Univerzita Karlova v Praze

Přírodovědecká fakulta

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Localization matters: function of paxillin and phosholipids in the cell nucleus

Význam lokalizace: funkce paxillinu a fosfolipidů v buněčném jádře

Dizertační práce

Školitel: Prof. RNDr. Pavel Hozák, DrSc.

Praha, 2015

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne .....

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. RNDr. Pavel Hozák, DrSc.

It started about seven years ago, when my supervisor Pavel Hozák gave me the opportunity to join the lab. He deserves my thanks, because I was a rookie and he gave me the opportunity to develop my abilities and blossom into an independently thinking scientist.
I am especially grateful to Rasťo Dzijak who kick-started my project and kept giving me impulses and pieces of advice to move both the project and myself forward. I am sending him many „greetings".
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I want to express special appreciation to Margarita Sobol, who enabled me to collaborate on her projects, which gave rise to two interesting papers. I am thankful to all other members of the lab for creating a friendly atmosphere. I will not name them, because I could miss someone, but they certainly know!
I would like to thank here to all the people who came to my life during preparation of this masterpiece. Even though some of them do not know, they helped me to realize who I am and what I want from life. The names will remain personal.
And biggest thanks belong to my family. My parents and my sister Ivana who supported me primarily mentally in whatever I decided to do and the older I am, the more I realize how great they are.

"You know you've worked in the lab too long when…"

Linguistics:

You say "conjugation" instead of "sex", and "pili" sounds dirty.
You refer to your children as the F1.
You think the following is a quality insult: "I've seen cells more competent than you!"
For you, media is something which increases your culture.

Entertainment:

You've seen how far away you can hit a target with a squirty water bottle or seeing how far away from the bin you can fire pipette tips.
You decide the courses and conference you want to go on by the quality of the food served.
When you start making patterns in your pipette tip box as you take the tips out.
You've used, "I'd like to get into your genes" as a pickup line.
You have made some kind of puppet out of a nitrile glove and kept it as a pet.

Couture:

Safety equipment is optional unless it makes you look cool.
A timer clipped to the hip is not only practical, but dead sexy.
You've never worn a clean lab coat.
You've left the lab wearing a piece of PPE (personal protective equipment) because you forgot you had it on.
You own Invitrogen t-shirts and actually wear them.

Kitchen and home skills:

You're also very good at transferring small amounts of liquid between containers.
When your fruits go bad and you get fruit flies, you can't help but check their eye color.
You open the toothpaste with one hand.

Professional:

You've worked out that a trained chimp could probably do 90% of your job.
You always seem to use the microscope after the person with the impossibly close-set eyes.
When you say goodnight to your microscope on a Friday night and tearfully hug it goodbye as you won't see it all weekend.
You can identify organs on roadkills.
You can't watch CSI without cursing at least one scientific inaccuracy.

C'est la vie:

No one in your family has any idea what you do.
The front page of Science is your light reading.
When a non-scientist asks you what you do for a living, you roll your eyes and talk science at them until they've lost the will to live.
When you rejoice when grabbing a handful of eppendorfs/bijous/anything and it turns outs to be the exact number you needed.

Health and Hygiene:

You wash your hands before and after using the washroom.
You've suffered carpal tunnel from the pipetman.
You've used Kimwipes as Kleenex.
You are slightly too fond of the smell of (pick one or many) Xylene/ Agar/ Ethanol/ Undergraduates/ Alcoholic hand-wash.

"Science is like sex: sometimes something useful comes out, but that is not the reason we are doing it."

Richard P. Feynman

ABBREVIATIONS

ATP	adenosine triphosphate
BAF	Brg/Brm-associated factor
BASP	brain acid soluble protein
ChIP	chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DMR	differentially methylated region
EGFP	enhanced green fluorescent protein
FAK	focal adhesion kinase
H1	histone 1
HDAC1	histone deacetylase 1
ICR	imprinting control region
IGF1	insulin-like growth factor 1
IGF2	insulin-like growth factor 2
IGF2R	insulin-like growth factor 2 receptor
MED	Mediator of RNA polymerase II
NES	nuclear export signal
NLS	nuclear localization signal
PABP1	poly(A) binding protein 1
PIP	phosphoinositiol phoshate
PIPK	phosphoinositol phosphate kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PS	phosphatidyl serine
PT	phosphatidyl threonine
PTEN	phosphate and tensin homolog protein
QPCR	quantitative polymerase chain reaction
shRNA	short hairpin (small hairpin) RNA
SMC1	structural maintenance of chromosomes 1
SMC3	structural maintenance of chromosomes 3
SNP	single nucleotide polymorphism

