATCTACCTTTCTCTTCTTTTTTGGAGCCATGGTGGCG-3′. Then NLS was cloned into N-terminus of mRFP- FKBP-5-ptase-dom using Nhe1 and Age1 restriction sites.

SV40 NLS was released from pEYFP-Nuc vector by cutting at BsrGI and AflII restriction sites, and inserted in the C-terminus of PLCδ1PH-GFP vector which was a kind gift from Dr. Tamas Balla (Varnai and Balla, 1998). PLCδ1PH-GFP-NLS was then cloned to XbaI and AfeI restriction sites of pPSG-IBA-103 vector (Iba BioTagnology) after amplification using following primers: forward primer 5`-GTCTTATCTAGAATGGACTCGGGCCGGGA-3′ and reverse primer 5`- GCTTCGAGCGCTTCCTACCTTTCTCTTCTTTTTTGGATCTAC-3′. For FRAP experiments mCherry was exchanged with GFP using AgeI and BsrGI restriction sites. PIP2-binding PLCδ1PH mutant was done by site directed mutagenesis by using following primers:

R40A forward primer 5`-GCTCATGGAGGAGAGAGGCGTTCTACAAGTTGCAGG-3´ and reverse primer 5`CCTGCAACTTGTAGAACGCCTCTCTCCTCCATGAGC-3´.

NLS sequence of NM1/Myo1C, IQ3 and tail domains of NM1/Myo1C was constructed from STR-GFP-Q123-Tail (Dzijak et al., 2012) by ligation of the fragment containing N-terminal OneStrep tag, EGFP and a headless portion of NM1/Myo1C into lentiviral expression vector pCDH-EF1-Puro. Point mutations were produced by site-directed mutagenesis. Recombinant lentiviruses were generated as described before (Naldini et al., 1996).

Expression and purification of recombinant proteins

NM1 and K908A mutant were fused with C-terminal Flag tag at the C-terminus and stably expressed in H1299 cell line. Cells were washed with PBS and extracted with pH 8 E-buffer (50 mM HEPES, 300 mM NaCl, 4 mM $MgCl_2$ and 1 % Triton X-100). The extract was filtered through 0,45 μm filter, and incubated 2 h with pre-equilibrated anti-Flag-M2 agarose beads (A2220, Sigma Aldrich). The beads were then washed several times with E-buffer, and the bound proteins were then eluted five times with 100 μg/ml Flag peptide in pH 8 buffer (10 mM Tris, 100 mM NaCl, 0.5 % EDTA and 20 % glycerol). Eluted fractions were pooled and analyzed for protein content by SDS-PAGE. For visualization of the protein on the membranes, anti-Flag antibody was used (200471, Stratagene, Agilent Technologies, Santa Clara, CA, USA).

GST-PLCδ1 (1-140) (pGST3) and GST-PLCδ1 (1-140)-R40A which lacks binding sites to PIP_2 were received from Dr. Hitoshi Yagisawa (Yagisawa et al., 1998). Escherichia coli BL21 (Stratagene) was transformed with constructed plasmids and transformants were grown at 37°C in LB medium in the presence of 100 μg/ml ampicilin for 4 h. The culture was induced for high protein expression by addition of 1M IPTG until the OD600 is approximately 1. Bacterial pellets

were resuspended in BC100 buffer (20 mM Tris, pH 8, 0.1 mM EDTA, 20% glycerol, 100 mM NaCl) with 0.1% NP40, 1 mM DTT, and protease inhibitors (Complete, EDTA-free, Roche Diagnostics GmbH). Bacterial lysates were loaded onto glutathione agarose column (G4510, Sigma Aldrich) equilibrated with BC100. After washes with BC100, proteins were eluted with 50 mM Tris-HCl, pH 8 having 10 mM reduced L-glutathione (G4251, Sigma Aldrich).

FRAP

Transiently transfected U2OS cells were photobleached at nucleoplasm with a 488 -nm laser for 20 m.s. GFP fluorescence intensities were monitored every 0.175 seconds on an Olympus microscope (IX71) with a 60x 1.42 NA objective. Approximately 15 cells were analyzed for each condition. All images were corrected for overall photobleaching and analyzed by DeltaVision software SoftWoRx 5.5 (Applied Precision). Two-dimensional diffusion of the fluorescent molecules is calculated according to a previously published model (Axelrod et al., 1976).

FCS

Instrumental setup: FCS measurements were performed on MicroTime 200 inverted confocal microscope (PicoQuant, Germany). We used a pulsed diode laser (LDH-P-C-470, 470 nm, PicoQuant) providing 80 ps pulses at 40 MHz repetition rate, dichroic mirror 490 DRLP, band-passfilter 515/50 (Omega Optical), and a water immersion objective (Olympus, 1.2 NA, 60x). In order to minimize the photobleaching and saturation effects, low laser power was applied (2 mW at the back aperture of the objective). At the detection plane, the pinhole was 50 μm in diameter. The signal was detected by single photon avalanche diodes (SPAD, Perkin Elmer). For calculating fluorescence correlation curves, we corrected the data for the detector afterpulsing.

Data acquisition and processing: Photon arrival times were stored using fast electronics (Picoharp 300, PicoQuant) in time-tagged time-resolved recording mode. Every detected photon was assigned i), a time after the beginning of the measurement, and ii), a time after the previous laser-pulse. The data were correlated using home-built software correlator yielding the auto-correlation curve *G(t)* where *t* corresponds to the delay time. The G(t) were fitted to the one-particle model:

$$G(\tau) = 1 + \left(1 - T + T \cdot e^{-\frac{\tau}{\tau_{triplet}}}\right) \frac{1}{N} \cdot \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right)} \cdot \frac{1}{\sqrt{1 + \left(\frac{r_0}{z_0}\right)^2 \cdot \frac{\tau}{\tau_D}}}$$

where t_D corresponds to the diffusion time, T to the triplet fraction, $t_{triplet}$ to the relaxation time, N to the average number of the particles present in the confocal volume, and r_0/z_0 to the ratio of the size of the confocal volume in the lateral and axial direction, respectively.

Antibodies and immunofluorescence

U2OS cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min, respectively. After washes with PBST, they were incubated with the primary and secondary antibodies for 1 h at RT. Images were taken in confocal microscope (Leica TCS SP5 AOBS TANDEM) with 100x (NA 1.4) oil immersion objective lense.

Primary antibodies used in this study: anti-PIP2 (MM, IgM, clone 2C11, Abcam, Cambridge, UK; 16 µg/ml), anti-lamin A (MM, IgG, clone JOL4, gift from Dr. C. J. Hitchinson; 20 µg/ml), anti-farnesyl (RP, AB4073, Millipore, Billerica, MA, USA; 20 µg/ml).

Secondary antibodies used in this study: goat anti-rabbit IgG conjugated with Alexa 647 (A21245, Invitrogen, Grand Island, NY, USA; 5 µg/ml), donkey anti-mouse IgM conjugated with Cy3 (715-165-140, Jackson ImmunoResearch, West Grove, PA, USA; 10 µg/ml), and donkey anti-mouse IgG conjugated with Alexa 488 (A21202, Invitrogen, Grand Island, NY, USA; 5 µg/ml).

Pull-down experiments

***In vivo* pull-down:** U2OS stable cells were harvested and nuclear extracts were prepared according to an earlier published protocol (Dignam et al., 1983) and incubated with Strep-Tactin sepharose beads (2-1201, Iba BioTagnology) at 4°C for 4 h. The proteins bound to beads were eluted in 1x Strep-Tag elution buffer (2-1000, Iba BioTagnology), denaturated, loaded into SDS-PAGE gel and excised for mass spectrometric analysis.

***In vitro* pull-down :** Hela cells nuclear extract (CC01-20-25, Accurate Chemical) pre-incubated with agarose beads (Echelon Biosciences Inc.) was incubated with PIP2-coated beads (P-B045a, Echelon Biosciences Inc.) for 3 h. After washes with BC100, beads were boiled in 1x Laemmli SDS buffer for 5 min and resolved by SDS-PAGE for immunoblotting detection. For detection of NM1 and Myo1C, an antibody against to tail domain of NM1/Myo1C was used (a gift from Dr. Peter Gillespie, clone R2652).

Co-immunoprecipitation of NM1 from HeLa nuclear extract

Co-immunoprecipitation of NM1 from HeLa nuclear extract was carried out using anti-NM1 antibody (M3567, Sigma Aldrich). Nuclear extracts were precleared with Protein A-agarose beads for 1 hour at 4^{o}C under rotation. NM1 immunoprecipitation was carried out by incubation of the nuclear extract with anti-NM1 beads (anti-NM1 antibody was crosslinked to beads as

described by Abcam and blocked for 30 minutes with 5% BSA in wash buffer) followed by several washes in a buffer containing 20 mM Tris HCl (pH 7.9), 10% glycerol, 0.1 mM EDTA (pH 8.0), 10 mM β-mercaptoethanol, 0.5 mM PMSF, 0.5 M KCl, 1 mM ATP and 0.2% NP40. Bound proteins were eluted in boiled Laemmli buffer and loaded into 10% PAGE. Rabbit serum was used as a control for the co-immunoprecipitation. Proteins immunoprecipitated with only NM1 were analyzed on Bruker APEX-Q FTMS instrument (Bruker Daltonics) coupled to MALDI source. Spectra were interpreted using the MASCOT software (Matrix Science).

Western blot analysis

Proteins were transferred to nitrocellulose membrane (Pall Corporation, USA). After 1 h of blocking with 5% non-fat milk in PBST, the membrane was incubated with primary antibody in PBST with 5% non-fat milk for 1 h and then washed with PBST. For enhanced chemiluminescence (ECL) detection, HRP-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories, 7500x dilution) was used. Alternatively, immunoreactive bands were detected by Odyssey Infrared Imaging System (LI-COR Biosciences) after the incubation with the appropriate IRDye coupled to goat anti- mouse or goat anti-rabbit antibodies. Primary antibodies used: anti-NM1 antibody (M3567, Sigma Aldrich, St. Louis, MO, USA), anti-lamin A (gift from Dr. C. J. Hitchinson), anti-farnesyl (AB4073, Millipore, Billerica, MA, USA).

Nuclear extract fractionation and dot blot analysis

Hela nuclear extracts were fractionated by sucrose gradient method. Briefly, 250 µl of nuclear extracts were mixed with the same amount of 80% sucrose in TKM buffer containing 50 mM Tris (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 % Brij 98, and EDTA-free protease inhibitor. Samples were transferred to centrifuge tubes, and sequentially overlaid with 4.3 ml of

36%, then 0.2 ml of 5% sucrose solution to a total volume of 5 ml. The mixture was subjected to equilibrium density gradient centrifugation at 50,000 rpm for 18 h at 4°C in a MLS50 rotor (Beckman Coulter, Fullerton, CA). 200 µl from each of the collected fractions were spotted on nitrocellulose membrane and probed for PIP2 using anti-PIP2 antibody (2C11, Abcam, Cambridge, UK). Visualization was performed by Odyssey Infrared Imaging System (LI-COR Biosciences) after incubation with the appropriate secondary antibody.

Mass spectrometric analysis

After incubations with nuclear extracts, Strep-Tactin sepharose beads were washed twice with 1 ml of deionized water. The bound lipids were extracted by 20 µl of chloroform/methanol (1:2) mixture. One µl of the extract was mixed with one µl 2,5-Dihydroxybenzoic acid matrix solution (30 mg of 2.5 dihydroxybenzoic acid in 30% TA– 0.1 % trifluoroacetic acid in 30% aqueous MeCN). One µl of resulting mixture was deposited on the MALDI plate.

Samples were analysed on an APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and a Dual II ion source (Bruker Daltonics, Billerica, MA). The analysis was performed using a matrix-assisted laser desorption/ionization (MALDI) and the spectra were acquired in positive ion mode. The cell was opened for 1.8 msec, accumulation time was set at 0.05 s and four experiments were collected for one spectrum in which one experiment corresponds to 200 laser shots. The acquisition data set size was set to 512k points with the mass range starting at m/z 400 a.m.u., resulting in a resolution of 260,000 at m/z 400. The instrument was externally calibrated using DHB matrix clusters and PepMix II peptide standard (Bruker Daltonics, Bremen, Germany) which typically results in mass accuracy below 2 ppm. The acquired spectra were apodized with a square sine bell function and Fourier transformed with one

zero-fill. The interpretation of mass spectra was done using DataAnalysis version 4.0 software package (Bruker Daltonics, Billerica, MA) and mMass 3.0 (www.mMass.org).

Figure legends

Figure 1. NM1 and Myo1C bind to PIP2 in the cell nucleus.

(A) NM1and Myo1C were pulled down by PIP2 coupled beads from nuclear extract (i). NM1 was found in the same fractions together with PIP2 (ii). Direct binding of NM1 to PIP2 was proved in PIP2 spotted membranes, while mutant in PH domain (K908A) lacked the binding (iii). NE, nuclear extract; Ag B, agarose beads; PIP2 B, PIP2-coupled beads; NM1-Fl, NM1-Flag; K908A-Fl, K908A-Flag.

(B) NM1 mobility measurements in U2OS cells which were transfected with plasmids expressing wild type or PIP2-binding deficient mutants K908A and R919A by FRAP (i) and FCS (ii). Results indicate that NM1 binding to PIP2 is via PH domain in tail region and when this binding is disrupted NM1 moves significantly faster. ***p value $\leq$ 0.001.

(C) NM1 mobility measurements in U2OS cells which were transfected with only NM1 or with NM1 and inositol 5-phosphatase.When PIP2 was depleted by inositol 5-phosphatase, NM1 became faster due to loss of PIP2-binding. ***p value $\leq$ 0.001. IN 5-PTASE, inositol 5-phosphatase.

(D) NM1 mobility measurements in U2OS cells which were co-transfected with NM1 and PLCδ1PH or NM1 and mutant R40A domain. NM1 and PLCδ1PH competed for the binding to

NM1 and therefore NM1 mobility is increased when PIP2 preferentially bound to PLCδ1PH. ***p value ≤ 0.001. PLCPH, PLCδ1PH.

(E) Myo1C mobility measurements in U2OS cells which were transfected with plasmids expressing wild type or PIP2-binding deficient mutants K892A and R903A. Results indicate that Myo1C binding to PIP2 is via PH domain in tail region and when this binding is disrupted Myo1C moves significantly faster. ***p value ≤ 0.001.

(F) Myo1C mobility measurements in U2OS cells which were transfected with only Myo1C or with Myo1C and inositol 5-phosphatase.When PIP2 was depleted by inositol 5-phosphatase , Myo1C became faster due to loss of PIP2-binding. ***p value ≤ 0.001. IN 5-PTASE, inositol 5-phosphatase.

Figure 2. NM1 mobility in the cell nucleus is dependent on active transcription and polymerized actin.

(A) NM1 mobility measurements in U2OS cells which were treated with AMD (2 μg/ml) for 2 h. Inhibition of transcription by all three polymerases causes a decrease in NM1 mobility. ***p value ≤ 0.001.

(B) NM1 mobility measurements in U2OS cells which were treated with AMD (0.04 μg/ml) for 2 h. Inhibition of transcription by RNA polymerase I causes a decrease in NM1 mobility. ***p value ≤ 0.001, **p value ≤ 0.01, *p value ≤ 0.05.

(C) NM1 mobility measurements in U2OS cells which were treated with Latrunculin A (2 μM) for 1 h. Inhibition of actin polymerization causes a decrease in NM1 mobility. ***p value ≤ 0.001.

(D) RAB6 mobility measurements in U2OS cells. CONT, non-treated cells; LAT, latrunculin A (2 μM, 1h) treated cells; HIGH, AMD (2 μg/ml, 2 h) treated cells; LOW, AMD (0.04 μg/ml, 2 h) treated cells. No statistical significance detected.

Figure 3. NM1/Myo1C-PIP2 complex is anchored in the nucleus via lamin A and farnesylated proteins.

(A) *In vivo* pull-down of NM1/Myo1C-PIP2 complexes resulted in lamin A binding.

(B) Farnesylated proteins were found in the NM1/Myo1C-PIP2 complexes as shown in western blot.

Figure 4. NM1 colocalizes with PIP2, lamin A and farnesylated proteins.

(A) Immunofluorescence staining of NM1 overexpressing cells with PIP2, lamin A and farnesylated proteins.

(B) Intensity profiles demonstrate the colocalization between proteins.

(C) Nuclear colocalization plots show the pixels from both channels with yellow pixels indicating colocalization between investigated molecules. Bar, 5 μm.

Figure 5. Myo1C colocalizes with PIP2, lamin A and farnesylated proteins.

(A) Immunofluorescence staining of Myo1C overexpressing cells with PIP2, lamin A and farnesylated proteins.

(B) Intensity profiles demonstrate the colocalization between proteins.

(C) Nuclear colocalization plots show the pixels from both channels with yellow pixels indicating colocalization between investigated molecules. Bar, 5 μm.

Figure 6. NM1 and PIP2-binding partners identified by co-immunoprecipitation and *in vivo* pull-down experiments.

(A) Co-immunoprecipitation of NM1 from HeLa nuclear extract. NM1, anti-NM1 antibody used for co-immunoprecipitation; Control, rabbit serum used for co-immunoprecipitation; M, marker.

(B) *In vivo* pull-down of PIP2 by using PLCδ1PH domain (WT) and its mutant for PIP2 binding (R40A) stably expressed in the U2OS cells. Arrow shows the domain expressed in the cells as bait. M, marker.

Tables

Table 1. The list of proteins found to be precipitate with NM1.

➢ **Cytoskeleton arrangement** • Myosin 9 • Actin ➢ **DNA damage** • DNA topoisomerase • Non-pou domain octomer binding protein ➢ **Splicing** • Splicing factor SV2 • RNA nucleolar helicase	➢ **Ribosome synthesis and maturation** • Nucleolin • Vigilin ➢ **Genomic stability** • Nucleophosmin

Table 2. Proteins and lipids found to be bound to PIP2.