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ABSTRACT (English)

Both paxillin and PIP2 are well known components of the cell, although of a distinct origin. Focal adhesion protein paxillin spreads the signals from extracellular matrix via integrins and growth factor receptors to affect cellular motility and migration (Schaller, 2001). PIP2, a major structural component of cytoplasmic membrane, is utilized by phospholipase C to generate second messenger molecules (Hokin and Hokin 1953; Streb et al. 1983). Both molecules were recently shown to be localized in the nucleus. Their original functions have been well established, but together with other research colleagues we are now shedding more light on completely different functions of these biological molecules and moreover, in the different compartments than they were primarily believed to function in.

Here, we introduce paxillin as an important factor of the cell nucleus, where it regulates transcription of two important growth-related genes, *IGF2* and *H19*. It does not affect the allelic expression of these imprinted genes, it rather regulates long-range chromosomal interactions between *H19* or *IGF2* promoter, and the shared distal enhacer on an active allele. In detail, paxillin stimulates the interaction between the enhancer and the *IGF2* promoter, activating *IGF2* gene transcription, while it restrains the interaction between the enhancer and the *H19* promoter, downregulating the *H19* gene. We identified paxillin in complex with cohesin and Mediator of RNA polymerase II, and we propose that this complex mediates the activity of the *IGF2*/*H19* gene cluster. We present a novel mechanism for gene regulation by a focal adhesion protein paxillin. Our observations contribute to a mechanistic explanation of paxillin role in proliferation and fetal development.

We further present here two aspects of nuclear-localized PIP2. First, we show that PIP2 isa stable component of RNA Pol I transcription complex throughout the whole cell cycle indicating its wider role in nucleolar organization. Second, we state here for the first time that a significant portion of nuclear PIP2 is located in previously uncharacterized spherical structures, which are distinct from nuclear speckles and we refer to them as PIP2 islets. They are surrounded by nucleic acids and proteins, while the inner space seems to be mainly composed of carbon-rich compounds. We show that PIP2 islets are evolutionary conserved from protozoa to human and we link them to RNA Pol II transcription, since they co-localize with nascent RNA transcripts and their depletion by PLC enzyme reduces the level of transcription.

ABSTRAKT (Czech)

Paxillin i PIP2 jsou dobře známé součásti každé buňky, i když odlišné povahy. Paxillin, protein fokálních adhezí, šíří signál z extracelulární matrix prostřednictvím integrinů a receptory růstových faktorů, a tak reguluje buněčnou motilitu a migraci buněk (Schaller, 2001). PIP2, hlavní stavební kámen buněčných membrán, který je fosfolipasou C štěpen na tzv. druhé posly (Hokin and Hokin 1953; Streb et al. 1983). V nedávné době bylo prokázáno, že obě molekuly jsou také lokalizovány v jádře. Jejich dosud známé funkce jsou detailně prozkoumány, ale my nyní odhalujeme zcela odlišné funkce těchto biologických složek, a to navíc v úplně odlišných částech buňky, než kde se jejich lokalizace předpokládá.

V této práci ukazujeme, že paxillin je důležitým faktorem buněčného jádra, kde kontroluje expresi dvou důležitých genů *IGF2* a *H19*, které řídí růst buňky. Paxillin nemění alelickou expresi těchto imprintovaných genů, ale reguluje chromosomální interakce mezi promotory *IGF2/H19* a jejich společným enhancerem na aktivní alele. Přesněji řečeno, paxillin stimuluje interakci mezi enahncerem a promotorem genu *IGF2*, čímž zesiluje transkripci *IGF2*, zároveň paxillin brání interakci mezi enhancerem a promotorem *H19*, a tím snižuje transkripci *H19*. Nalezli jsme paxillin v komplexu s kohesiny a také Mediatorem RNA polymerasy II a navrhujeme, že tento komplex zprostředkovává působení na genový klastr *IGF2*/*H19*. Představujeme zde nový mechanismus genové regulace zprostředkovaný proteinem fokálních adhezí, paxillinem. Naše pozorování přispívá k vysvětlení mechanismu role paxillinu v proliferaci a při vývoji plodu.

Dále předkládáme dvě roviny funkce molekuly PIP2 lokalizovaného v buněčném jádře. Prvně ukazujeme, že PIP2 je stabilní součástí transkripčního komplexu RNA polymerasy I, a to během celého buněčného cyklu, což naznačuje, že má širší funkci v organizaci jadérka. Za druhé, poprvé uvádíme, že značná část jaderného PIP2 je lokalizována v dříve nepopsaných kruhových strukturách, které jsou odlišné od jaderných speckles a označili jsme je jako „ostrůvky“. Ty jsou obklopeny nukleovými kyselinami a proteiny, zatímco vnitřek je tvořen převážně látkami bohatými na uhlík. PIP2 ostrůvky jsou evolučně konzervované od prvoků až po člověka a jsou spojené s transkripcí RNA polymerasy II, jelikož se lokalizují do stejných míst jako nově vznikající RNA molekuly a jejich rozštěpení fosfolipasou C snižuje hladinu transkripce v buněčném jádře.

INTRODUCTION

One of the features which differentiate eukaryotic cells from prokaryotic cells is the presence of intracellular distinct compartments, organelles, such as mitochondria, ribosomes, and the nucleus. Each compartment has its own specific function, whereas collectively these functions are in harmony with each other and permit a cell to live. Cell function is dependent upon the coordinated and dynamic formation of complex interaction networks between molecules of diverse biochemical properties. These networks or interactomes are comprised of macromolecular biopolymers; proteins, DNA, RNA and Ípids. Chromatin or simply DNA plays a central role in the cells since it consists of genes. The careful orchestration of gene expression in a cell-type and developmental stage-specific manner assures the proper development of a cell. The multistep pathway of eukaryotic gene regulation involves a series of highly controlled events in the nucleus and also cytoplasm. Launching expression of a particular gene requires binding of a particular transcription factor, not only to gene promoter, but also to distant regulatory sequences. Apart from common ones, a cell exploits also "unusual" mechanisms to ensure proper control and these are just beginning to be understood. Cytoplasm and the nucleus are separated by a double nuclear envelope bearing nuclear pore complexes, which allow exchange of macromolecules between the two compartments. Once the proteins gain residence in the nucleus, some of them remain there throughout their life span, whereas others are exported back into the cytoplasm. This type of behaviour – a continuous movement back and forth across the nuclear envelope – is termed nucleocytoplasmic shuttling. The transport mechanism uses specific cellular factors and macromolecular complexes to accurately regulate such bi-directional trafficking of protein cargoes, since this can be a critical point in regulation of protein function. Thus, controlled nuclear entry and exit assures the physical separation of the nuclear genomic material from the other intracellular components and cells take an advantage of this barrier to control access of transcriptional regulators to target genes. To locate an activator protein to the cytoplasm, until a specific signal triggers its access to the nucleus is a simple way to regulate transcription of a particular gene in response to a signaling pathway. Thus, nucleocytoplasmic shuttling can be understood as an additional level of gene regulation and increasing number of focal adhesion proteins found in the nucleus, paxillin being among them, confirms this notion. The nuclear functions of these proteins remain to be discovered.

For many years, lipids were thought to be almost inert structural components of cell membranes and attention was focused on protein-protein interactions. Discoveries in 1980s advanced our understanding of phosphatidylinositol 4,5-bisphosphate (PIP2) as a signalling molecule, but the study of Lassing and Lindberg in 1985 showed for the first time that PIP2 can directly modulate activities of proteins to regulate cellular processes, thus providing the backbone of PIP2 biology. Over the last decade, number of evidence has demonstrated that the nucleus is the site of an autonomous lipid metabolism and predominantly phosphoinositides (PIPs) play here roles other than signaling molecules, and are involved in a direct regulation of nuclear structure and gene transcription in particular. The proposed cellular functions of PIPs have expanded remarkably over the past years but the story is only now emerging.