➢ **Chromatin regulation** • Histone H2A • Histone H2B ➢ **Transcription** • Far upstream element binding protein 2 • X ray repair cross complementing protein 5 • Nucleoside diphosphate kinase beta • Nuclease sensitive element binding protein 1 • Acidic leucine-rich nuclear phosphoprotein 32 family member A • GTP-binding nuclear protein
➢ **Lipids** ▪ Ceramide phosphocholines (sphingomyelins) ▪ Sulfoglycosphingolipids (sulfatides) ▪ 1-acyl,2-alkylglycerophosphoethanolamines ▪ 1-acyl,2-alkylglycerols ▪ Diacylglycerophosphocholines ▪ Diacylglycerophosphoethanolamines ▪ 1-alkyl,2-acylglycerophosphocholines ▪ Diacylglycerophosphates

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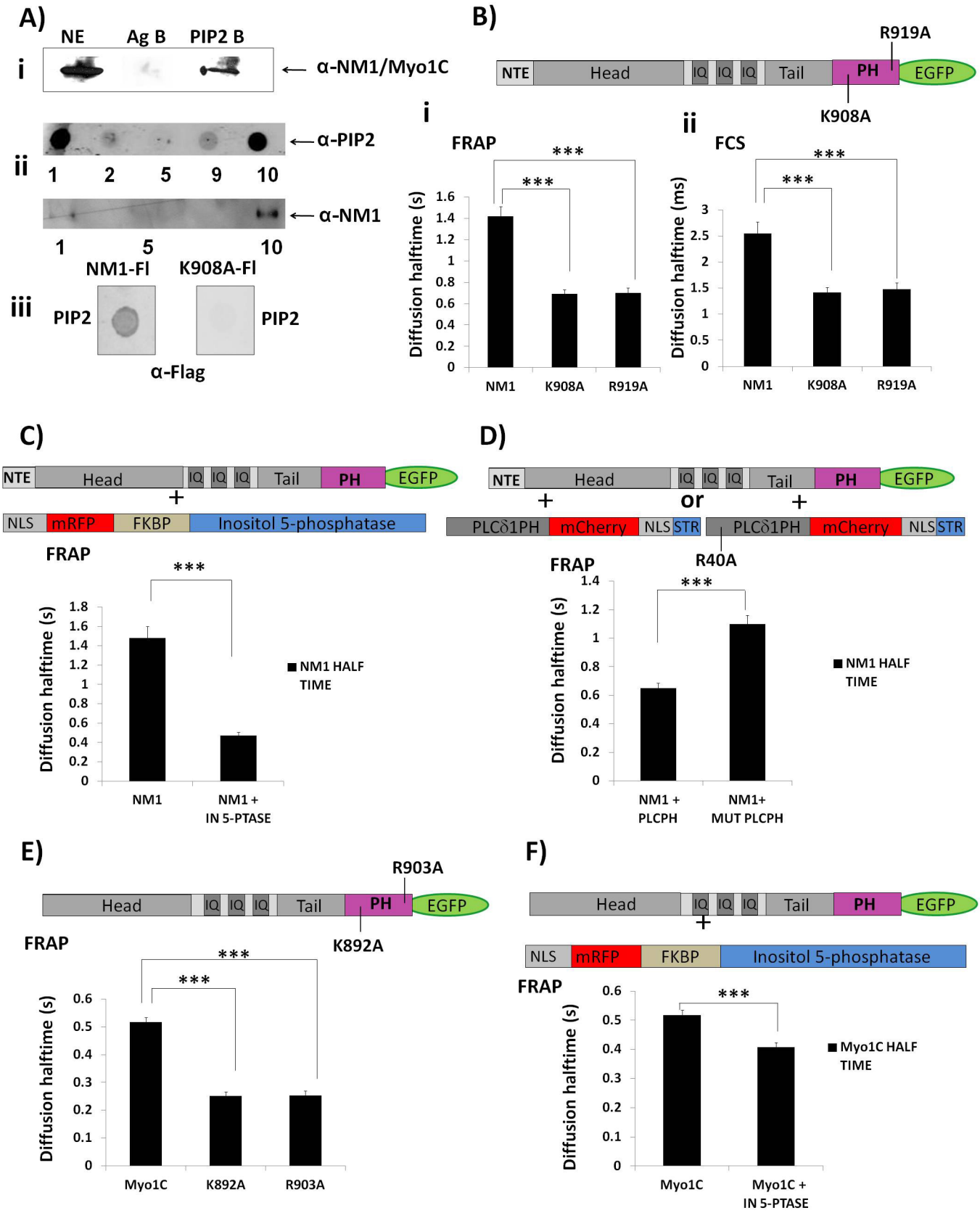
Figure 1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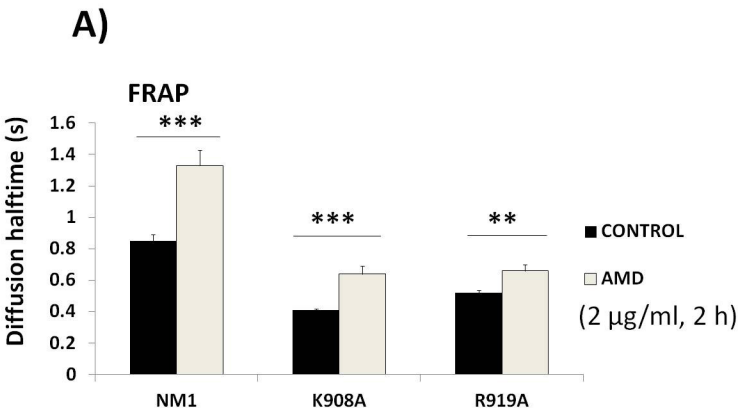
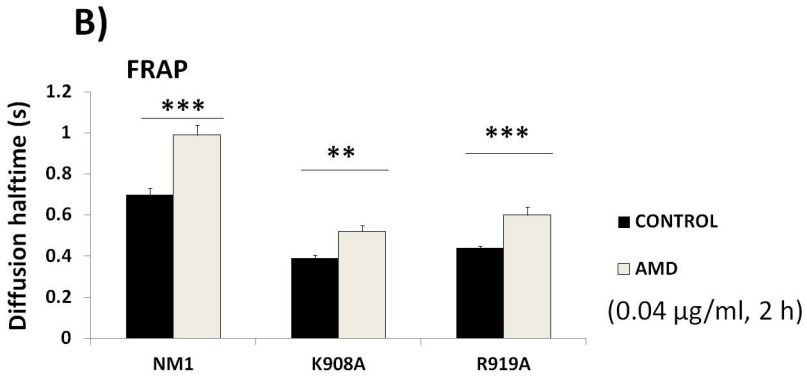
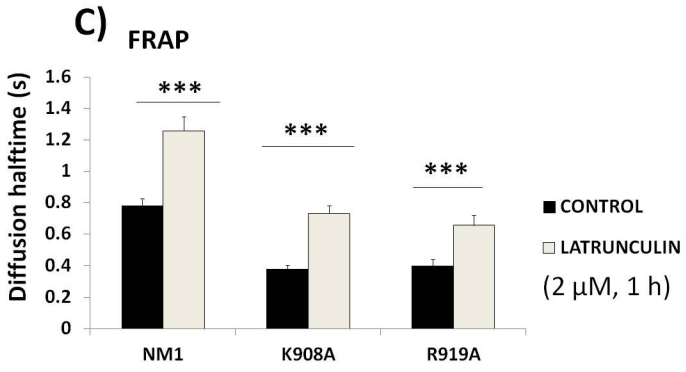
NE
Ag B
PIP2 B
i
α-NM1/Myo1C
α-PIP2
ii
1 2 5 9 10
α-NM1
1 5 10
NM1-Fl
K908A-Fl
iii
PIP2
PIP2
α-Flag
B)
R919A
NTE Head IQ IQ IQ Tail PH EGFP
K908A
i
FRAP
Diffusion halftime (s)
NM1 K908A R919A
ii
FCS
Diffusion halftime (ms)
NM1 K908A R919A
C)
NTE Head IQ IQ IQ Tail PH EGFP
+
NLS mRFP FKBP Inositol 5-phosphatase
FRAP
Diffusion halftime (s)
NM1 HALF TIME
NM1
NM1 + IN 5-PTASE
D)
NTE Head IQ IQ IQ Tail PH EGFP
+ or +
PLCδ1PH mCherry NLS STR
PLCδ1PH mCherry NLS STR
R40A
FRAP
Diffusion halftime (s)
NM1 HALF TIME
NM1 + PLCPH
NM1+ MUT PLCPH
E)
R903A
Head IQ IQ IQ Tail PH EGFP
K892A
FRAP
Diffusion halftime (s)
Myo1C K892A R903A
F)
Head IQ IQ IQ Tail PH EGFP
+
NLS mRFP FKBP Inositol 5-phosphatase
FRAP
Diffusion halftime (s)
Myo1C HALF TIME
Myo1C
Myo1C + IN 5-PTASE

Figure 2


A)
FRAP
Diffusion halftime (s)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
**
NM1
K908A
R919A
CONTROL
AMD
(2 µg/ml, 2 h)


B)
FRAP
Diffusion halftime (s)
1.2
1
0.8
0.6
0.4
0.2
0
***
**
***
NM1
K908A
R919A
CONTROL
AMD
(0.04 µg/ml, 2 h)


C)
FRAP
Diffusion halftime (s)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
NM1
K908A
R919A
CONTROL
LATRUNCULIN
(2 µM, 1 h)

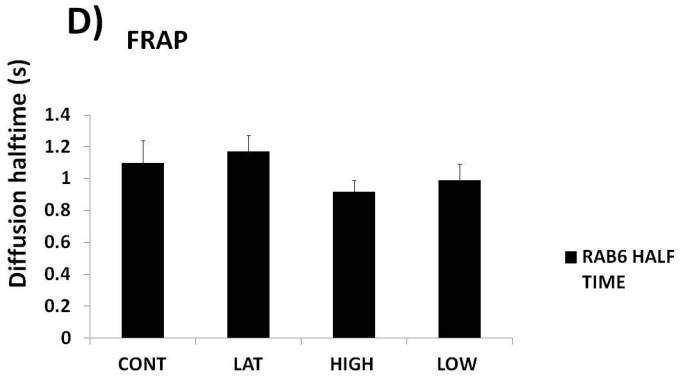
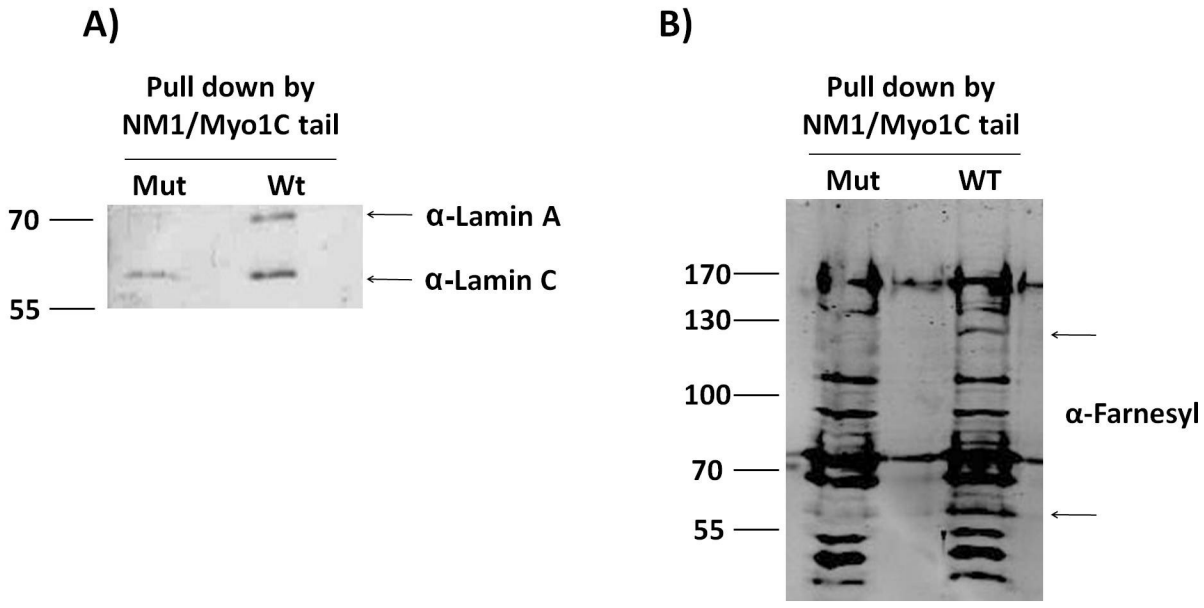

D)
FRAP
Diffusion halftime (s)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONT
LAT
HIGH
LOW
RAB6 HALF TIME

Figure 3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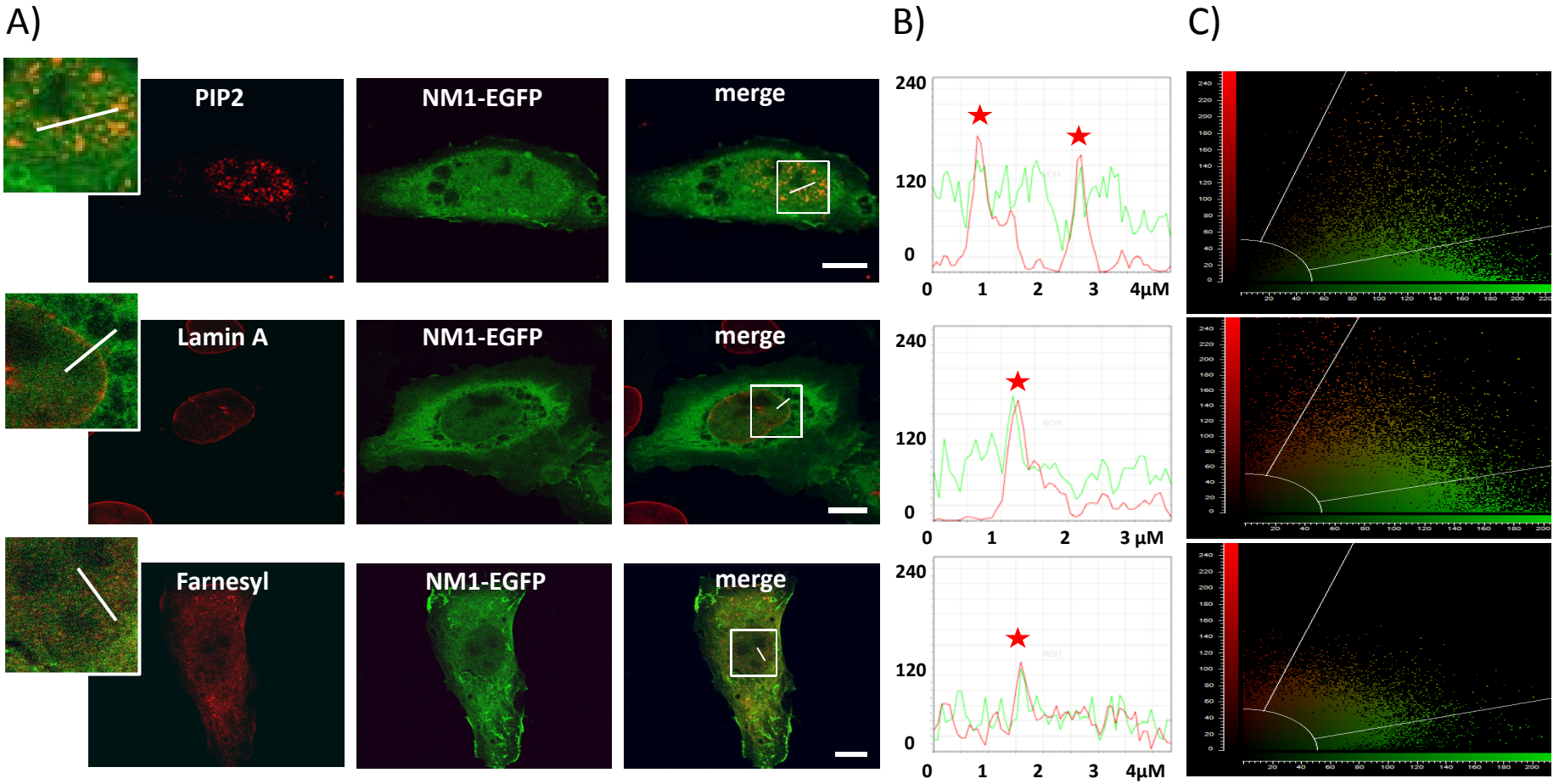
Pull down by
NM1/Myo1C tail
Mut
Wt
70
55
α-Lamin A
α-Lamin C
B)
Pull down by
NM1/Myo1C tail
Mut
WT
170
130
100
70
55
α-Farnesyl

Figure 4


A)
B)
C)
PIP2
NM1-EGFP
merge
Lamin A
NM1-EGFP
merge
Farnesyl
NM1-EGFP
merge
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120
0
0 1 2 3 4µM
240
120
0
0 1 2 3 µM
240
120
0
0 1 2 3 4µM

Figure 5

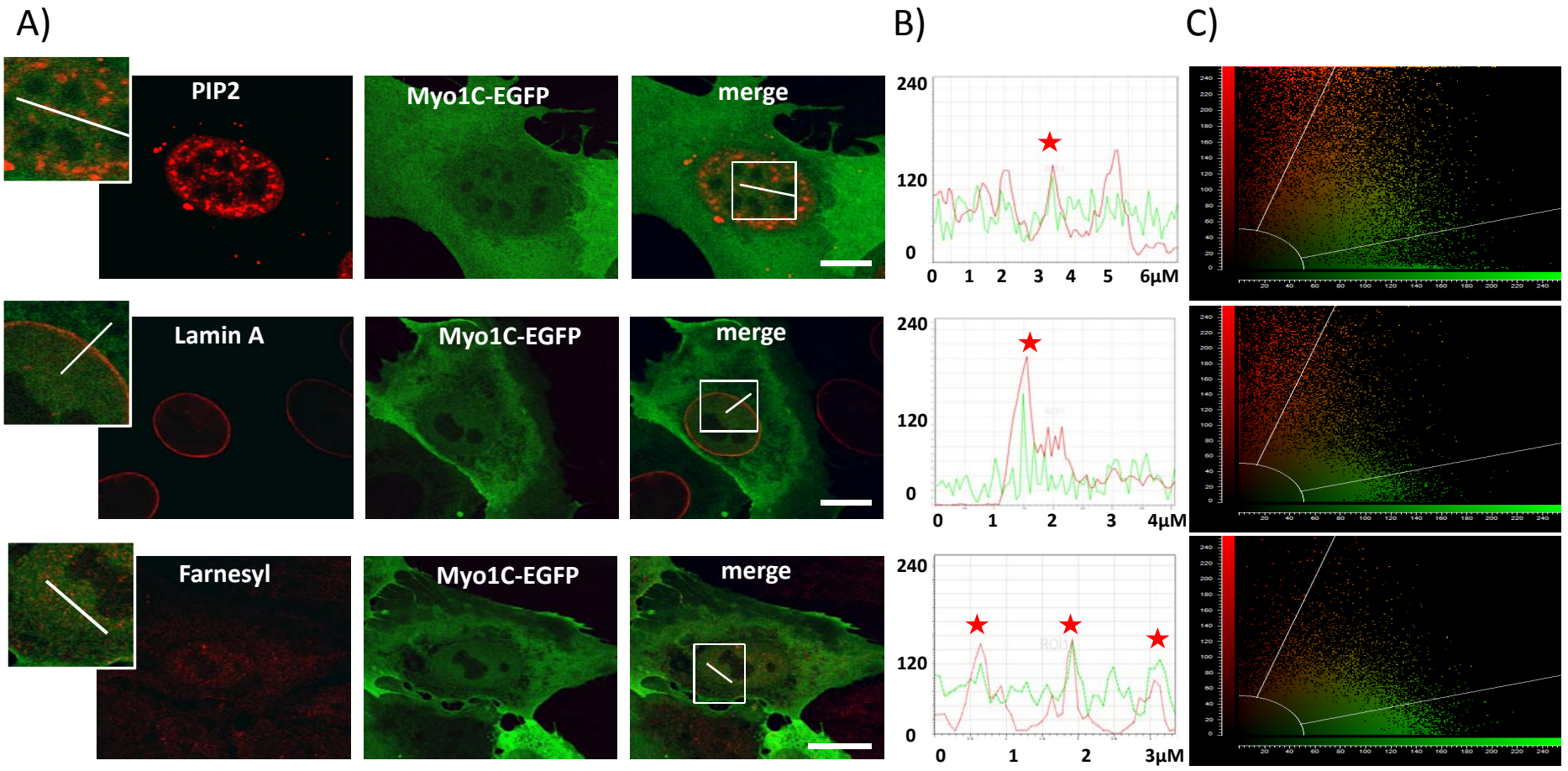
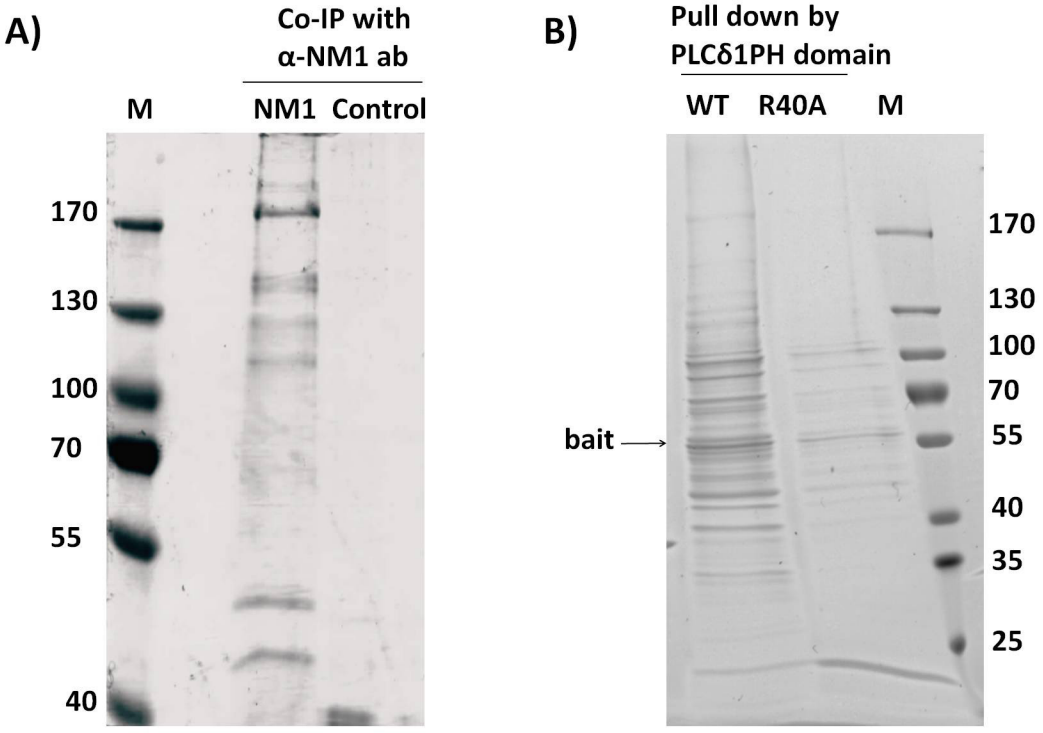

A)
B)
C)
PIP2
Myo1C-EGFP
merge
Lamin A
Myo1C-EGFP
merge
Farnesyl
Myo1C-EGFP
merge
240
120
0
0 1 2 3 4 5 6µM
240
120
0
0 1 2 3 4µM
240
120
0
0 1 2 3µM

Figure 6


A)
Co-IP with
α-NM1 ab
M
NM1
Control
170
130
100
70
55
40
B)
Pull down by
PLCδ1PH domain
WT
R40A
M
bait
170
130
100
70
55
40
35
25

Involvement of PIP2 in RNA Polymerase I transcription

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Running title: PIP2 in RNA Pol I transcription

Keywords: Nucleolus, transcription, PIP2, UBF, fibrillarin

Word count: 8199

Summary

RNA polymerase I (Pol I) transcription is essential for the cell cycle, growth, and overall protein synthesis in eukaryotes. We found that phosphatidylinositol 4,5-bisphosphate (PIP2) is a part of the protein complex on the active ribosomal promoter during the transcription. PIP2 makes a complex with Pol I and Pol I transcription factor UBF in the nucleolus. PIP2 depletion reduces Pol I transcription which can be rescued by the addition of exogenous PIP2. In addition, PIP2 also binds directly to the pre-rRNA processing factor, fibrillarin (Fib), and co-localizes with nascent transcripts in the nucleolus. PIP2 binding to UBF and Fib modulates their binding to DNA and RNA, respectively. In conclusion, PIP2 interacts with a subset of Pol I transcription machinery, and promotes Pol I transcription.