We bring here new insights into understanding the functions of new members of vast family of transcription regulators: focal adhesion protein paxillin, and the major cellular phospholipid PIP2 which in spite of significant difference in their molecular nature are similar in their functional duality.

CURRENT STATE OF KNOWLEDGE

1. Focal adhesion proteins

The first indication of role for focal adhesions in the regulation of gene expression has come from studies of mammary epithelial cell cultures, which in response to integrin-matrix interaction upregulated transcription of caseine gene and developed a differentiated phenotype (Schmidhauser et al., 1992). The focal adhesions, consisting of more than 50 proteins, are known to participate in substratum anchorage, integrin-dependent signaling and actin dynamics (Zamir and Geiger, 2001). Recently, it appears clear that some focal adhesion components have also ability to travel to the nucleus (Figure 1), where they affect distinct nuclear processes (Hervy et al., 2006).

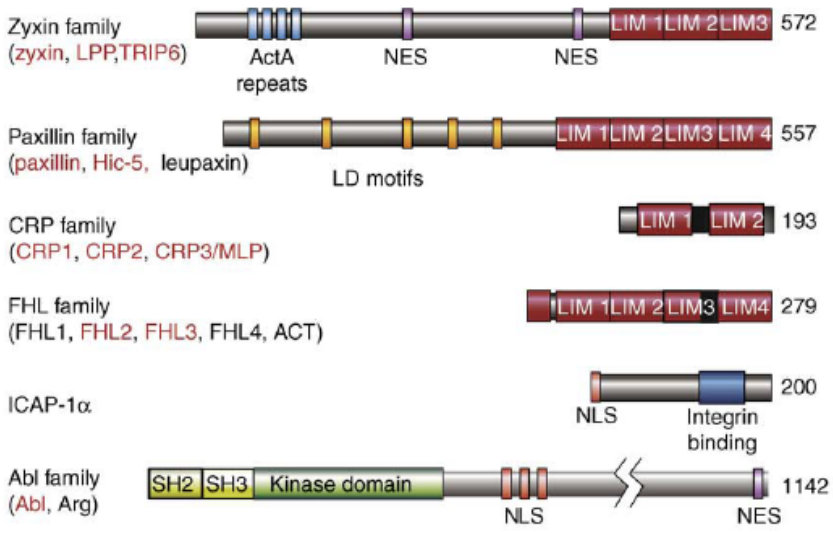


Figure 1 Major families of focal adhesion proteins and their schematic structure

One or more members known to shuttle to the nucleus are shown in red; number of amino acids is shown at the end of each protein; LD - leucine rich motifs, NES - nuclear export signal, NLS - nuclear localization signal, SH2 and SH3 - src homology domains (Hervy et al., 2006).

1.1. Paxillin

Paxillin was first identified as a 68 kDa protein exhibiting a significant phophorylation increase on tyrosine in cells transformed by the src oncogene (Glenney Jr and Zokas, 1989).

Paxillin preferentially localizes to sites of cell adhesion to the extracellular matrix (ECM), which are called focal complexes or focal adhesions. They form a structural link between the ECM and the actin cytoskeleton and are important sites of signal transduction, especially arising from activation of integrins or resulting from stimulation of grow factor receptors (Hynes, 1992).

Paxillin does not exhibit enzymatic activity, but it serves as an adapter that provides platform with multiple docking sites for signalling and structural proteins, and thus facilitates the assembly of multi-protein complexes to coordinate signaling (Schaller, 2001).

Paxillin has significant impact on development, differentitation and tissue morphogenesis. Overexpression of paxillin in cultured myoblasts blocks differentiation instead of promoting cell proliferation (Sastry et al., 1999; Azuma et al., 2005; Mehta and Griendling, 2007). To affect the paxillin activity is main target in several deseases, e.g. fibrosarcoma, chronic myelogenous leukemia, cervical carcinoma, lung and breast cancer. Paxillin is phosphorylated by oncogenic kinases which disrupt focal adhesions as well as actin cytoskeleton, and consequently the interaction of the cell with extracellular matrix promoting invasion and tumorigenesis (reviewed in Sattler et al., 2000).

1.2. Structure of paxillin

The C-terminal half of paxillin contains four LIM domains (Figure 2; motif shared by three transcriptional factors Lin11, Isl-1, Mec-3). They are double-zinc-finger motifs each comprised of two antiparallel β-sheets that are separated by a tight turn. Domain ends with a short α-helix. In addition, two neighbouring zinc fingers pack together due to hydrophobic interactions (Dawid, 1998; Schmeichel and Beckerle, 1994; Perez-Alvarado et al., 1994). The integrity of LIM3 is essential for targeting paxillin to focal adhesions, LIM2 plays a supporting role (Brown et al., 1996). The LIM domains are found in all eukaryotes but are absent in prokaryotes (Kadrmas and Beckerle, 2004).

The N-terminus controls most of its signaling activities. It consists of SH2, SH3-binding domains (Weng, 1993) and five Leu- and Asp-rich LD motifs (LD1-LD5) which have LDxLLxxL consensus sequence (Tumbarello et al., 2002). Despite their small size and high level of sequence conservation, the individual paxillin LD motifs are able to mediate multiple protein interactions that are both overlapping and specific (Brown and Turner, 2004; Turner, 2000; Turner et al., 1999). The LD peptides probably form amphipatic alfa-helices in which

the leucine side chains are arranged on a single face of the helix, presenting a hydrophobic protein-binding surface (Brown et al., 1998a; Sattler et al., 2000; Tumbarello et al., 2002).
In higher eukaryotes, three alternative splice isoforms have been identified. The first isolated isoform, referred to as paxillin α, is ubiquitously expressed. Another two described variants, paxillin β and γ, exhibit restricted expression. (Mazaki et al., 1997).

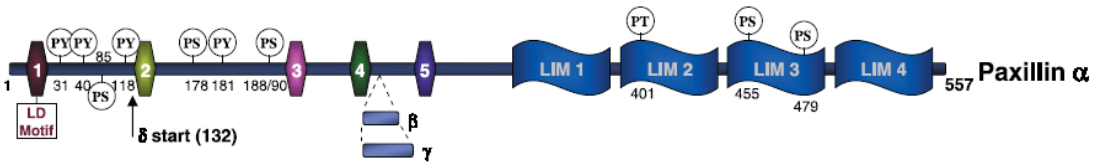


Figure 2 Paxillin isoforms and their structure
All paxillin isoforms contain LIM1–4 domains in the C-terminal half, Leu- and Asp-rich LD1-LD5 motifs in the N-terminal part; paxillin isoform α is shown in full length, isoforms β and γ differs by short insertions between LD4 and LD5; the main phosphorylated (PY – phosphotyrosine, PS – phosphoserine, PT – phosphothreonine) sites are highlighted ; (Brown and Turner, 2004).

Throughout the paxillin molecule, there exist multiple tyrosine, serine and threonine phosphorylation sites (Brown and Turner, 2004; Turner and Miller, 1994; Webb et al., 2005). The phosphorylation of paxillin regulates the interactions of various proteins with their protein-binding motifs (Brown and Turner, 2004). The sites of phosphorylation are in most cases not known.

1.3. Paxillin-binding proteins

Paxillin binds to many proteins that affect focal adhesion dynamics, changes in the organization of actin cytoskeleton, which are necessary for cell motility during embryonic development, wound repair and tumor metastasis (Table I; Turner, 2000; Deakin and Turner, 2008). Two of the earliest paxillin-binding partners identified were vinculin and focal adhesion kinase (FAK). A characterization of the paxillin-binding sites on these two proteins revealed a conserved paxillin-binding sequence (PBS) RSNDKVYENVTGLVKAVIEM VDAKNLLDVIDQARL, that allowed searching for PBS sequences within paxillin binding partners such as PKL, ILK or actopaxin. A mutation of conserved amino acids in this region

eliminates the localization of either protein to focal adhesions, indicating a role for paxillin in determining their subcellular localization (Wood et al., 1994; Tachibana et al., 1995; reviewed in Brown and Turner, 2004). Paxillin also binds to important mediators of Rho-family GTPases, namely Cdc42, Rac, Rho, which regulate the rearrangements of the actin cytoskeleton – activation of Cdc42 stimulates formation of filopodia. Rac stimulates production of lamellipodia, Rho the formation of focal adhesions and actin stress fibers. Balance of these signals coordinates cell motility (Hall, 1998).