Introduction

The eukaryotic nucleus is a highly structured organelle composed mainly of proteins and nucleic acids. However, in addition to these abundant molecules, the nuclear interior was also shown to contain minor components such as lipids (Rose and Frenster, 1965). Several biochemical studies (Boronenkov et al., 1998; Cocco et al., 1987; Divecha et al., 1991; Vann et al., 1997) have shown that purified nuclei contain enzymes involved in the production and degradation of phosphoininositides (PI). These studies also suggested that PIs could be divided into physically separate pools within the nucleus. Initial experiments with the antibody against PIP2 and the pleckstrin homology domain of phospholipase C δ1 (PLCδ1PH) as probes (Osborne et al., 2001; Watt et al., 2002) revealed the presence of their cognate lipid in distinct nuclear compartments such as the interchromatin granule clusters and the nucleolus. While there have been no reports on the function of nucleolar PIP2, a number of studies have shown PIP2 function in nuclear speckles (Boronenkov et al., 1998; Mellman et al., 2008; Osborne et al., 2001). Phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha and a non-canonical poly (A) polymerase, called Star-PAP, were detected in the nuclear speckles where 3'-end processing of select mRNAs by Star-PAP was stimulated by PIP2 (Mellman et al., 2008). PIP2 immunodepletion from HeLa nuclear extracts caused inhibition of precursor mRNA splicing due to the loss of PIP2 and its binding partners (Osborne et al., 2001). Moreover, apart from mRNA processing, mRNA export was also shown to be regulated by PIP2 in speckles. PIP2 binding to mRNA export protein Aly directed the protein to nuclear speckles, while the disruption of PIP2 binding caused a reduction in its mRNA export activity (Okada et al., 2008). A few studies showed the involvement of PIP2 in RNA polymerase II (Pol II) transcription. In *Drosophila*, PIP2 binding to histone H1 reversed the inhibitory effect of histone H1 on transcription (Yu et al., 1998). Similarly, a Drosophila trithorax group protein, ASH2, binding to nuclear phosphatidylinositol 4-phosphate 5-kinase, called SKTL, was shown to be involved in maintaining transcriptionally active chromatin via reducing histone H1 hyperphosphorylation (Cheng and Shearn, 2004). In mammalian cells, PIP2 was found to bind only the hyperphosphorylated active form of Pol II (Pol IIO), implying its role in Pol II transcription (Osborne et al., 2001). PIP2 was required for activation of SWI-SNF like BAF complex binding to nuclear matrix/chromatin in actin-dependent manner (Zhao et al., 1998). PIP2 also facilitated

the synthesis of filamentous actin in the nucleus by interfering with BRG1-actin binding at the C-terminus of BRG1 (Rando et al., 2002). Even though PIP2 presence was shown in the nucleolus (Mortier et al., 2005; Osborne et al., 2001), nucleolar PIP2 function has not been yet investigated. The nucleolus is composed of well-defined subdomains such as fibrillar centers (FCs), a dense fibrillar component (DFC), and a granular component (GC). Transcription of pre-ribosomal genes takes place at the FC/DFC border while ribosomal subunits assemble in the GC (Nemeth and Langst, 2011). Nucleoli are formed around nucleolar organizer regions (NORs), which contain tandem rRNA gene repeats. Each ribosomal gene unit usually consists of a transcribed sequence and an external non-transcribed spacer (Liau and Perry, 1969). Even though 400 copies of rDNA exist in diploid somatic cells, only a small fraction is transcribed and the transcription is driven by Pol I upon binding to UBF together with the SL1 complex at the enhancer region of rDNA. UBF binds to rDNA not only at the promoter, but also in the transcribed region, and it is involved in the formation of open chromatin structures at actively transcribed genes (Denissov et al., 2011). Since UBF recruitment to UBF-binding site arrays outside the nucleolus forms pseudo-NORs (Mais et al., 2005), it is suggested that UBF is involved in structural organization of rDNA for the assembly of FC and DFC regions.

Upon transcription initiation, rRNA transcripts proceed through several maturation stages before ribosomal subunit assembly (Mayer and Grummt, 2006). In the early stages of rRNA processing, Fib, being a component of ribonucleoprotein complex called box C/D small nucleolar RNP, binds to precursor rRNA in the DFC region and functions in site-specific 2'-*O*-methylation of rRNA (Hernandez-Verdun, 1991). Mutations affecting Pol I elongation also have an effect on precursor rRNA cleavage by the Spt4-Spt5 complex in yeast linking both machineries (Anderson et al., 2011; Schneider et al., 2006; Schneider et al., 2007). Fib is recruited to nucleoli upon transcription initiation in telophase, and its presence at this point of the cell cycle was shown to be essential for cell survival (Dundr et al., 1997; Kopp et al., 2007).

Here we document that PIP2 interacts directly or indirectly with Pol I in the nucleolus. We also show that direct binding of PIP2 to UBF and Fib may change their respective conformation and thus the ability to bind nucleic acids. Moreover, nascent rRNAs co-localize with PIP2 in vivo, and in vitro ribosomal gene transcription is compromised when PIP2 is depleted. Addition of exogenous PIP2 can rescue the transcription inhibition while exogenous IP3 [Ins (1,4,5)P3] and

DAG have no effect. Pre-incubation with anti-PIP2 antibodies and PIP2 depletion by addition of PLC before transcription initiation abolishes nucleolar transcription. These results indicate that PIP2 might be involved in Pol I transcription by interacting with pre-rRNA production and processing machineries.

Results

PIP2 is required for optimal Pol I transcription in vitro

Based on existing evidence suggesting involvement of PIP2 in Pol II transcription (Yu et al., 1998), we investigated PIP2 influence on Pol I transcription using several strategies for in vitro transcription assays. When anti-PIP2 antibody was added to in vitro transcription assay, the level of Pol I transcription was reduced by more than 80% (Fig. 1A, lane 2). On the other hand, anti-histone H3 antibody, which was used as a control antibody at similar concentration, had a minor inhibitory effect on transcription (Fig. 1A, lane 3). It was possible to neutralize the inhibitory effect of anti-PIP2 antibody in transcription by pre-blocking the antibody with PIP2 before its addition to the transcription reaction in a dose-dependent manner (Fig. S1). We then tested if degradation of existing nuclear PIP2 by phospholipase C (PLC) enzyme has an effect on transcription. Indeed, the addition of purified PLC to the nuclear extract prior to transcription initiation (before the addition of nucleotides) caused almost 60% inhibition in transcription, while the addition of the PLC at the time of transcription initiation (after the addition of nucleotides) showed no significant effect as seen in Fig. 1B. To further test the effect of PIP2 on transcription we compared Pol I transcription levels using nuclear extracts in which PIP2 was depleted using GST-tagged PLCδ1PH domain. The PLCδ1PH domain binds to the PIP2 head group with a high affinity and a single basic amino acid replacement in the N-terminal part of the domain (R40A) results in the abolishment of PIP2 binding (Yagisawa et al., 1998). When PLCδ1PH domain was added to the nuclear extract, Pol I transcription was reduced significantly as compared to nuclear extract where PIP2-binding mutant PLCδ1PH domain was added (Fig. 1C). PIP2 cleavage by nuclear PLCs results in the production of second messengers (IP3 and DAG) in the nucleus (for review see Irvine, 2003). In order to confirm that PIP2 or products of PIP2 cleavage are the executive molecules in Pol I transcription modulation, PIP2, IP3 or DAG were added into PIP2

depleted extract and used for in vitro transcription assays. While IP3 and DAG addition showed no effect on transcription, PIP2 addition significantly restored (~50%) Pol I transcription in PIP2-depleted extracts However, PI3P or PI4P addition resulted in only 15% increase in transcription (Fig. 1D). These results clearly show that PIP2 acts in Pol I transcription directly, but not as a source for second messengers. The PIP2 presence on the promoter along with the transcription machinery was checked at different stages of transcription using the rDNA promoter bound to magnetic beads. Addition of GST-tagged PLCδ1PH domain to the transcription reaction showed the presence of PIP2 at the transcription machinery on the promoter when successful transcription was achieved by the addition of all four rNTPs (N) as shown in Fig. 1E, lane 9. Alternatively, when only ATP (A) was added to the transcription mixtures during transcription preinitiation to promote the phosphorylation required before the transcription, there was no detectable PIP2 level on the promoter as seen in Fig. 1E, lane 6. When PIP2-binding mutant PLCδ1PH domain was added to the transcription reaction, there was no staining with anti-GST antibody, indicating the inability of mutant domain to bind to PIP2 (Fig. 1E, lanes 7 and 10). The amounts of Fib, BRG1, and TBP on the promoter were approximately 2x higher during transcription compared to preinitiation reactions. The presence of PIP2 in transcription complexes on the promoter region was also directly shown in TLC after lipid extraction (Fig. 1F). In summary, this is the first report showing that PIP2 is a part of Pol I transcription machinery on the promoter and promotes Pol I transcription in vitro.

PIP2 participates in the formation of Pol I transcription foci

PIP2 presence in the nucleolus has been previously shown by Osborne et al. (2001) and Mortier et al. (2005), however, there has been no report on the interacting partners and functions of nucleolar PIP2. Since PIP2 was found to be a part of the transcription machinery on the promoter during rDNA transcription, we continued to identify the components of Pol I transcription machinery that interact with PIP2. For this purpose, GST-tagged PLCδ1PH domain and its PIP2-binding mutant were added to the nuclear extract and pulled down by glutathione beads. Since wild type PLCδ1PH domain can bind to PIP2, but not the mutant form, proteins pulled down by only wild type PLCδ1PH domain were considered as interacting partners of PIP2. As a second approach, PIP2-coupled agarose beads and control agarose beads were used for pull-down experiments. For microscopy studies, anti-PIP2 antibody or GST-tagged PLCδ1PH domain and

anti-GST antibody were used. The use of recombinant PLCδ1PH domain as a PIP2 probe provided a reliable and consistent staining of PIP2 on the plasma membrane (Fig. 2A) and in the nucleus and nucleolus (Fig. 2B), while the mutant PLCδ1PH domain which does not bind to PIP2 did not provide a signal with immunofluorescent staining (Fig. 2B), thus proving the specificity of PLCδ1PH domain binding.

Pull-downs with PLCδ1PH domain showed that PIP2 and the largest subunit of Pol I (RPA116) are present in the same complex (Fig. 2C and Fig. S2). In agreement, the immunofluorescence detection in U2OS cells showed PIP2 in concrete foci in the nucleolus together with Pol I (Fig. 2D). The co-localization of PIP2 and Pol I is documented by the intensity profile showing that fluorescence maxima of both proteins along the line crossing nucleolus clearly coincide (Fig. 2D). On the other hand, TBP and TAF 95/110 which are also members of transcription initiation machinery were not detected in the protein complex along with PIP2 (Fig. 2E and Fig. S3), indicating that PIP2 interacts with a subset of proteins of Pol I transcription machinery. This selective composition of PIP2-bound protein complex is also reflected in PIP2 localization in the nucleolus. Fib is localized to DFC where transcription takes place while B23 is localized in GC where ribosomal subunits assemble (Nemeth and Langst, 2011). Since Fib is present in PIP2-protein complex and B23 is absent (Fig. 2E), we suggest that the restricted PIP2 localization to the transcriptionally active sites of the nucleolus might be dictated by the binding partners of PIP2.

UBF and Fib are both essential components of rRNA biogenesis during Pol I transcription initiation and early steps of rRNA maturation, respectively. We pulled both of them down from nucleolar extracts via PIP2-coupled agarose beads (Fig. 3A and Fig. S4). After cheking the specificity of the antibodies (Fig. S5), co-localization studies of PIP2 with UBF and Fib were performed. PLCδ1PH domain showed a prominent co-localization of PIP2 with UBF and Fib in the nucleoli of interphase cells as documented by the corresponding intensity profiles (Fig. 3B). In addition, superresolution structured illumination microscopy (SIM) allowed us to demonstrate PIP2 co-localization with UBF and Fib in subnucleolar, due to its higher resolution as compared to confocal microscopy (Fig. 3C). In order to improve the resolution even further, immunoelectron microscopy (IEM) revealed PIP2 co-localization with UBF in the inner space of FCs where proteins involved in rDNA transcription reside, and on the border between FC and

DFC where rDNA transcription takes place. PIP2-Fib co-localization was detected in the DFC region (Fig. 3D). To reveal the fine details of nucleolar compartmentalization in terms of PIP2 localization, 3D electron tomography was used, which demonstrated that PIP2-containing structures are found between individual FCs through the DFC and stretch out to the nucleoplasm as seen in Fig. 3E and in Movie 1 in supplementary material. These results support the data showing PIP2 involvement in rRNA biogenesis. In accordance with our previous observation showing the absence of B23 in PIP2 pull-down (Fig. 2E), PIP2 was not detected in GC, suggesting that PIP2 is not involved in the late maturation of pre-ribosome particles.

Even though proteins involved in ribosomal gene transcription and rRNA processing were shown to be interacting with PIP2, it was not clear if these interactions were direct. We therefore probed for direct interactions of PIP2 with recombinant UBF and Fib (Fig. 4A) on nitrocellulose membranes where PIP2 and PI4P were spotted. The results clearly showed direct binding of UBF and Fib to PIP2, but not to PI4P (Fig. 4B). As a control, we used recombinant OSH1PH domain (Fig. 4A), which binds to PI4P with high affinity (Roy and Levine, 2004), and importin 5 (Imp 5, Fig. 4A). OSH1PH was found to bind to PI4P with greater affinity than to PIP2, while Imp 5 did not show any binding to either PI4P or PIP2 (Fig. 4B). To clearly show direct protein-lipid interaction, we performed pull-down experiments with the recombinant proteins (UBF, Fib and Imp 5) using PIP2-coupled agarose beads and control agarose beads. Recombinant UBF and Fib were pulled down by PIP2, while Imp 5 failed to bind to PIP2 (Fig. 4C, Fig. S6-1, Fig. S6-2 and Fig. S6-3). In addition, trypsin digestion of UBF and Fib showed that PIP2 binding blocked a particular region of UBF for trypsin accessibility (Fig. 4D), while PIP2 binding to Fib resulted in higher accessibility for trypsin (Fig. 4E). These changes in digestion patterns point to the alteration of conformation or protection at certain sites of UBF and Fib due to binding to PIP2.

In order to understand the effect of PIP2 binding on UBF-rDNA interaction, footprinting experiment was carried out by incubating purified recombinant UBF with PIP2, IP3 or DAG. Upon PIP2 binding, the overall binding of UBF to rDNA was reduced and a more selective footprint to the UBF binding sequence was observed (Fig. 4F, lane 4) compared to DAG (Fig. 4F, lane 2) or IP3 (Fig. 4F, lane 3). Normalized densitometric profiles of the footprint (left panel) show tighter binding of UBF with PIP2 at the UBF footprint sequence as compared to all the other conditions (Fig. 4F).

The influence of PIP2 on the binding of Fib to U6 small nuclear RNAs (snRNAs) was further investigated in gel shift mobility assays (Fig. 4G and Fig. S7). The mobility of the Fib/U6 snRNA complex was altered in the gel during electrophoresis upon addition of PIP2, thus suggesting changes in RNA topology or other alterations resulting from PIP2 binding to Fib (Fig. 4G). Densitometric profile of the gel shift assay (below) clearly shows the increase in mobility of U6 snRNA/Fib complex upon binding to PIP2 as shown by the arrows at the peaks in the density profile (Fig. 4G).

PIP2 co-localizes with rRNA nascent transcripts in nucleoli

To assess the presence of PIP2 during early steps of rDNA transcription, we visualized nascent rRNA transcripts by short pulse incorporation of BrUTP in permeabilized cells. A clear co-localization between PIP2 and Br-rRNA was observed by immunofluorescence (Fig. 5A) and IEM (Fig. 5B). The labeling of nascent rRNA and PIP2 showed that both localize at the border between FC and DFC and in the DFC. PIP2 and Br-rRNA clusters were intermingled; most of the transcription signals were associated with PIP2 signal. However, there were also zones in DFC where only PIP2 labeling was present (Fig. 5B). These results demonstrate the presence of PIP2 at the sites of nucleolar transcription in situ and, in parallel with the in vitro data, show the importance of PIP2 for rDNA transcription and possibly nucleolar compartmentalization.

Discussion

PIP2 is the source of the second messengers IP3 for intracellular Ca^{2+} mobilization and DAG for protein kinase C activation. In addition to the role of PIP2 in cytoplasmic signal transduction, the presence of its biosynthetic machinery inside the nucleus indicates a distinct nuclear signaling pathway (Irvine, 2003). The presence of phospholipids in the nucleus was shown more than 70 years ago (Stoneburg, 1939), however, little is known about their physico-chemical properties and functions in the nucleus. Since there are no membranous structures inside of the nucleus, it is suggested that proteins with hydrophobic pockets bind to PIPs to protect them from the hydrophilic environment (for review see Irvine, 2003). Here we demonstrate that PIP2 binds to some of the principal components of the Pol I transcription machinery and is anchored via these interactions in the fibrillar regions of the nucleolus where rDNA transcription occurs.

Over the years, only few studies reported the involvement of PIP2 in Pol II transcription. PIP2 addition was shown to promote Pol II transcription (Yu et al., 1998) and the binding of chromatin remodeling complex to DNA (Zhao et al., 1998). However, there have been no reports on the modulation of Pol I transcription by PIP2. Therefore, we first tested if PIP2 is required for Pol I transcription. Pol I transcription inhibition by the addition of either anti-PIP2 antibody or PLC enzyme indicated the involvement of PIP2 in Pol I transcription. We also observed a reduction in Pol I transcription upon depletion of PIP2, which was reversed by the addition of exogenous PIP2 to the depleted extract, but not by the addition of IP3 and DAG. Taken together, our data suggest that PIP2 acts as itself in Pol I transcription rather than as a substrate of nuclear PI-PLC, since neither IP3 nor DAG rescue the inhibitory effect of PIP2 depletion in Pol I transcription.

The presence of PIP2 on the Pol II promoter was recently shown by Toska et al. (2012). BASP1 binding to PIP2 was shown to be required for the interaction with HDAC1 which resulted in the recruitment of HDAC1 to the promoter of WT1-targeted genes to repress transcription of Pol II (Toska et al. 2012). Here we report for the first time the presence of PIP2 in the transcription complex on the promoter during Pol I transcription. Pol I, UBF and Fib were detected in PIP2-bound protein complex while TBP and TAF 95/110 were not. This result indicates that instead of binding to the whole transcription machinery, PIP2 selectively binds to a subset of proteins involved in transcription. We found that PIP2 binds directly to UBF and they co-localize in FC region. UBF is a scaffold protein that binds to rDNA promoter and bends it to establish proper DNA-protein structure (Stefanovsky et al. 2001). According to the model proposed by Denissov et al. (2011), together with other components of SL1 complex, UBF creates a core-helix DNA structure where the transcribed regions are cylindrically wrapped around. Pol I initiates the transcription in the core and elongates along the cylindrical helix (Denissov et al., 2011). In addition, UBF is required for the formation of secondary constrictions of NORs (Mais et al., 2005). Since PIP2 binding to UBF results in a somewhat tighter binding to rDNA promoter, it is plausible that the interaction between PIP2 and UBF has a regulatory role in the formation of transcription initiation complex at the rDNA promoter.

Fib is known to have a role in nucleolar assembly by gathering prenuclear bodies together (Fomproix et al., 1998). Direct binding of PIP2 to Fib caused an increase in the mobility of U6 snRNA/Fib complex, but did not significantly affect the amount of bound complex under the

assay conditions. The change in mobility may arise from the altered physico-chemical properties of the complex due to PIP2 addition, or from conformational changes in Fib as suggested by the trypsin digestion in Fig. 4E. Similarly, it was reported that conformational opening of ezrin upon binding to PIP2 results in more extensive contacts with F-actin (Jayasundar et al., 2012). Since PIP2 localization is restricted to the transcriptionally active regions in the nucleolus, we suggest that a particular hydrophobic protein-lipid-RNA enviroment might exist in that particular region and contribute to the detergent-resistant nuclear PIP2 pools, which were shown to make up to 40% of the total PIP2 mass (Vann et al., 1997). The morphology of nuclear speckles was shown to be dependent on PIP2 binding to PDZ domain of syntenin-1 (Mortier et al., 2005). Therefore, it is likely that PIP2 also contributes to the formation of transcriptionally active sites of the nucleolus and acts as a structural interface between the nucleolar skeletal elements (like FC) and the macromolecular complexes involved in rDNA transcription and in early rRNA processing (FC/DFC interphase and DFC).

Our observation that nascent rRNA co-localizes with PIP2 contributes to a conclusion that PIP2 is associated with transcription machinery on active rDNA and has a role in Pol I transcription as well as in early stages of rRNA maturation. RNA was shown to be required for the localization of PIP2 to nuclear speckles (Osborne et al., 2001), and therefore it was suggested that the interaction with RNA/protein complex might stabilize PIP2 in the absence of intranuclear membranous structures. Similarly, PIP2 might be tethered within the nucleolus by the interaction with nascent rRNA transcripts and Fib, bridging the processes of nascent transcript production and early processing. To our knowledge, this is the first report on PIP2 involvement in Pol I transcription and rRNA processing.

All these findings led us to suggest a model in which PIP2 modulates UBF binding to rDNA, and Fib binding to rRNA. Upon active transcription Fib binds to PIP2 and associates with nascent rRNA in a Fib complex to enforce methylation for further rRNA processing. PIP2 may act as a bridge between Pol I, UBF and Fib to connect transcription initiation and early maturation steps. After the initial methylation, the Fib complex may release the rRNA to be further processed in the GC region (Fig. 6). The link between RNA synthesis and maturation may dictate nucleolar structures in the FC and DFC regions, where PIP2 may form a framework which allows gathering of proteins to work in concert for efficient transcription of ribosomal RNA genes.

Materials and methods

Cell culture

Human osteosarcoma (U2OS) cells and cervical carcinoma (HeLa) cells were kept in DMEM with 10% fetal calf serum in 5% CO_2/air, 37°C, humidified atmosphere.

Plasmids

GST-tagged PLCδ1PH (1-140) (pGST3) and PLCδ1PH-Mut (R40A), which lacks binding to PIP2, were provided by Dr. Hitoshi Yagisawa (Yagisawa et al., 1998). pECHU plasmid was constructed by PCR of the human rDNA promoter (-514 to +20) from prHU3 with EcoRI-XhoI site into pEC111/80 (Castano et al., 2000) after the HIV LTR promoter removal. prHU3 was a kind gift from Dr. Lucio Comai. UBF1 (gift from Dr. Sui Huang) and Fib were cloned into pET15b vector for protein purification studies. In order to produce recombinant protein, OSH1PH domain (gift from Dr. Tamas Balla) was cloned into pET42a(+) vector. Imp 5 in pQE30 vector was received from Dr. Dirk Görlich (Jäkel and Görlich, 1998).

Expression and purification of recombinant proteins

Recombinant Fib, UBF, PLCδ1PH and PLCδ1PH-Mut were expressed in *Escherichia coli (E. coli)* BL21 (DE3)-pLysS (Stratagene, La Jolla, CA, USA). Both UBF and Fib had histidine tags and were purified over Ni-agarose in a buffer (20mM Tris pH 8, 0.1 mM EDTA, 20% glycerol, 500 mM NaCl, 0.1% NP40, 1 mM DTT). UBF was further purified by passing through Q sepharose fast flow column (17-0510-10, GE Healthcare, Uppsala, Sweden) followed by SP sepharose fast flow column (17-0729-10, GE Healthcare, Uppsala, Sweden). For GST-tagged PLCδ1PH and PLCδ1PH-Mut, the purification was carried on glutathione-agarose column (G4510, Sigma Aldrich, St. Louis, MO, USA), which had been equilibrated with BC100 (20 mM Tris pH 8, 0.1 mM EDTA, 20% glycerol, 100 mM NaCl). Proteins were eluted with 50 mM Tris-HCL, pH 8 having 0.1g reduced L-Glutathione (G4251, Sigma Aldrich, St. Louis, MO, USA).

11 mg of partially purified PLC from *Clostridium perfrigens* (*C. welchii*) (P7633-125 UN, Sigma Aldrich, St. Louis, MO, USA) was further purified by passing through SP sepharose fast flow column followed by Q sepharose fast flow column. Proteins were eluted in a KCl gradient at 500 mM.

Recombinant OSH1PH domain was expressed in Rosetta™(DE3)pLysS competent cells (70956, Millipore EMD, Billerica, MA, USA) and purified over glutathione-agarose column followed by Ni-agarose column. Elution from Ni-agarose column was performed in a buffer consisted of 25 mM HEPES, pH 8, 100 mM NaCl and 250 mM imidazole.

Recombinant Imp 5 was expressed in Qiagen Rep4 strain of *E. coli* and purified over Ni-agarose column as described previously (Kutay et al., 1997).

Confocal microscopy

Primary antibodies: Anti-GST antibody (gift from Dr. Igor Shevelev; 5 µg/ml), anti-PIP2 antibody (2C11, Abcam, Cambridge, UK; 16 µg/ml), anti-Fib antibody (38F3, Abcam, Cambridge, UK; dilution 1:100), anti-UBF antibody (sc13125, Santa Cruz Biotechnology, Inc, CA, USA; 2 µg/ml), anti-RPA116 antibody (gift from Dr. Ingrid Grummt; 2µg/ml). Secondary antibodies: Donkey anti-mouse IgG conjugated with Alexa 488 (Invitrogen, CA, USA), goat anti-rabbit IgG conjugated with Alexa 647 (Invitrogen, CA, USA) and donkey anti-mouse IgM conjugated with Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Images were taken using a confocal microscope (Leica TCS SP5 AOBS TANDEM, Germany) with 100x (NA 1.4) oil immersion objective lense. In order to test the specificity of the primary antibodies, anti-UBF, anti-Fib and anti-PIP2 antibodies were preblocked with an excess amount of purified UBF, Fib and PIP2, respectively. Preblocking incubations were performed in PBS with 1% BSA. After 30 min incubation, immunostaining was performed with these preblocked primary antibodies.

IEM

Primary antibodies used in IEM were already described in confocal microscopy section. Secondary antibodies: Goat anti-mouse IgG (H+L chains) antibody coupled with 6 nm colloidal gold particles, goat anti-mouse IgM (µ-chain specific) antibody coupled with 12 nm colloidal gold particles, goat anti-rabbit IgG (H+L chains) antibody coupled with either 6 nm or 12 nm colloidal gold particles (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The thin sections (70-90 nm) were examined by a FEI Morgagni 268 transmission electron microscope at 80 kV. The images were captured with Mega View III CCD camera. Multiple sections of at least three independent immunogold labeling experiments were analyzed.

Detection of transcription sites by confocal microscopy and IEM

Bromouridine triphosphate (BrUTP; Sigma Aldrich, St. Louis, MO, USA) incorporation was performed as described previously (Pombo et al., 1999). For Br-RNA labeling anti-bromodeoxyuridine antibody IgG1 (BMC9318, Roche Diagnostics GmbH, Mannheim, Germany; 20 µg/ml) was used.

SIM

Images were taken with a superresolution structured illumination microscope (ELYRA PS.1, Carl Zeiss, Munich, Germany) with Plan-Apochromat 63x/1.4 Oil DIC M27 oil immersion objective lens using the parameters as follows: number of SIM rotations - 5; SIM grating periods varied according to the excitation wavelength from 34.0 µm to 42.0 µm

PIP strips

Purified UBF, Fib, OSH1PH domain and Imp 5 were tested on PIP Strips (Echelon Biosciences Inc, CA, USA). The membranes were blocked with 5 ml of blocking buffer (PBST, 3% BSA) for 1 h at RT. UBF and Fib were added in 5 ml blocking buffer over night at 4°C. After binding, membranes were washed 3 times with PBST and followed the same protocol as Western blots.

Limited protease digestion assay

Purified UBF (100 ng) and Fib (200 ng) were incubated for 30 min at RT with or without PIP2 (1 µg) in BC100 buffer. After the incubation, trypsin (0.5 ng) was added and the mixtures were incubated for 1 min at 4°C, as indicated. The digestion reactions were stopped by adding 10 µl Laemmli buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% bromophenol blue) and the samples were denatured at 95 °C for 5 min, electrophoresed through a 15% SDS-PAGE. Western blot was carried out as described previously.

Footprint

10 ng of radioactively end labeled PCR reactions from rDNA -514 to 100 from prHU3 were used as template for binding to purified UBF (100 ng) in the presence or absence of added compounds with the amounts mentioned in the legends for 30 min at 4°C in a buffer containing 20 mM Tris pH 7.4, 1 µg pUC 18, 5% glycerol, protease inhibitors. After binding, the reactions were digested with 1 unit of DNAse I (Fermentas, MA, USA) for 1 min at 4°C, followed by phenol/chloroform

extraction and ethanol precipitation. Purified DNA was run on a 6% PAGE with 6M urea. Footprint image was analyzed as previously published (Hofmann et al. 2011). For quantitative analysis of the footprint, ImageJ 1.42q software was used. We normalized the plot using the measurements from the non-UBF binding region of the footprint for each lane.

Gel shift

Radioactively labeled U6 snRNA (40 Kcpm, gift from Dr. Gary R. Kunkel) was used as a template for binding to purified Fib (200 ng). The reactions were incubated on ice for 30 min then loaded onto a 1% agarose gel and ran in cold room for 2 h at 40 V. Bands were visualized by autoradiography. For quantitative analysis of the gel shift, ImageJ 1.42q software was used. Photoshop was used to assign a particular color for each plot profile and compiled into one plot for easier comparison.

In vitro pull-down of PIP2

Nuclear and nucleolar lysates were prepared from HeLa cells as previously described (Andersen et al., 2002). Nuclear lysates were incubated with glutathione agarose beads (G4510, Sigma Aldrich, St. Louis, MO, USA) in the presence of GST-tagged PLCδ1PH and PLCδ1PH-Mut for 2 h or overnight at 4^{o}C and washed thoroughly with 10 mM HEPES pH 7.9, 1 mM $MgCl_2$, 0.5 mM DTT. Nuclear and nucleolar lysates were incubated with control agarose beads or agarose beads coupled with PIP2 (Echelon Biosciences Inc, CA, USA) for 2 h at 4^{o}C and thoroughly washed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DTT and protease inhibitors). Beads were boiled in Laemmli buffer for 5 min and resolved by SDS-PAGE for immunoblotting detection. In order to check direct interactions between proteins and PIP2, recombinant proteins (approximately 2 μg of recombinant protein used in each incubation) were incubated with control agarose beads or PIP2-coupled agarose beads which were previously blocked with 1% BSA in PBS. The beads were then thoroughly washed with BC100 buffer and were boiled in Laemmli buffer for 5 min and resolved by SDS-PAGE for immunoblotting detection.

In vitro transcription assays

Run-off transcription assays were performed using HeLa nuclear extracts or HeLa nuclear extracts depleted for PIP2 using PLCδ1PH coated beads and as a control PLCδ1PH-Mut coated

beads used for depletion. The reactions contained 15 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 100 μg/ml of α-amanitin, 100 mg/ml β-cyclodextrin, in the presence or absence of added compounds with the amounts mentioned in the legends. Extracts were incubated at 25°C for 20 min before initiating the transcription reaction by adding 500 μM ATP, GTP, CTP, 50 μM UTP, [α-32P] UTP (8 Ci/mmol) and 100 ng of prHU3 plasmid digested with SalI endonuclease. PLC was added before or after nucleotides as stated in the figure legend. The reactions were incubated for 1 h at 30°C and transcripts were purified by phenol extraction and ethanol precipitation. The RNA was electrophoretically resolved on a 6% acrylamide sequencing gel with 6M urea and visualized and quantified with a PhosphorImager. For quantitative measurement of each band, background was subtracted and each lane was normalized to a labeled primer, which was added as an internal loading control. The data were compared in two independent experiments. Having set the initial control condition at 100%, the plots represent the mean of each measurement and standard error of the mean for each condition.

In order to assay if PIP2 was bound to the transcription machinery on the promoter during the in vitro transcription, rDNA promoter region was produced by using biotinylated F-primer 5`CCAACGCGTTGGATGCATAGCTT 3´ and R-primer 5`ATCCTTTTTGATAATCTCATGACC3, bound to streptavidin coupled magnetic beads (Dynabeads MyOne Streptavidin C1, 650.01, Invitrogen, CA, USA) and blocked with 5% BSA. The beads were then incubated with HeLa nuclear extract in a transcription buffer without nucleotides. For PIP2 detection purified PLCδ1PH or PLCδ1PH-Mut domains were also added to the nuclear extracts. The beads were incubated with nuclear extracts for 1 h. After incubation of the beads, half of the reactions were allowed to transcribe in the presence of only ATP or all the rNTPs to distinguish between transcription initiation and elongation, respectively. Afterwards, the beads were washed six times with wash buffer and loaded onto a SDS-PAGE for Western blot analysis.

Thin layer chromatography for PIP2 in cell free system

In vitro transcription reactions were carried out using rDNA promoter region or control DNA which is a part of the vector pGEM7z+ bound to magnetic beads and blocked with 5% BSA. The beads were then incubated with nuclear extract in a transcription buffer with only ATP or all the rNTPs. After transcription, the beads were washed and lipid extractions were processed. We used

an earlier published protocol (Yu et al., 1998) to extract PIP2 from bound protein followed by loading on TLC plates that were gently soaked in 1% (w/v) potassium oxalate and dried. The solution for separation was 90:90:7:22 (CHCl3:MeOH:NH3OH:H20; v/v/v/v). TLC was then stained with acidic phosphomolibdate solution.

3D electron tomography of PIP2 in nucleolus

For 3D electron tomography, HeLa cells were grown on coverslips and fixed with 4% PFA for 15 min, permeabilized in 0.5% Triton-X-100 for 5 min, washed in PBS and blocked in the mixture of 5% BSA, 5% NGS, and 0.1% fish gelatin for 10 min. Then, cells were washed with the incubation buffer (0.1% BSA, pH 7.4) and incubated with a primary antibody overnight at +4^{o}C. After thorough washes, cells were incubated with a secondary antibody conjugated to ultra-small (0.8 nm) gold particles (Aurion, Wageningen, The Netherlands). Then, cells were washed in the incubation buffer, additionally fixed in 2% glutaraldehyde, washed in distilled water and silver-enhanced using the Aurion kit for 45 min. After washing in distilled water, cells were dehydrated in ethanol series and embedded in epon resin by standard procedure. 400-nm sections were cut with ultra microtome for single-axis electron tomography. The tilt series were acquired using TECNAI G2 20 LaB6 electron microscope (FEI, Eindhoven, The Netherlands) operated at 200 kV. The tilt series were aligned using the Inspect 3D software (FEI). Visualization was done using the Amira software (Visage Imaging GmbH, Berlin, Germany).