Table I Proteins directly interacting with paxillin

MOTIF	BINDING PROTEIN	REFERENCES
LD1	actopaxin vinculin integrin linked kinase (ILK) papillomavirus E6 protein	Nikolopoulos and Turner, 2000 Brown et al., 1996 Nikolopoulos and Turner, 2001 Tong and Howley, 1997
LD2	vinculin focal adhesion kinase (FAK)	Brown et al., 1996
LD3	none actopaxin focal adhesion kinase (FAK)	Nikolopoulos and Turner, 2000 Turner et al., 1999
LD4	Arf GTPase activating proteins (GIT1, GIT2) clathrin poly(A)-binding protein 1 (PABP1)	Turner et al., 1999 Woods et al., 2002
LD5	none	
LIM1	none	
LIM2	tubulin	Herreros et al., 2000
LIM3	tubulin tyrosine phosphatase PTP-PEST	Shen et al., 1998
LIM4	tyrosine phosphatase PTP-PEST	

1.4. Nuclear activity of paxillin

Although paxillin primarily localizes to focal adhesions, recently several evidence appeared detecting translocations of paxillin into the nucleus and proposing its nuclear functions.

The treatment of fibroblasts with leptomycin B caused retention of paxillin in the nucleus, providing evidence for shuttling between cytoplasm and the nucleus (Woods et al., 2002). This observation evoked discovering paxillin NES and NLS. Paxillin does not contain a traditional NLS. Paxillin promotes unconventional translocation sequence, which is not determined yet, or it enters the nucleus in association with other proteins containing a regular NLS (Thomas et al., 1999; Wang and Gilmore, 2003). Recently the paxillin NES was uncovered within the LD4 motif. Its deletion leads to retention of paxillin inside the nucleus. In addition, phosphorylation of Ser272 within LD4 blocks nuclear export and also reduces GIT1, but not FAK1 binding (Figure 3). Curiously, LD4 requires LD3 to behave as a functional NES. There is a hypothesis that LD3 might provide a platform to promote Ser272 dephosphorylation (Dong et al., 2009).

Less is known about regulative activity of paxillin in the nucleus. Translocation of paxillin into the nucleus promotes DNA synthesis and cell proliferation. Whereas increased levels of paxillin (predominantly cytoplasmic) are not able to regulate these processes, the N-terminal-truncated form, which is nuclear-enriched, is sufficient to enhance the number of cells entering the cell cycle and thus cell proliferation (Dong et al., 2009). In the regulation of transcription, paxillin is involved in the regulation of the *H19* gene expression. This finding was discovered after paxillin overexpression (Dong et al., 2009). The *H19* was the first identified imprinted gene located within the *H19*/*IGF2* (insulin-like growth factor 2) locus. The *H19* gene encodes a 2.5 kb fully capped, spliced and polyadenylated, but untranslated RNA (Arney, 2003). In spite of significant interest, the *H19* gene function is not understood. It has been suggested to function as a tumor suppressor as well as promoter of tumorigenesis (Steenman et al., 1994; Okamoto et al., 1997; Berteaux et al., 2005).

More direct function provides the ability to bind steroid receptors, it contributes to transactivation of androgen, glucocorticoid and progesterone receptors, but not the estrogen receptor (Fujimoto et al., 1999; Kasai et al., 2003; Yang et al., 2000).

It si postulated that paxillin iteracts with polyA-binding protein 1 (PABP1), facilitating the export of PABP1-bound mRNAs into the cytoplasm and targeting the ribonucleoprotein complex to sites of protein synthesis at the endoplasmatic reticulum and the leading edge of migrating cells. Paxillin directs targeting of specific mRNAs to nascent focal adhesions, where localized translation affects efficient cell locomotion. The spatial restriction of mRNAs

and their translation control provides a mechanism for regulated gene transcription. In addition, association of PABP1 with paxillin is necessary for efficient nuclear export of PABP1 to the cytoplasm and abolishment of this association interfere with remodeling of focal adhesions during cell migration (Woods et al., 2005).