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Figure Legends

Fig. 1. PIP2 promotes Pol I transcription in vitro. (A) Run-off transcription reaction showed that addition of anti-PIP2 antibody (clone 2C11, Abcam, Cambridge, UK; 0.8 μg) decreases transcription levels by more than 80%. On the other hand, anti-histone H3 antibody (H0164, Sigma Aldrich, St. Louis, MO, USA; 0.5 μl) had a minor effect on Pol I transcription. (Lane 1: control transcription reaction; lane 2: transcription reaction in the presence of anti-PIP2 antibody; lane 3: transcription reaction in the presence of anti-histone H3 antibody). The charts show relative activities (mean ± SEM) normalized to an internal DNA control from two independent experiments. (B) The effect of PLC enzyme on in vitro transcription is shown. PLC (100 ng) addition before the addition of nucleotides (PLC B) inhibited Pol I transcription, while PLC addition after the addition of nucleotides (PLC A) did not inhibit transcription. (Lane 1: control transcription reaction; lane 2: transcription reaction in the presence of PLC added after nucleotides; lane 3: transcription reaction in the presence of PLC added before nucleotides). The charts show relative activities (mean ± SEM) normalized to an internal DNA control from two independent experiments (C) Nuclear extracts were depleted for PIP2 using PLCδ1PH coated beads. As a control, PLCδ1PH-Mut coated beads were used for depletion, since PLCδ1PH-Mut fails in binding to PIP2 (Yagisawa et al. 1998). Transcription intensities were normalized by 700 bp PCR product labeled with [α-^{32}P]. PIP2 depletion by PLCδ1PH domain caused 90% inhibition in transcription while mutant domain did not show such a pronounced inhibitory effect (~40% inhibition). (Lane 1: Non-depleted nuclear extract; lane 2: nuclear extract depleted with PLCδ1PH; lane 3: nuclear extract depleted with PLCδ1PH-Mut domain). The charts show relative activities (mean ± SEM) normalized to an internal DNA control from two independent experiments (D) PIP2-depleted nuclear extracts supplemented with DAG, IP3, PIP2, PI3P and PI4P were tested for Pol I transcription. PIP2 supplementation resulted in the most dramatic rescue of transcription compared to other compounds tested. (Lane 1: PIP2-depleted nuclear extract; lane 2: PIP2-depleted nuclear extract supplemented with DAG (100 ng); lane 3: PIP2-depleted nuclear extract supplemented with IP3 (100 ng); lane 4: PIP2-depleted nuclear extract supplemented with PIP2 (100 ng); lane 5: PIP2-depleted nuclear extract supplemented with PI3P (100 ng); lane 6: PIP2-depleted nuclear extract supplemented with PI4P (100 ng); lane 7: non-depleted control nuclear extract). The charts show relative activities (mean ± SEM) normalized to

an internal DNA control from two independent experiments. (E) In order to visualize PIP2 during transcription; we used rDNA promoter bound to Dynabeads. GST-tagged Wt or Mut PLCδ1PH domains were purified from bacteria, added to in vitro transcription mixtures and probed with anti-GST antibody. There was no detectable binding of Wt PLCδ1PH domain to the promoter in the presence of ATP solely, and only after the addition of all four rNTPs binding was detected; indicating that PIP2 binds to the promoter region only when transcription is active, in vitro. (Lane1: PLCδ1PH domain; lane 2: PLCδ1PH-Mut domain; lane 3 and 4: nuclear extract; lane 5: transcription reaction in the presence of ATP; lane 6: transcription reaction with ATP and PLCδ1PH domain; lane 7: transcription reaction with ATP and PLCδ1PH-Mut domain; lane 8: transcription reaction in the presence of all four rNTPs; lane 9: transcription reaction with rNTPs and PLCδ1PH domain; lane 10: transcription reaction with rNTPs and PLCδ1PH-Mut domain). (F) In order to prove the presence of PIP2 at the transcription machinery on the promoter during transcription, we used rDNA promoter bound to Dynabeads. PIP2 was found on the rDNA promoter upon the addition of all four rNTPs [N] but not on the control DNA after extracting the lipids with chloroform/methanol/HCl and analyzing them by TLC. (Lane 1: purified PIP2; lane 2 and 4: lipids from transcription reactions with all four rNTPs; lane 3 and 5: lipids from in vitro transcription reactions with only ATP added). Lipids were stained with acidic phosphomolibdate solution.

Fig. 2. PIP2 binds to the largest subunit of Pol I. In order to test the suitability of PLCδ1PH domain for PIP2 detection, we performed ultrastructural immunolabelling and immunofluorescence. (A) Immunogold electron microscopy was carried out using PLCδ1PH domain as a PIP2 sensor. PIP2 was localized at the plasma membrane of HeLa cells as expected. Scale bar represents 500 nm. (B) There is no staining in the nucleus when U2OS cells are incubated with PLCδ1PH-Mut domain as a control. Scale bar represents 5 μm. (C) PLCδ1PH domain pulled down RPA116 in vitro but Mut form of PLCδ1PH domain failed in pulling down RPA116. (Lane 1: input; lane 2: protein pulled-down with Wt PLCδ1PH domain; lane 3: protein pulled-down with Mut PLCδ1PH domain). (D) In vivo, anti-PIP2 antibody showed co-localization with Pol I subunit RPA 116 in nucleoli of U2OS cells. Scale bar represents 5μm. (E) Nuclear extract was incubated with agarose beads coupled to PIP2 in order to pull-down proteins interacting with PIP2. Pol I transcription machinery and nucleolar proteins were checked in the

pull-down and fibrillarin was found to be present, while B23, TAF 95/110 and TBP were absent in the PIP2-protein complex. (Lane 1: input; lane 2: pull-down with PIP2-coupled agarose beads; lane 3: pull-down with control agarose beads). In, input; Ctrl, control.

Fig. 3. PIP2 co-localizes with Pol I transcription factor UBF and rRNA early processing factor Fib in intact cells. (A) When nucleolar extract was incubated with PIP2-coupled agarose beads, UBF and Fib were found to be present in the PIP2-bound protein complex. (Lane 1: input; lane 2: protein unbound to PIP2-coupled agarose beads (flow-through); lane 3: protein pulled-down with PIP2-coupled agarose beads; lane 4: protein unbound to control agarose beads (flow-through); lane 5: protein pulled-down with control agarose beads). In, input; FT, flow-through; Ctrl, control. (B) PLCδ1PH domain, used as a PIP2 marker, co-localized with UBF and Fib in U2OS cells. Scale bars represent 5μm. (C) SIM revealed PIP2 co-localization with UBF and Fib in the subnucleolar components, which can be identified as FC and DFC, respectively. Scale bars represent 0.5μm. (D) IEM precisely distinguished PIP2 co-localization with UBF inside and at the periphery of FC, and with Fib in the DFC of HeLa cells. N, nucleus; NL, nucleolus. Scale bars represent 200 nm. (E) Ultrastructural architecture of PIP2 clusters in nucleolar subcompartments by TECNAI G2 20 LaB6 tomography. PIP2 is localized in HeLa cells using pre-embedding procedure with 0.8 nm immunogold particles pseudocoloured in green. Fibrillar center is pseudocoloured in yellow, dense fibrillar component in orange.

Fig. 4. UBF and Fib bind to PIP2 in vitro. (A) Western blot of purified UBF, Fib, OSH1PH and Imp 5 used in binding assays. (B) PIP2 and PI4P strips were incubated with recombinant UBF, Fib, OSH1PH and Imp 5 proteins for direct binding analysis. (C) Control agarose beads or PIP2-coupled agarose beads were incubated with purified UBF, Fib and Imp 5 proteins to study the direct binding to PIP2. (Lane 1: input; lane 2: protein unbound to PIP2-coupled agarose beads (flow-through); lane 3: protein pulled-down with PIP2-coupled agarose beads; lane 4: protein unbound to control agarose beads (flow-through); lane 5: protein pulled-down with control agarose beads). In, input; FT, flow-through; Ctrl, control. (D) Limited protease digestion assays show a difference in the digestion pattern (arrow) of UBF due to a conformational change or specific binding of PIP2. (Lane 1: UBF as an input; lane 2: UBF treated with trypsin; lane 3: UBF treated with trypsin in the presence of PIP2). (E) Limited protease digestion assays show difference in digestion pattern of Fib (arrow) due to the conformational change or specific

736 binding of PIP2. (Lane 1: Fib as an input; lane 2: Fib treated with trypsin; lane 3: Fib treated with
737 trypsin in the presence of PIP2). (F) Footprinting experiment was done using purified
738 recombinant UBF incubated with 100 ng DAG, IP3 and PIP2. (Lane 1: template incubated with
739 UBF; lane 2: template incubated with UBF in the presence of DAG; lane 3: template incubated
740 with UBF in the presence of IP3; lane 4: template incubated with UBF in the presence of PIP2;
741 lane 5: template only). UBF binding sites are indicated in the figure. Normalized densitometry
742 plot analysis of the footprint is shown on the left. (G) PIP2 binding to Fib was tested on mobility
743 assays with U6 snRNA where PIP2 association with Fib altered the mobility of RNA, suggesting
744 an additional conformational bend or loop on RNA. (Lane 1: template; lane 2: template incubated
745 with Fib; lane 3, 4, 5 and 6: template incubated with Fib in the presence of decreasing amounts of
746 PIP2 (0.1 μg, 0.05 μg, 0.025 μg, 0.0166 μg). Lane 7, 8, 9 and 10 are the duplicates of lane 3, 4, 5
747 and 6, respectively. See Fig. S7 for the entire gel pattern). Different conformations of RNA-Fib
748 complexes are reflected in the altered mobility of U6 snRNA shown by arrows. Normalized
749 densitometry plot analysis of the gel shift shows Fib complex with U6 snRNA in blue, the Fib
750 and PIP2 complex with U6 snRNA in black at different concentrations of PIP2 and U6 snRNA
751 only as an orange plot. The peak marked as N.S. shows a nonspecific radioactive signal.

752 **Fig. 5. PIP2 positive foci co-localize with rRNA nascent transcripts in nucleoli.** (A) α-
753 amanitin treated U2OS cells showed very high co-localization of PIP2 and anti-BrdU positive
754 nascent transcripts. Scale bar represents 5μm. (B) Immunogold detection revealed intermingled
755 clusters and strings of PIP2 and rRNA transcripts in the DFC of HeLa cells. Major portion of Br-
756 rRNA transcripts co-localized with PIP2 molecules, while some DFC-zones contained only PIP2.
757 NL, nucleolus. Scale bar represents 200 nm.

758 **Fig. 6. Model for Pol I transcription**. UBF and Pol I interacts with PIP2 during the transcription
759 where PIP2 directs UBF to bind to a more specific site on the promoter compared to its
760 promiscuous binding to the rDNA (Transcription assembly). This specific promoter binding
761 occurs in FC/DFC region. Fib interacts with PIP2 only when RNA is newly synthesized and this
762 interaction takes place in DFC region close to UBF (Transcription initiation/elongation). PIP2 is
763 not involved in further processing of RNA and riboproteins since it is not localized in the GC
764 region where maturation of rRNA takes place. Differences in the hydrophobicity of the
765 complexes may have a role in the formation of subnucleolar structures (Processive transcription).

Supplementary Data Legends

Supplementary Movie 1. **PIP2 localization in nucleolus by 3D electron tomography.** Ultrastructural architecture of PIP2-clusters in nucleolar subcompartments by TECNAI G2 20 LaB6 tomography. PIP2 is localized using pre-embedding procedure with 0.8 nm immunogold particles pseudocoloured in green. Fibrillar center is pseudocoloured in yellow, dense fibrillar component is pseudocoloured in orange.

Supplementary Figures: Fig. S1. Preincubation of PIP2 with anti-PIP2 antibody neutralizes the inhibitory effect of antibody in Pol I transcription. Run-off transcription reaction showed that PIP2 blocked the inhibitory effect of anti-PIP2 antibody (clone 2C11, Abcam, Cambridge, UK; 0.8 μg) in transcription in a dose-dependent manner. (Lane 1: control transcription reaction; lane 2: transcription reaction in the presence of anti-PIP2 antibody; lane 3 and 4: transcription reaction in the presence of anti-PIP2 antibody with the addition of 50 ng and 100 ng PIP2, respectively).

Fig. S2. Ponceau staining of blotted proteins from in vitro pull-down wherein recombinant Wt and Mut PLCδ1PH domains were incubated with nuclear lysates.

Fig. S3. Ponceau staining of blotted proteins from in vitro pull-down performed by incubation of nuclear lysate with control agarose beads or PIP2-coupled agarose beads.

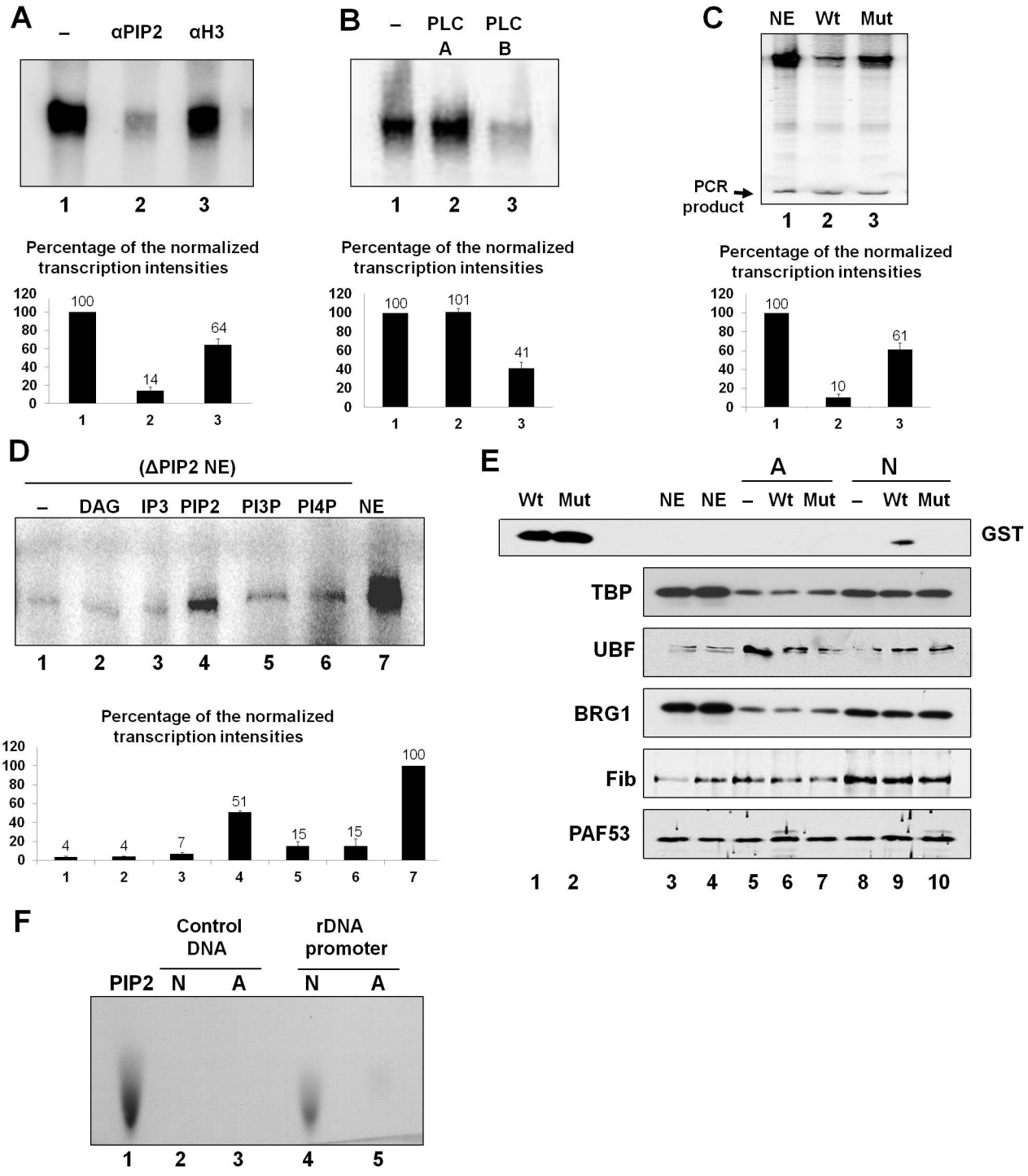
Fig. S4. Ponceau staining of blotted proteins from in vitro pull-down performed by incubation of nucleolar lysate with control agarose beads or PIP2-coupled agarose beads.

Fig. S5. Control experiment showing the specificity of the antibodies used. Specific signals were diminished after blocking the primary antibodies with excess amount of relevant proteins or lipids. When primary antibodies were omitted, secondary antibodies did not produce any visible signal. Scale bar represents 5 μm.

Fig. S6-1,2,3. Ponceau staining of blotted proteins from in vitro pull-down experiments performed by incubation of control agarose beads or PIP2-coupled agarose beads with purified UBF, Fib and Imp 5 proteins, respectively.

Fig. S7. Gel shift assay in which aggregates formed after fibrillarin addition can be seen in the wells. This aggregation leads to an apparent loss of radioactivity in the corresponding gel lanes.

Fig.1


A
–
αPIP2
αH3
1 2 3
Percentage of the normalized transcription intensities
120 100 80 60 40 20 0
100
14
64
1 2 3
B
–
PLC A
PLC B
1 2 3
Percentage of the normalized transcription intensities
120 100 80 60 40 20 0
100
101
41
1 2 3
C
NE
Wt
Mut
PCR product
1 2 3
Percentage of the normalized transcription intensities
120 100 80 60 40 20 0
100
10
61
1 2 3
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(ΔPIP2 NE)
– DAG IP3 PIP2 PI3P PI4P NE
1 2 3 4 5 6 7
Percentage of the normalized transcription intensities
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51
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E
A
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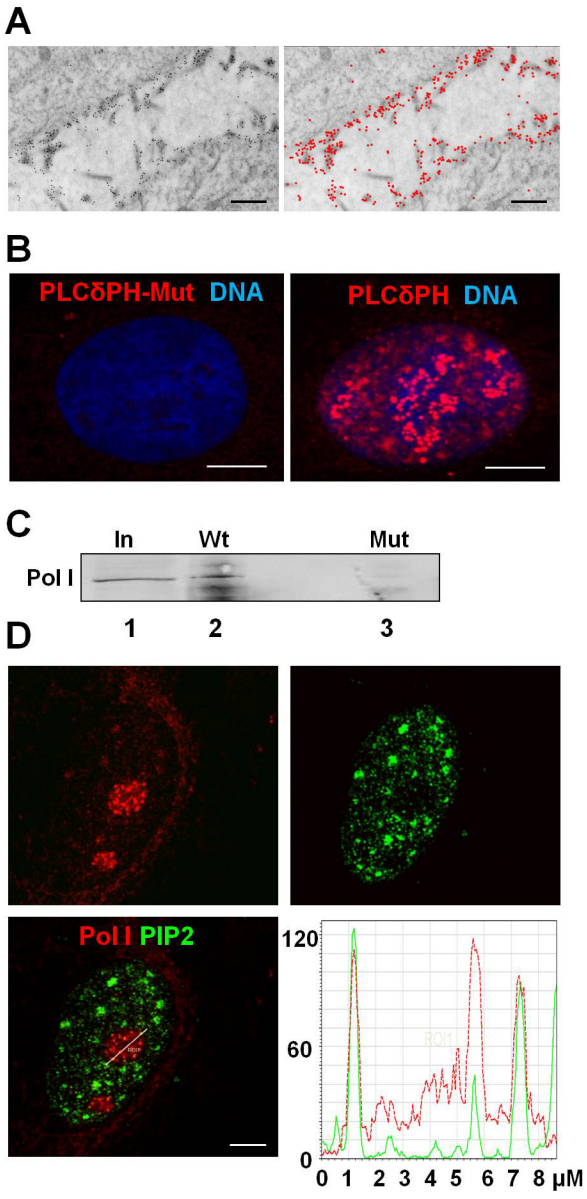
Wt Mut NE NE – Wt Mut – Wt Mut
GST
TBP
UBF
BRG1
Fib
PAF53
1 2 3 4 5 6 7 8 9 10
F
Control DNA
rDNA promoter
PIP2 N A N A
1 2 3 4 5

Fig. 2


A
B
PLCδPH-Mut DNA
PLCδPH DNA
C
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Wt
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Pol I PIP2
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PIP2 beads
Ctrl beads
B23
TAF 95/110
TBP
Fib
1
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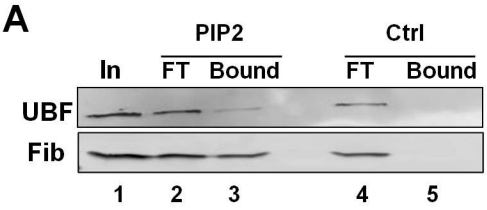
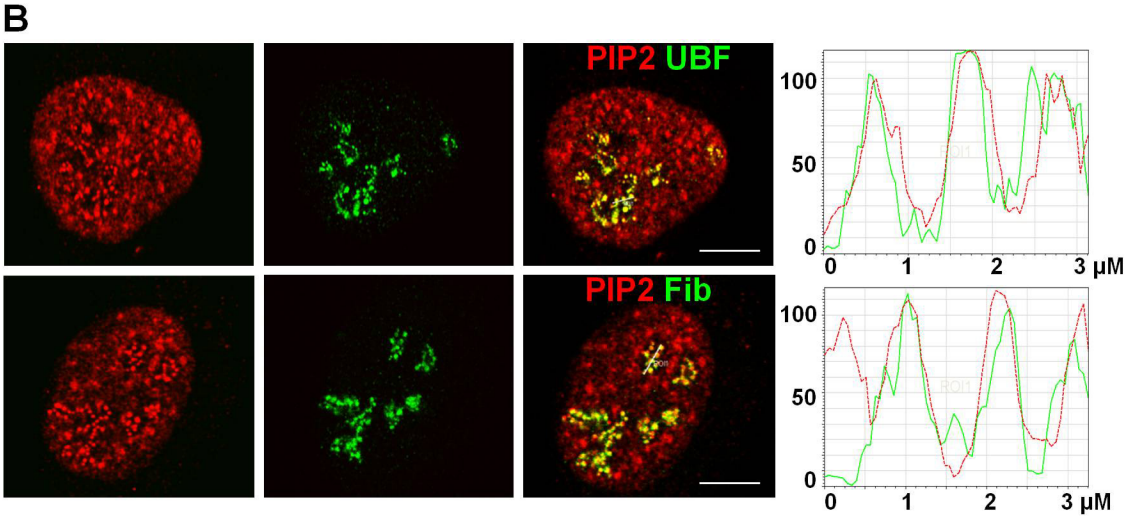
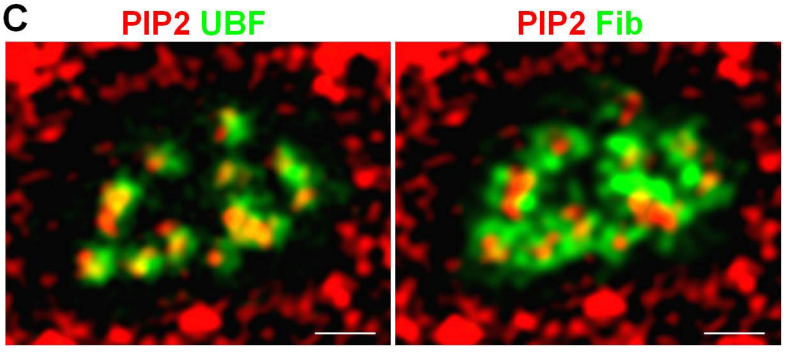
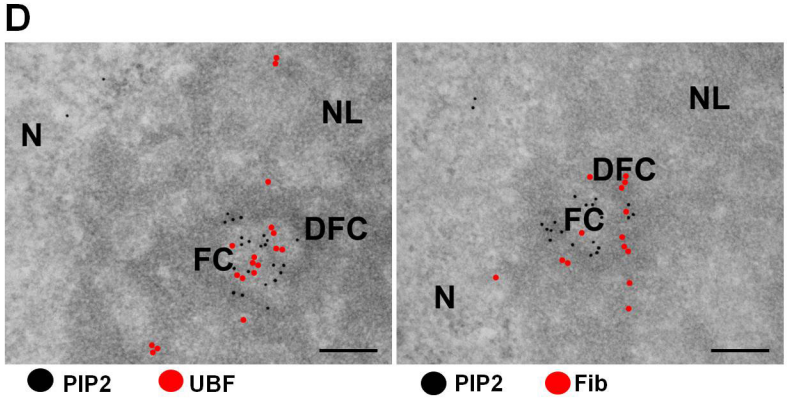
3

Fig. 3


A
PIP2
Ctrl
In
FT
Bound
FT
Bound
UBF
Fib
1
2
3
4
5


B
PIP2 UBF
PIP2 Fib
100
50
0
0
1
2
3 μM


C
PIP2 UBF
PIP2 Fib


D
N
NL
DFC
FC
PIP2
UBF
N
NL
DFC
FC
PIP2
Fib

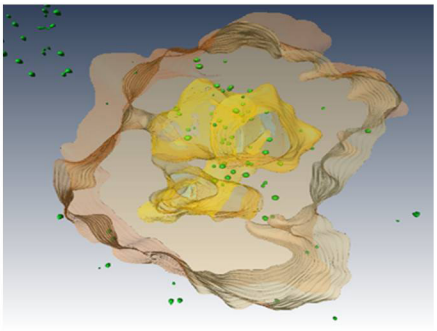
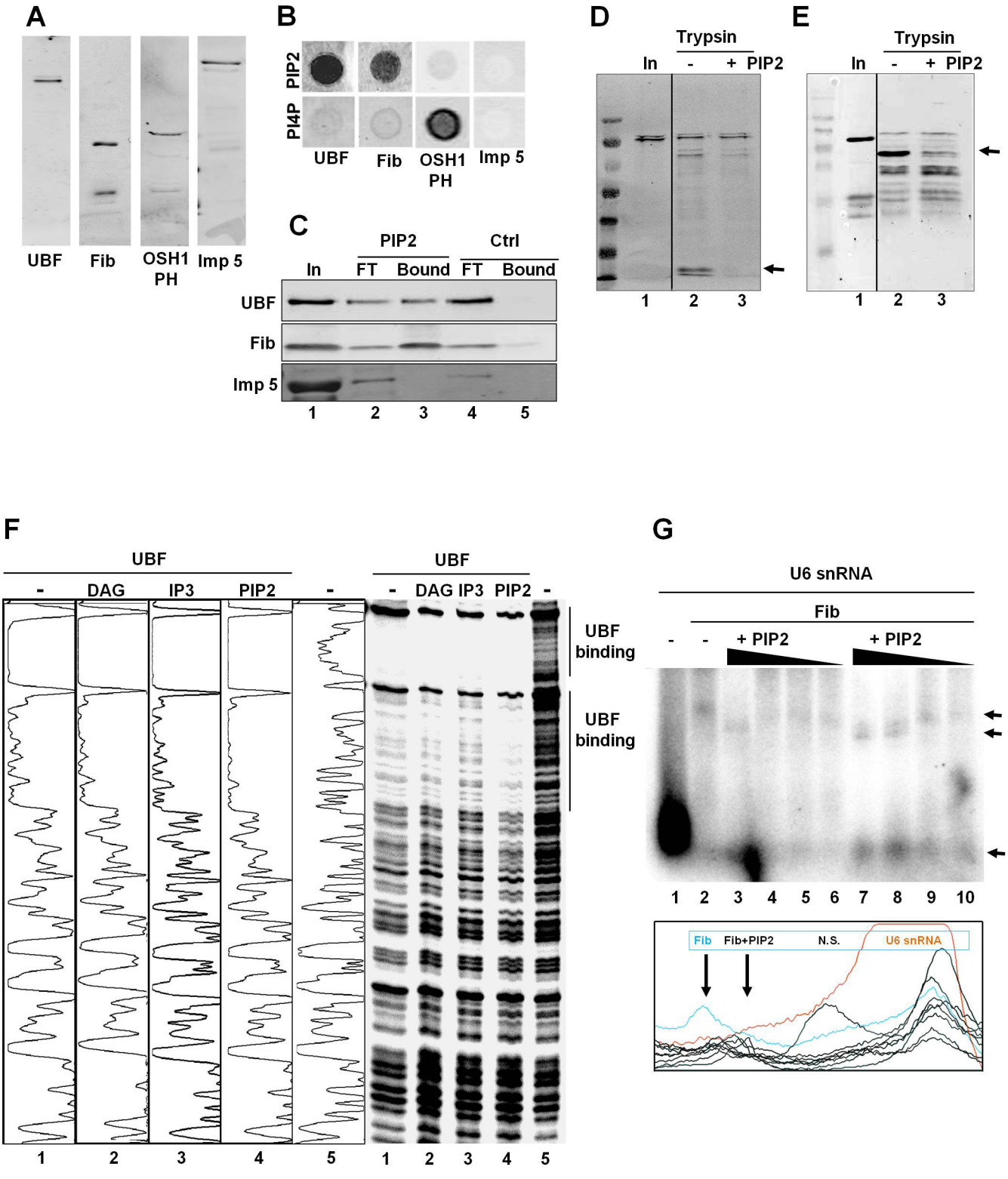

E

Fig. 4


A
UBF
Fib
OSH1 PH
Imp 5
B
PIP2
PI4P
UBF
Fib
OSH1 PH
Imp 5
C
PIP2
Ctrl
In
FT
Bound
FT
Bound
UBF
Fib
Imp 5
1 2 3 4 5
D
Trypsin
In
-
+
PIP2
1 2 3
E
Trypsin
In
-
+
PIP2
1 2 3
F
UBF
- DAG IP3 PIP2 -
UBF
- DAG IP3 PIP2 -
UBF binding
UBF binding
1 2 3 4 5
1 2 3 4 5
G
U6 snRNA
Fib
- - + PIP2 + PIP2
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Fib
Fib+PIP2
N.S.
U6 snRNA

Fig. 5

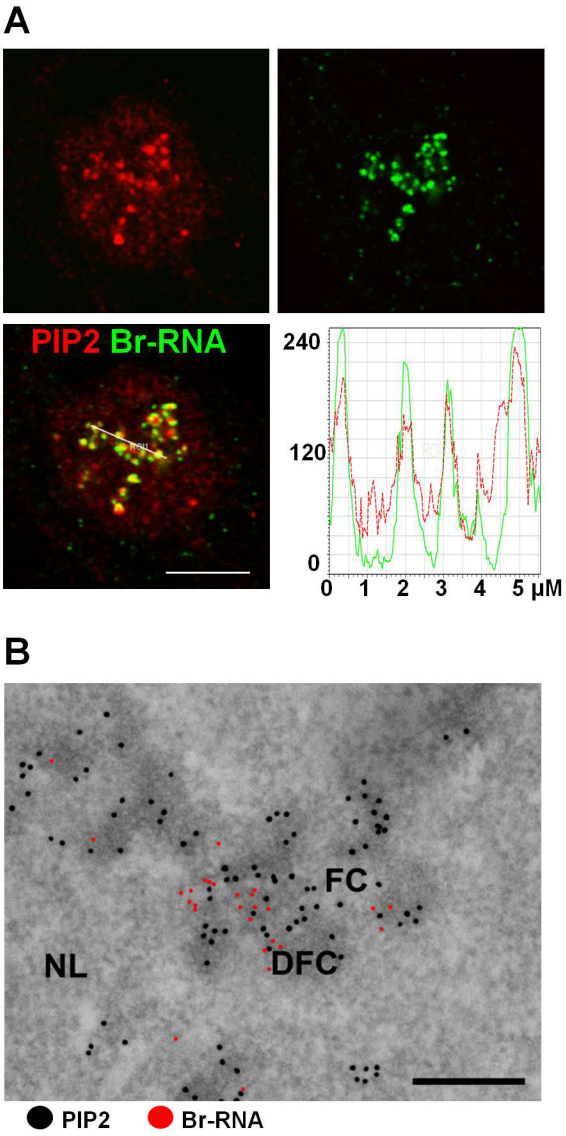
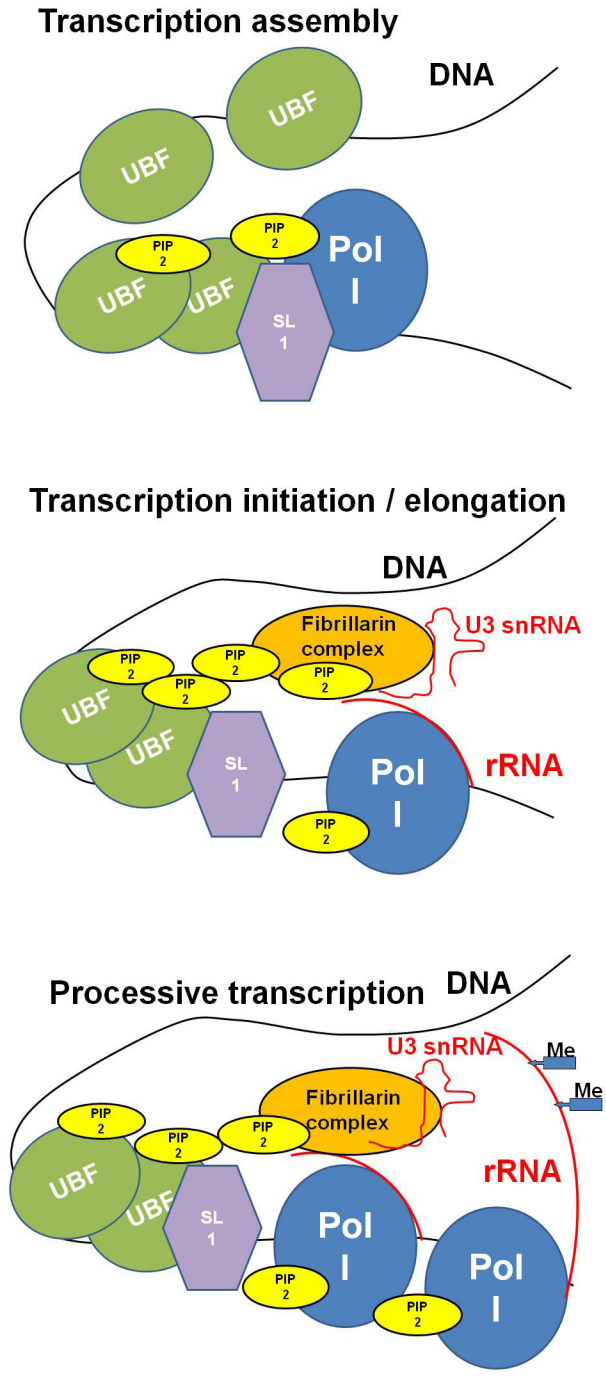


Fig. 6


Transcription assembly
DNA
UBF
UBF
PIP 2
PIP 2
UBF
UBF
Pol I
SL 1
Transcription initiation / elongation
DNA
Fibrillarin complex
U3 snRNA
PIP 2
PIP 2
PIP 2
PIP 2
UBF
UBF
SL 1
Pol I
rRNA
PIP 2
Processive transcription
DNA
U3 snRNA
Me
Me
Fibrillarin complex
PIP 2
PIP 2
PIP 2
rRNA
UBF
UBF
SL 1
Pol I
Pol I
PIP 2
PIP 2

Supplementary Figures

Fig. S1

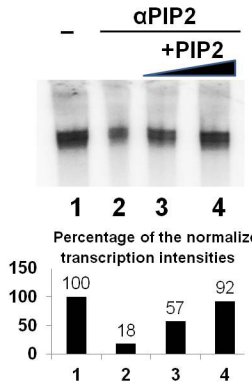


Fig. S2

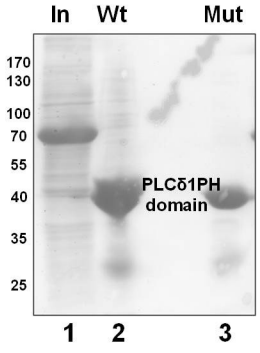


Fig. S3

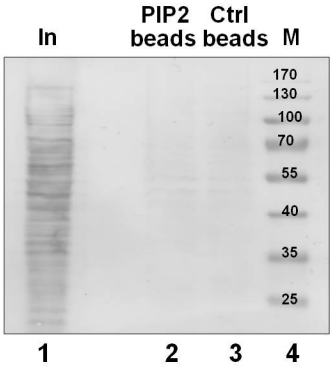


Fig. S4

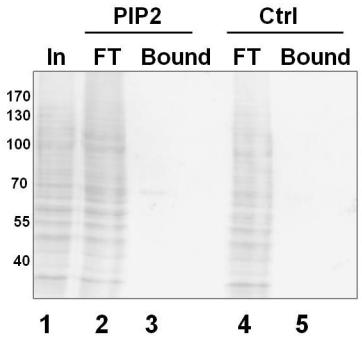


Fig. S5

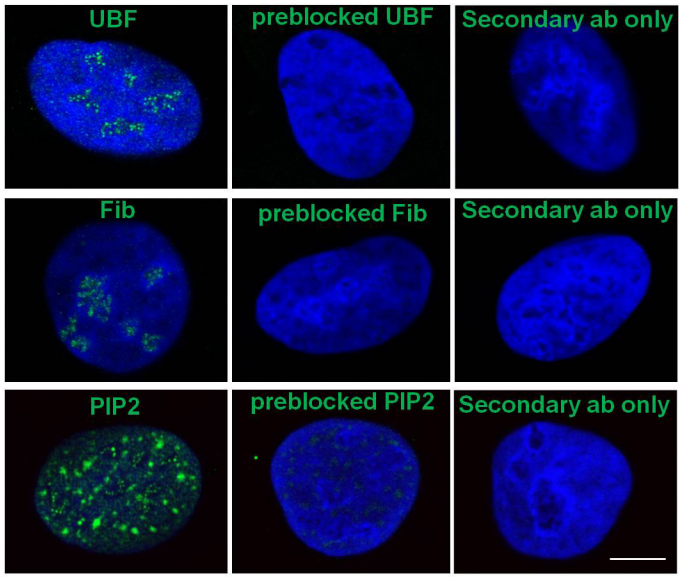
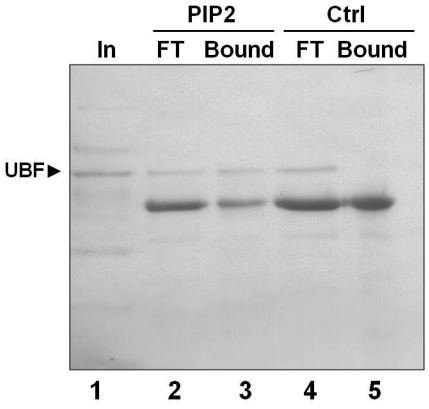


Fig. S6-1



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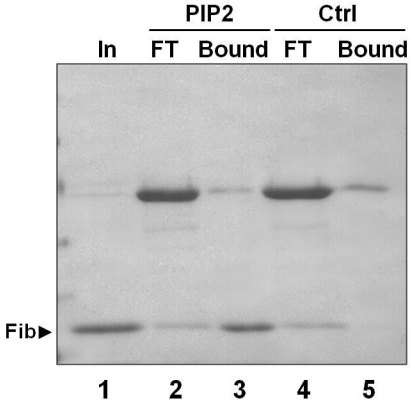


Fig. S6-3

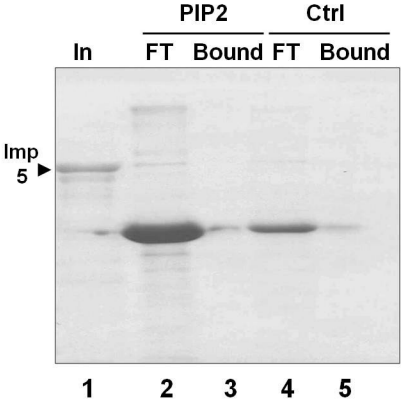
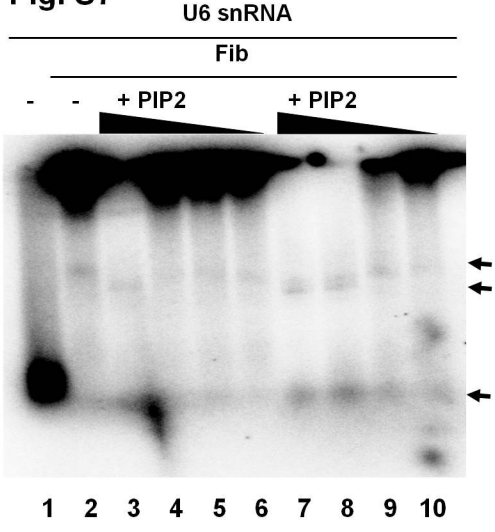


Fig. S7



Transcription-dependent PIP2 distribution in the nucleolus

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Abstract

The nucleolus is a non-membrane bound structure composed of three distinguishable regions: fibrillar centers (FCs), dense fibrillar component (DFC), and granular component (GC). rRNA transcription takes place at the boundary of the FC and DFC while ribosomal subunits assemble in GC of the nucleolus. Recent studies indicate the presence of phosphatidylinositol 4,5-bisphosphate (PIP2) within the nucleolus. Here we studied the connection between transcriptional activity of the cell and sub-nucleolar localization of PIP2 and rRNA transcription machinery. We blocked transcription with Actinomycin D (AMD) and 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole (DRB) treatments which are known to induce particular changes in the nucleolar architecture. AMD treatment results in the formation of nucleolar caps and separation of fibrillarin and UBF. DRB treatment leads to the spatial separation of rDNA genes from preribosomal particles and formation of nucleolar necklaces. Here we show that upon inhibition of transcription by both inhibitors, PIP2 maintains its colocalization with RNA polymerase I (Pol I) and UBF in the FC regions of nucleolar caps and nucleolar necklaces. On the other hand, upon AMD treatment fibrillarin is concentrated in the dense part of the caps and does not colocalize with PIP2. Similarly, DRB treatment also causes a reduction in the colocalization between PIP2 and fibrillarin. These results show that PIP2 binds to Pol I transcription machinery regardless of active transcription, and to fibrillarin only upon synthesis of rRNA.

Keywords: PIP2, Transcription, Nucleolus, RNA Polymerase I, UBF, Fibrillarin

1. Introduction

Nucleolus is the most prominent structure within the cell nucleus. It is formed around the nucleolar organizing regions (NORs) which contain the 5.8S, 18S, and 28S rRNA genes in tandem repeats. After each cell cycle, the nucleolus is formed by the fusion of small pre-nucleolar bodies that contain processing factors for ribosomes.

Nucleolus has three well defined subcompartments: fibrillar centers (FCs), dense fibrillar component (DFC), and granular component (GC). While transcription of pre-ribosomal genes by RNA Polymerase I (Pol I) takes place at the FC/DFC border, assembly of the ribosomal subunits into ribosomes takes place in the GC. Pol I transcription requires binding of UBF and SL1 complex to the enhancer region of rDNA. UBF binding to rDNA leads to the creation of open chromatin structure by displacing linker Histone H1 (Kermekchiev et al., 1997). Before ribosomal subunit assembly, rRNA transcripts proceed through several stages of maturation (Mayer and Grummt, 2006). Fibrillarin is a rRNA 2'-O-methyltransferase that localizes in DFC region and is involved in the early stages of rRNA processing (Hernandez-Verdun, 1991).

Phosphatidylinositol 4,5-bisphosphate (PIP2) is a minor lipid of the cellular membranes that binds and regulates the activity of numerous proteins. When PIP2 is cleaved by phospholipase C (PLC), IP3 [Ins (1,4,5)P3] is released into the cytoplasm resulting in Ca^{2+} mobilization, and DAG remains bound to the membrane where it activates protein kinase C leading to a cellular response (for a review see, Martelli et al., 2002). PIP2 has been shown to be also present in the nucleus and to regulate nuclear functions in nuclear speckles (Mellman et al., 2008; Okada et al., 2008; Osborne et al., 2001). We have recently shown that PIP2 binds to transcription factor UBF and makes complex with Pol I on the promoter of ribosomal genes. Moreover, depletion of PIP2 resulted in reduction of Pol I transcription. PIP2 colocalization with fibrillarin and nascent

transcripts in the nucleolus indicates that PIP2 might link transcription and early processing of rRNA (Yildirim et al., 2013). Here we wanted to extend the study by investigating transcription-dependent rearrangement of PIP2 and Pol I transcription machinery. Transcription can be inhibited by different compounds that cause changes in nucleolar morphology. Actinomycin D (AMD) is known to inhibit Pol I transcription at low doses and redistribute the components of the nucleolus to produce nucleolar caps and intranuclear inclusions (Sirri et al., 2008). RNA Polymerase II (Pol II) inhibitor 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole (DRB) produces a dramatic change in the nucleolus by forming bead-like structures (Panse et al., 1999).We have shown that PIP2 colocalizes with Pol I and UBF in a transcription-independent manner. On the other hand, PIP2-fibrillarin colocalization is sensitive to the changes in transcription. Our data for the first time show the dynamic changes of lipid/protein complexes in the nucleolus reflected by the changes in the transcription activity.

2. Material and methods

2.1. Cell culture

Human osteosarcoma (U2OS) cells and cervical carcinoma (HeLa) cells were kept in DMEM with 10 % fetal calf serum in 5 % CO_2/air, 37°C, humidified atmosphere.

2.2. Plasmids

GST tagged PLCδ1PH (1-140) was received from Dr. Hitoshi Yagisawa (Yagisawa et al., 1998).

2.3. Cell treatments

Cells were treated with AMD (0.02 μg/ml) for 2 hours and with DRB (50 μg/ml) for 1 hour.

2.4. Antibodies

2.4.1. *Confocal microscopy:* Primary antibodies: anti-GST antibody (RP, gift from Dr. Igor Shevelev; 5 µg/ml), anti-PIP2 antibody (MM, 2C11, Abcam, Cambridge, UK; 16 µg/ml), anti-fibrillarin antibody (MM, 38F3, Abcam, Cambridge, UK; dilution 1:100), anti-UBF antibody (MM, sc13125, Santa Cruz Biotechnology, Inc, CA, USA; 2 µg/ml), anti-RPA116 antibody (RP, gift from Dr. Ingrid Grummt; 2µg/ml). Secondary antibodies: donkey anti-mouse IgG conjugated with Alexa 488 (A21202, Invitrogen, Grand Island, NY, USA; 5 µg/ml), goat anti-rabbit IgG conjugated with Alexa 647 (A21245, Invitrogen, Grand Island, NY, USA; 5 µg/ml) and donkey anti-mouse IgM conjugated with Cy3 (715-165-140, Jackson ImmunoResearch, West Grove, PA, USA; 10 µg/ml). Images were taken by a confocal microscope (Leica TCS SP5 AOBS TANDEM) with 100x (NA 1.4) oil immersion objective lense.

2.4.2. Immunoelectron microscopy (*IEM):* Primary antibodies: anti-UBF antibody (Sigma Aldrich, St. Louis, MO, USA; 2.6 µg/ml), anti-fibrillarin antibody (RM, C13C3, Cell Signaling Technology Inc., Danvers, MA, USA; 0.3 µg/ml), anti-PAF53 antibody (MM, 1, BD Transduction Laboratories, Franklin Lakes, NJ, USA; 2.5 µg/ml), anti-PIP2 antibody (MM, 2C11, Abcam, Cambridge, UK; 32 µg/ml), anti-GST antibody (RP, gift from Dr. Igor Shevelev; 5 µg/ml). Secondary antibodies: goat anti-mouse IgG (H+L chains) antibody coupled with 6 nm colloidal gold particles, goat anti-mouse IgM (µ-chain specific) antibody coupled with 12 nm colloidal gold particles, goat anti-rabbit IgG (H+L chains) antibody coupled with either 6 nm or 12 nm colloidal gold particles (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA); dilution 1:30. The thin sections (70-90 nm) were examined in a FEI Morgagni 268 transmission electron microscope at 80 kV. The images were captured with Mega View III CCD camera. Multiple sections of at least three independent immunogold labeling experiments were analyzed.

2.5. Expression and purification of recombinant proteins

For GST tagged PLCδ1PH, the purification was carried on glutathione-agarose column (G4510, Sigma Aldrich) which had been equilibrated with BC100 (20mM Tris pH 8, 0.1 mM EDTA, 20% glycerol, 100 mM NaCl). After washes with BC100, 0.1% NP40, 1 mM DTT, and protease inhibitors (Complete, EDTA-free, Roche Diagnostics GmbH) proteins were eluted with 50mM Tris-HCL, pH 8 having 0.1g reduced L-Glutathione (G4251, Sigma Aldrich).

3. Results

3.1. PIP2 colocalization with Pol and UBF in nucleoli is transcription-independent

Even though PIP2 presence was shown in the nucleolus (Mortier et al., 2005; Osborne et al., 2001), sub-nucleolar PIP2 distribution had not been investigated. For colocalization studies we used either anti-PIP2 antibody or GST-tagged PLCδ1PH domain and anti-GST antibody. PLCδ1PH domain as a PIP2 probe showed the presence of PIP2 in the nucleus and the nucleolus. We used antibodies directed against Pol I (anti-RPA 116 or PAF53 antibodies) and UBF as markers for FC and showed that PIP2 colocalizes with Pol I and UBF in interphase cells, as seen by intensity profiles (Figure 1A). In agreement, IEM also showed the PIP2 localization in close proximity to Pol I and UBF in the FC region of nucleoli, where the inactive components of Pol I transcription machinery are assembled (Figure 2A).

We used low concentration of AMD in order to investigate the effect of Pol I transcription inhibition on the colocalization of PIP2 with Pol I and UBF. Pol I inhibition by AMD treatment results in nucleolar segregation and the formation of a central body associated with caps (Puvion-Dutilleul et al., 1997). Immunofluorescence microscopy showed that PIP2 still colocalizes with Pol I and UBF, even though Pol I transcription is blocked (Figure 1B). IEM further demonstrated

intermingling clusters of PIP2 with Pol I and UBF in the light part of the caps as seen in Figure 2B.

DRB, an inhibitor of the phosphorylation of CTD domain of Pol II, was used to block the activity of Pol II (Bird et al., 2004). DRB induced Pol II inhibition causes alterations in the nucleolar architecture and results in "beads on a string" morphology. Even though there are prominent changes in the structure of nucleolar subdomains, rRNA genes remain transcriptionally active (Scheer et al., 1984). After the DRB treatment, nucleolar beads were assembled and PIP2 colocalization with Pol I and UBF were unchanged as documented in the corresponding intensity profiles (Figure 1C). IEM also demonstrated the localization of PIP2 in very close vicinity to Pol I and UBF in DRB treated cells (Figure 2C).

These results show that the colocalization of PIP2 with Pol I and UBF in the FC region of nucleoli is not dependent on active transcription.

3.2. PIP2 colocalization with fibrillarin in nucleoli is transcription-dependent

Fibrillarin methylates pre-rRNAs (Hernandez-Verdun, 1991) and its recruitment to the DFC region of the nucleolus is dependent on active transcription (Dundr et al., 1997; Kopp et al., 2007). We investigated the effect of transcription inhibition on the localization of PIP2 and fibrillarin in the DFC region of nucleoli. PIP2 and fibrillarin colocalize in untreated control cells (Figure 1A) and PIP2-fibrillarin clusters can be readily seen in the DFC region by IEM (Figure 2A). However; upon the inhibition of Pol I transcription, PIP2-fibrillarin colocalization is lost as seen in the intensity profile (Figure 1B). Fibrillarin is concentrated in the dense part of the caps and in most cases lacks the colocalization with PIP2 after AMD treatment (Figure 2B). Pol II inhibition by DRB treatment also causes a reduction in the colocalization between PIP2 and fibrillarin as demonstrated by the corresponding intensity profile (Figure 1C). Using IEM, we

clearly distinguished DFC where PIP2 is intermingled with fibrillarin. Furthermore, we showed that the majority of PIP2 clusters are localized separately from fibrillarin clusters in the DFC (Figure 2C). These results indicate that PIP2-fibrillarin colocalization is dependent on active transcription.

4. Discussion

It has been known for many years that after removal of nuclear membranes, significant amounts of lipids still remain in the nucleus (Vann et al., 1997). Due to the lack of membranous structures inside of the nucleus, it has been suggested that other hydrophobic molecules maintain a particular environment for phospholipids to be localized in the nucleus (Irvine, 2003; Irvine, 2006). The nucleolus is not separated from the nucleoplasm by a membrane, yet it has a unique protein-nucleic acid composition. Nucleolar proteins are rich in basic residues which allow them to translocate into the nucleolus upon binding to GTP protein (Tsai and McKay, 2005). Therefore, the nucleolus can harbor acidic phospholipids such as PIP2. Here we investigated the dependence of sub-nucleolar localization of PIP2 and specific Pol I transcription and pre-rRNA processing machinery proteins on the transcriptional activity of the cells.

The nucleolus is subdivided into FC, DFC, and GC regions, the functions and features of which are known but the mechanisms of their formation still remain unclear (Leung et al., 2004). We found that PIP2 is not localized in the GC region, where the assembly of pre-ribosome particles takes place, but rather clusters in the FC and DFC regions where transcription and processing of rRNA occur, respectively. We have already shown PIP2 colocalization with Pol I, UBF, and fibrillarin in nucleoli of interphase cells (Yildirim et al., 2013). The effect of inhibition of transcription was further tested with AMD and DRB treatments which are known to induce particular changes in the nucleolar architecture. AMD treatment leads to Pol I inhibition by

reducing the amount of Pol I associated with nucleolar structures (Scheer and Rose, 1984) thus resulting in the formation of nucleolar caps and separation of fibrillarin- and UBF- positive structures (Puvion-Dutilleul et al., 1997). DRB treatment leads to a spatial separation of rDNA genes from preribosomal particles (Scheer et al., 1984). DRB treatment was shown to reduce the level of early rRNA precursors in the cells, leading to impairment of ribosome biosynthesis (Granick, 1975). DRB-induced reduction in pre-rRNA production can be due to the inhibition of UBF1 and TIF1A phosphorylation since DRB inhibits casein kinase II (Zandomeni et al., 1986). Indeed, we found that PIP2 maintains its colocalization with Pol I and UBF in nucleolar caps and nucleolar necklaces but not with fibrillarin, reinforcing the view that PIP2 colocalizes with fibrillarin only upon active transcription of pre-rRNA while its interaction with UBF or Pol I factors are not dependent on the synthesis of rRNA. It was reported that mutations affecting Pol I elongation in yeast cells also affect the cleavage of precursor rRNA by the Spt4-Spt5 complex, indicating the link between transcription and processing of rRNA (Schneider et al., 2006; Schneider et al., 2007). Our data support the notion that coordination of Pol I transcription and pre-rRNA processing factors is mediated by the production of rRNA.

PIP2 can be generated in the nucleus via type I phosphatidylinositol 4-phosphate 5-kinases (Mellman et al., 2008) and its accumulation may contribute to the detergent-resistant nuclear PIP2 pools which makes approximately 40% of the total PIP2 mass (Vann et al., 1997). This particular hydrophobic enviroment may help mediate the formation of FC and DFC components in the nucleolus. Based on our data, we suggest that PIP2 might be a structural-functional interface between the nucleolar skeletal elements and macromolecular complexes involved in rDNA transcription as well as in early rRNA processing. However, further experiments are

needed to elucidate the role of PIP2 and its binding partners in the structure and function of the nucleoli.

5. Acknowledgements

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7. Figure Legends

Figure 1: PIP2 colocalization with Pol I and UBF is not influenced by transcription inhibition while PIP2 colocalization with fibrillarin is disrupted during transcription inhibition as shown by confocal microscopy. (A) Immunocolocalization studies show the colocalization of PIP2 with Pol I, UBF and fibrillarin in control cells as seen in the intensity profiles. Scale bars: 5 μm. (In graph: x-axis in is μm, y-axis is in arbitrary units). **(B)** Pol I transcription inhibition did not affect the colocalization of PIP2 with Pol I and UBF. On the other hand, PIP2-fibrillarin colocalization was disrupted by Pol I transcription inhibition. **(C)** Pol II transcription inhibition by DRB did not change the colocalization of PIP2 with Pol I and UBF in nucleoli. On the other hand, PIP2-fibrillarin colocalization was disrupted upon inhibition of Pol II transcription.

Figure 1: PIP2 colocalization with Pol I and UBF is not influenced by transcription inhibition while PIP2 colocalization with fibrillarin is disrupted by transcription inhibition as shown by IEM. (A) IEM results show that PIP2 is in close proximity to Pol I in the nucleolus and colocalizes with UBF in the FC and with fibrillarin in DFC regions, respectively. Scale bars: 200 nm. **(B)** PIP2 localizes to the light part of the caps together with Pol I and UBF while fibrillarin localizes mainly to the denser part of the caps after the inhibition of Pol I transcription. Scale bars: 200 nm. **(C)** Upon DRB treatment, PIP2 colocalizes with Pol I and UBF in the inner space of FCs as well as on the border between FC and DFC, while in the DFC PIP2 and fibrillarin were arranged as internal necklace-like structures. Scale bars: 200 nm.

Figure 1

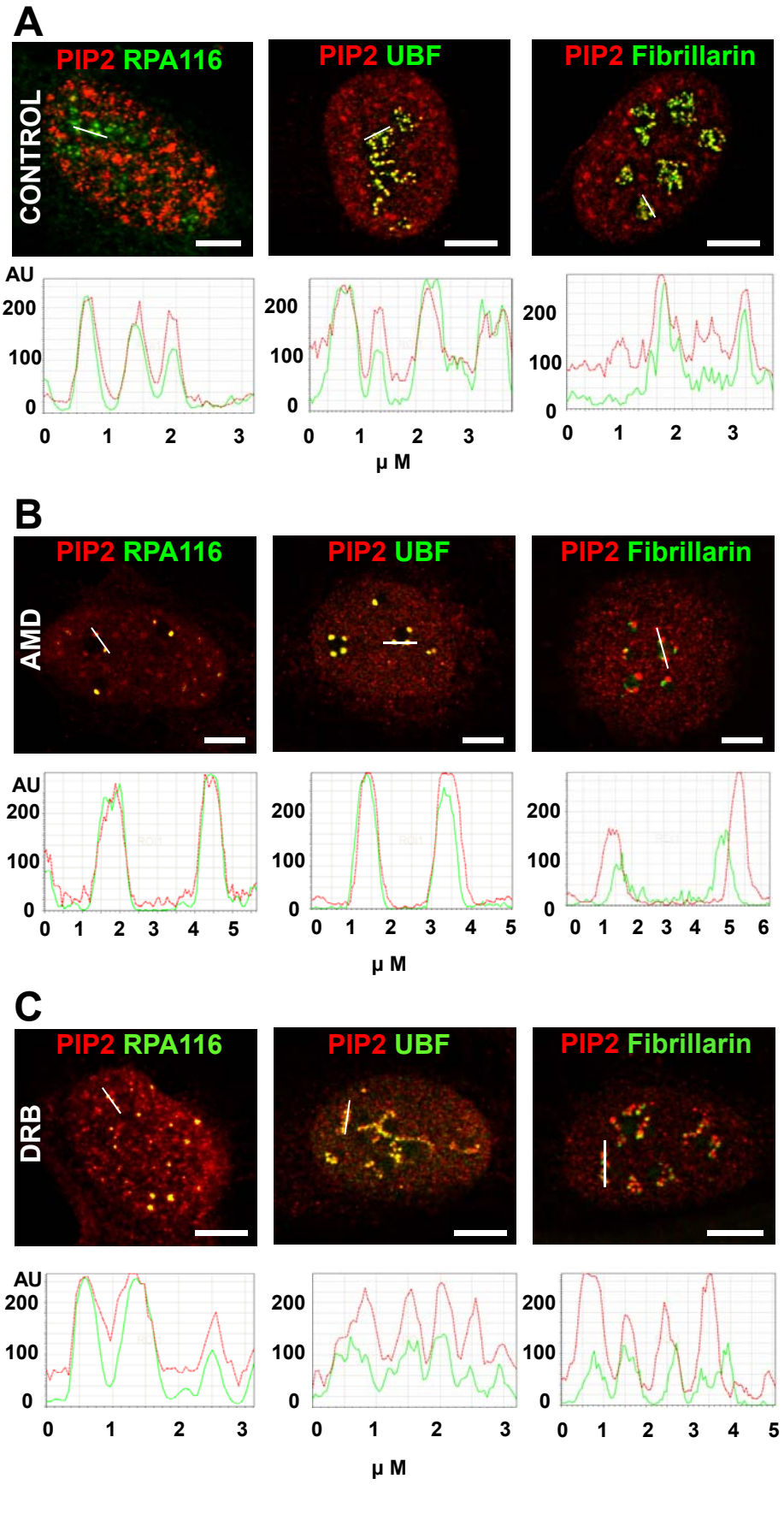
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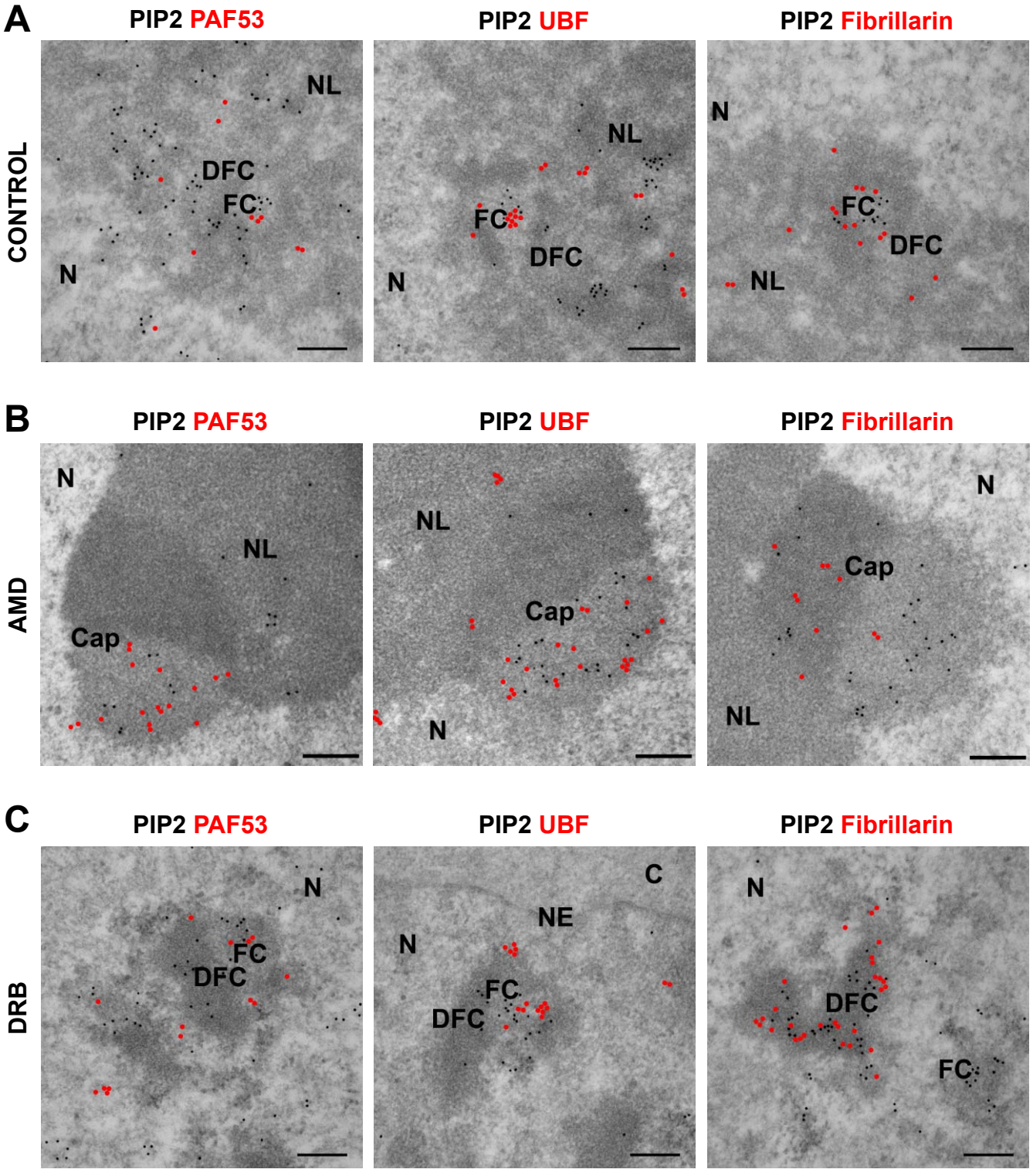
Figure 2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IV. Discussion

1. Translocation of Myo1C/NM1 into nucleus

Except the 16 amino acids at the N-terminus, Myo1C and NM1 have identical amino acid sequence. Therefore, it was suggested that this 16 amino acid at the N-terminus can serve as nuclear localization signal (NLS; Pestic-Dragovich et al., 2000). Since there was no study on the NLS of NM1, we prepared different truncated and mutant forms of NM1 tagged with GFP and our results showed that NLS of NM1 resides in the second IQ motif of the neck domain. Neck domain of Myo1C isoforms has three IQ motifs which had been already shown to bind to CaM in a calcium-dependent manner. In the presence of calcium, CaM disassociates from the first IQ motif and this abolishes the motility of NM1 (Manceva et al., 2007). The second IQ motif binds to cadherin 23 and PIP2 (Cyr et al., 2002; Phillips et al., 2006; Tang et al., 2002) in a CaM dependent manner (Phillips et al., 2006). Based on our findings, we propose that in case of low levels of calcium second IQ motif is occupied by CaM, and when the calcium levels go higher in the cell, CaM disassociates from the second IQ motif. This motif can then associate with the importins for the active transport to the nucleus. Since we did not know which importins are involved in the transclocation of NM1, we performed then pull-down and immunoprecipitation experiments. We found that importin 5, importin 7 and importin beta interact with NM1.

Taken together, these results indicate that all the Myo1C isoforms can localize to the nucleus, since they contain the same IQ motif which is responsible for nuclear translocation. On the other hand, previous studies claimed that Myo1C is a cytoplamic protein, while NM1 is nuclear. However, here we proved that Myo1C is present in the cell nucleus along with NM1. NM1 has already been shown to promote transcription by Pol I and Pol II (Hofmann et al., 2006b; Philimonenko et al., 2004; Ye et al., 2008). Based on our data, we suggest that Myo1C and NM1 can replace each other in transcription by Pol I and Pol II. Further studies are needed to elucidate the interchangeability among Myo1C isoforms in general process of transcription.

2. PIP2 interaction with Myo1C/NM1 in the cell nucleus

PIP2 is a plasma membrane phospholipid with a capability of anchoring various proteins to plasma membrane. For instance, Myo1C is tethered to plasma membrane via binding to PIP2 through its hydrophobic residues within PH domain of tail region (Hokanson and Ostap, 2006; Hokanson et al., 2006). Interestingly, PIP2 was also shown to be present at the nuclear envelope

(Mazzotti et al., 1995; Tran et al., 1993) and within the nucleus (Boronenkov et al., 1998; Mellman et al., 2008; Osborne et al., 2001; Yildirim et al., 2013). It was claimed that PIP2 is anchored within the nucleus via interactions with proteins and lipids (Irvine, 2006).

In order to find out how PIP2 is retained within the nucleus, we investigated the interaction of Myo1C and NM1 with PIP2 in the nucleus. When we compared diffusion properties of EGFP-labeled myosins and their PIP2 binding mutants, we detected a significant increase in the mobility of the mutant myosins compared to wild type myosins, indicating that Myo1C and NM1 bind via PIP2 to some larger intranuclear immobile structures. Therefore, we were interested in identifying the proteins that associate with myosins via PIP2. When we performed pull-down assays using tail domain of Myo1C and NM1, we were able to show that lamin A binds to myosin-PIP2 complex. It was suggested that lamin structures might anchor chromatin and modulate its state (Dechat et al., 2008; Simon and Wilson, 2011), since lamin A binds to DNA and mutations in lamins alter the positions of chromosomes and disrupt their epigenetic state. Thus, we suggest that lamin A might also anchor PIP2-myosin complex within the nucleus. Moreover, farnesylated proteins which carry 15C fatty acid chain at the C-terminus were pulled-down with the tail domain of Myo1C and NM1, but not with the PIP2-binding mutant. We speculate that Myo1C and NM1 interaction with farnesylated proteins might attract proteins with hydrophobic domains and facilitate the formation of nuclear lipo-protein complexes.

NM1 and actin have already been shown to involve in transcription by Pol I and Pol II (Hofmann et al., 2006b; Hofmann et al., 2004; Pestic-Dragovich et al., 2000; Philimonenko et al., 2004; Ye et al., 2008). In particular, polymerized actin and NM1 motor domain are required for the activation of Pol I transcription (Ye et al., 2008). We have recently shown that PIP2 also makes complex with RNA polymerase I and promotes transcription for ribosomal RNA genes (Yildirim et al., 2013). PIP2 also facilitates the synthesis of filamentous actin in the nucleus by displacing actin from the actin binding site of ATP-dependent chromatin remodeling protein BRG1 (Rando et al., 2002), which is analogous to uncapping of actin via PIP2 in cytoplasm (Yin and Janmey, 2003). However, nothing is known about the functional interplay between actin and NM1-PIP2 complex in the nucleus. Therefore, we studied the dependence of NM1-PIP2 complex mobility on active transcription and actin polymerization. Inhibition of transcription and disruption of actin polymerization caused a significant decrease on NM1 mobility in the cell nucleus. These data indicate that NM1-PIP2 complex mobility is dependent on polymerized actin and

transcriptional activity of the cells. However, the functional significance of myosin-PIP2 interaction in nuclear processes still needs to be investigated.

3. PIP2 involvement in Pol I transcription

Even though the presence of phospholipids in the nucleus has been shown by various research groups (Boronenkov et al., 1998; Cocco et al., 1987; Maraldi et al., 1995; Mazzotti et al., 1995; Mellman et al., 2008; Osborne et al., 2001), little is known about their nuclear functions. Few studies reported the involvement of PIP2 in Pol II transcription. PIP2 addition was shown to promote Pol II transcription via interfering with the binding of histone H1 to DNA (Yu et al., 1998). PIP2 addition also augmented the binding of chromatin remodeling complex to DNA (Zhao et al., 1998). However, there has been no study on the modulation of Pol I transcription by PIP2. Therefore, here we focused on the involvement of PIP2 in transcription by Pol I.

Our results showed that PIP2 promotes Pol I transcription, since addition of anti-PIP2 antibody resulted in the reduction of Pol I transcription. In order to rule out the possibility of the involvement of PIP2 cleavage products in Pol I transcription, we compared the Pol I transcription levels upon addition of exogenous PIP2, DAG and Ins(1,4,5)P3 to PIP2 depleted extracts. The inhibitory effect of PIP2 depletion in Pol I transcription was reversed by the addition of exogenous PIP2, but not by the addition of Ins(1,4,5)P3 and DAG. Taken together, our data suggest that PIP2 acts as itself in Pol I transcription rather than as a substrate of nuclear PI-PLC.

We also showed that PIP2 is anchored in the fibrillar regions of the nucleolus via interactions with Pol I, UBF and fibrillarin. Since PIP2 did not show any interaction with the other Pol I transcription machinery proteins, such as TBP and TAF 95/110; we concluded that PIP2 selectively binds to a subset of proteins involved in transcription.

We showed that direct binding of PIP2 to UBF results in a tighter binding to rDNA promoter. UBF is a scaffold protein that binds to rDNA promoter and bends it to establish proper DNA-protein structure (Stefanovsky et al., 2001). In addition, UBF is required for the formation of secondary constrictions of NORs (Mais et al., 2005). Based on our data, we propose that the interaction between PIP2 and UBF might have a regulatory role in the formation of transcription initiation complex at the rDNA promoter.

Fibrillarin is a component of ribonucleoprotein complex, which binds and methylates precursor ribosomal RNA in the DFC region (Hernandez-Verdun, 1991). We showed that direct binding of

PIP2 to fibrillarin causes an increase in the mobility of U6 snRNA/Fib complex, indicating the conformational changes in fibrillarin upon PIP2 binding.

RNA is required for the localization of PIP2 to nuclear speckles, since RNase treatment abolished PIP2 staining (Osborne et al., 2001). PIP2 interaction with RNA was suggested to stabilize PIP2 in the absence of intranuclear membranous structures. Our results showing the colocalization of PIP2 with nascent ribosomal RNA transcripts, indicate the possibility of PIP2 association with intranuclear structures via interaction with nascent ribosomal RNA transcripts. We suggest that a particular hydrophobic protein-lipid-RNA enviroment might exist in the transcriptionally active regions of the nucleolus where PIP2 exclusively localizes. It was shown that the morphology of nuclear speckles is dependent on PIP2 (Mortier et al., 2005). Therefore, we believe that PIP2 might also play a role in the formation of transcriptionally active sites of the nucleolus. Indeed, PIP2 might act as a structural interface between the macromolecular complexes involved in rDNA transcription and in early ribosomal RNA processing, thus contributing to formation of nucleolar structures.

4. Transcriptional regulation of PIP2 in the cell nucleus

Even though the nucleolus is not separated from the nucleoplasm by a membrane, it contains unique proteins that are rich in basic residues (Tsai and McKay, 2005). Therefore, it is expected that nucleolus could harbor acidic phospholipids such as PIP2. We have previously shown that PIP2 activates Pol I transcription and colocalizes with some proteins belonging to Pol I transcription machinery in the nucleolus (Yildirim et al., 2013). Therefore, we extended the study to understand if PIP2 localization in the nucleolus is regulated by transcription activity.

The nucleolus is subdivided into three regions: fibrillar centers (FC), dense fibrillar component (DFC), and granular component (GC). We found that PIP2 is not localized in the GC region, where the assembly of preribosome particles takes place, but rather clusters in the FC and DFC regions where transcription and processing of ribosomal RNA occur, respectively.

Pol I and Pol II transcription can be inhibited by treatment with actinomycin D (AMD) and 5,6-dichloro-1β-d-ribofuranosyl-benzimidazole (DRB), respectively. Upon AMD treatment, fibrillarin and UBF are spatially separated and nucleolar caps are formed (Puvion-Dutilleul et al., 1997). DRB treatment also leads to morphological changes in the nucleolus (formation of nucleolar necklaces) and spatial separation of rDNA genes from preribosomal particles (Panse et

al., 1999; Scheer et al., 1984). In this study, we demonstrated that upon inhibition of trancription by AMD and DRB, PIP2 still colocalizes with Pol I and UBF in nucleolar caps and nucleolar necklaces but not with fibrillarin. These results indicate that PIP2 colocalizes with fibrillarin only upon active transcription of pre-ribosomal RNA and its interaction with UBF and Pol I is not dependent on the synthesis of ribosomal RNA. Based on these data, we suggest that ribosomal RNA is needed for connecting the processes of nascent transcript production and early processing.

V. Summary and conclusions

Myo1C and NM1 are monomeric actin-based molecular motors. Except the 16 amino acid extension at the N-terminus, they are identical. Myo1C is implicated in many diverse physiological and biochemical processes that take place in the cytoplasm and plasma membrane, mainly by interacting with PIP2 and actin cytoskeleton. On the other hand, NM1 is involved in transcription and chromain dynamics. Practically nothing is known regarding the interaction of these myosins with PIP2 in the cell nucleus. Moreover, PIP2 involvement in Pol I transcription is also unknown. Therefore, we aimed to extend our knowledge on the interaction between myosins and PIP2, and the involvement of PIP2 in Pol I transcription. Our findings presented in this work can be summarized as follows:

1. **Translocation of Myo1C and NM1 is directed by the second IQ domain in the neck region.** We showed that second IQ domain in the neck region is responsible for the nuclear import of Myo1C and NM1 via the interaction with nuclear transport receptors such as importin 5, importin 7 and importin beta. Moreover, we found that calmodulin binding to first and second IQ domains interferes with nuclear translocation of Myo1C and NM1. Our results indicate that all the Myo1C isoforms can localize to the nucleus, since they contain the same IQ motif which is responsible for nuclear translocation. Moreover, our data suggest that Myo1C and NM1 can replace each other in transcription by Pol I and Pol II. However, further studies are needed to elucidate the interchangeability among Myo1C isoforms in transcription.
2. **Myo1C and NM1 make a complex with PIP2 in the cell nucleus.** We demonstrated that both Myo1C and NM1 interact with PIP2 through their tail domains. We showed that

PIP2 binding causes a reduction in the mobility of Myo1C and NM1. Since PIP2 is a relatively small molecule, we propose that myosins make complexes with other proteins and lipids in the nucleus via interactions with PIP2. In paralel, we found that impairment in PIP2 binding to myosin leads to loss of interaction with lamin A and farnesylated proteins. We suggest that myosin-PIP2 complexes can be anchored within the nucleus via interactions with lamin A and farnesylated proteins. Moreover, interaction of myosin-PIP2 complexes with farnesylated proteins might attract proteins with hydrophobic domains and facilitate the formation of nuclear lipo-protein complexes.

3. **PIP2 promotes transcription of ribosomal RNA genes.** Here we focused on the involvement of PIP2 in Pol I transcription. Using an *in vitro* transcription assay, we demonstrated an involvement of PIP2 in the regulation of Pol I transcription. PIP2 depletion and add-back studies showed that a loss of PIP2 in nuclear extracts leads to a dramatic reduction in transcription levels which can be only rescued by PIP2 addition, but not by adding PIP2 precursors or cleavage products. These results indicate that PIP2 promotes transcription of ribosomal RNA genes. Furthermore, we showed that PIP2 also interacts with Pol I transcription machinery proteins, such as Pol I, UBF and early processing protein fibrillarin. We found that PIP2 is not localized in the GC region, where the assembly of preribosome particles takes place, but rather clusters in the FC and DFC regions where transcription and processing of ribosomal RNA occur. Since PIP2 also colocalizes with the nascent ribosomal RNAs in the nucleolus, we propose that PIP2 can link the ribosomal RNA gene transcription to processing machineries.
4. **Nucleolar PIP2 localization is dependent on the transcriptional activity of the cell.** We showed that the transcriptional state of the cell influences the localization of PIP2 in the nucleolus. Pol I and Pol II transcription can be inhibited by treatment with AMD and DRB, respectively. AMD treatment causes the formation of nucleolar caps and the separation of fibrillarin and UBF. DRB treatment results in spatial separation of rDNA genes from preribosomal particles, and formation of bead-like structures called nucleolar necklaces. In this study we demonstated that upon inhibition of trancription by AMD and DRB, PIP2 still colocalizes with Pol I and UBF in nucleolar caps and nucleolar necklaces but not with fibrillarin. Since PIP2 colocalization with fibrillarin is abolished upon

inhibition of ribosomal RNA synthesis, we suggest that ribosomal RNA might link ribosomal RNA synthesis to ribosomal RNA early processing.

VI. Prospects

In the near future we plan to address the following questions:

1. **Determining the function of myosin-PIP2 complex in the cell nucleus.** We know that NM1 and PIP2 are involved in Pol I and Pol II transcription. However, we still do not know if NM1-PIP2 interaction has any role in transcription. Therefore, we would like to perform an *in vitro* transcription assay to determine if PIP2 interaction with myosins has any function in the regulation of transcription. To do this, we will deplete the nuclear extracts with specific antibodies against myosins and add back the wild type and PIP2-binding mutants of myosins. We may also use the nuclear extracts prepared from NM1 knock-out cells (Venit et al., 2013) as a second approach.
2. **Mapping nuclear phospholipids and their binding partners.** The sub-nuclear localization of phospholipids has not been studied yet. Moreover, we still do not know the composition and function of nuclear lipo-protein complexes. We believe that identifying the nuclear phospholipids and their nuclear functions will help us to understand the mechanism of nuclear processes in detail. Therefore, we would like to map the sub-nuclear distribution of phospholipids and investigate their nuclear functions. To achieve this, we will create GST or FLAG-tagged protein domains specifically bound to phospholipids (Balla, 2005) and use them as sensors together with antibodies against phospholipids. We will use these tools to visualize the phospholipids by confocal, super-resolution and electron microscopy. We also would like to use these tools to perform pull down and co-immunoprecipitation experiments to identify the binding partners of phospholipids in the nucleus. Based on these results, we will investigate the specific nuclear functions of these lipo-protein complexes.
3. **Investigating the indispensability of phospholipids as structural elements in the nucleus.** Intranuclear structures are composed of proteins, nucleic acids and phospholipids. However, we do not know the impact of phospholipids on intranuclear morphology. Therefore, we would like to find out if nuclear phospholipids have any

roles in the formation and maintanence of nuclear structures. To to this, we will alter the levels of nuclear phospholipids by knocking-down or overexpressing phospholipid-effector enzymes, namely kinases, lipases and phosphatases and check the impact on nuclear structures.

4. **Questioning the involvement of other PIP2 species in the transcription.** We revealed that PIP2 precursor and cleavage products have no significant effect on transcription of ribosomal RNA genes. However, we still do not know if other PIP2 species such as PI(3,4)P2 and PI(3,5)P2 also have roles in the regulation of transcription. We will deplete PI(3,4)P2 and PI(3,5)P2 from the nuclear extracts and perform *in vitro* transcription experiments. We hope that these experiments answer the question whether all PIP2 species are involved in transcription, particularly in transcription by Pol I. This would be followed by a detailed mechanistic story.

VII. References

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