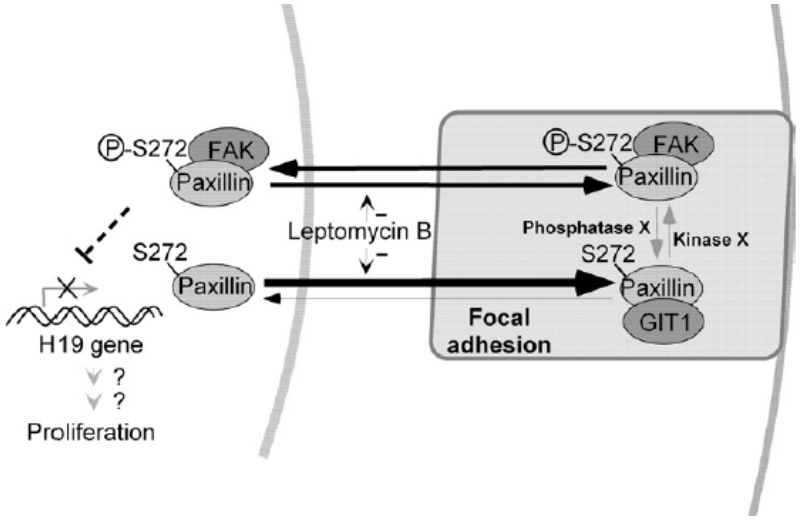


Figure 3 Proposed model for regulation of paxillin nucleocytoplasmic shuttling
Phosphorylation of paxillin at Ser272 favours association with focal adhesion kinase (FAK) and can contribute to nuclear retention of paxillin, where it might also be associated with nuclear FAK. In the nucleus paxillin can suppresses expression of *H19* gene, thus promoting proliferation (Dong et al., 2009).

2. Imprinting

Most genes are expressed biallelically, but a certain number of genes in the mammalian genome exhibit functional inequivalence of the two alleles - depending on the parent of origin, some genes are expressed exclusively from the maternal chromosome and others exclusively from the paternal chromosome. This phenomenon is called genomic imprinting (Figure 4; Tomizawa and Sasaki, 2012).

The imprinted genes play a crucial role in embryogenesis, fetal growth, sex determination, behavioral development, the totipotential state of the zygote, and pluripotency of developmentally early stem cells (Morison and Reeve 1998). The current papers indicate their importance also in the normal brain function and in the postnatal energy homeostasis (reviewed in Bartolomei and Ferguson-Smith, 2011).

The most common theory for explanation of imprinting is the „Haig parental conflict model". It says that imprinting evolved as a result of opposing interests of the maternal and paternal genomes. While the paternally derived genes (father) will favor fetal growth, its muscle mass and body size, at the expense of depleting maternal resources and disadvantaging the further offspring. On the other hand, the maternal genes (mother) will oppose the paternal effect and save resources to ensure the fitness of the mother for the birth and for the future offspring. This model predicts that paternally expressed imprinted genes should promote growth, maternally expressed genes should have opposite effects. Many, but not all imprinted genes identified to date, suit to these predictions (Haig, 1993; Hurst and McVean, 1997; Bartolomei and Ferguson-Smith, 2011).

2.1. Mechanism of imprinting

How does the transcription machinery of the nucleus distinguish between maternally and paternally inherited chromosome homologs and express only one of the two alleles of an imprinted gene?

One of the striking features of vertebrate genome is DNA methylation, the epigenetic modification that differs on the two parental chromosomes (Ferguson-Smith and Surani, 2001; McGrath and Solter, 1984; Reik and Walter, 2001; Surani et al., 1984; Tilghman, 1999; Verona et al., 2003). In the mammalian cells, it is well established that the 5-positioned carbon of cytosine in 5'-CpG-3' dinucleotide is frequently covalently modified by a methyl group and forms the 5-methylcytosine at CpG dinucleotides, which are often found as CG-rich sequences called CpG-islands, can influence transcription (Ohki et al. 2001). The CpG-islands are predominantly associated with the promoters of housekeeping genes (i. e. genes

that are ubiquitously expressed (Bird, 2002) and can also be found near the promoters of imprinted genes, where they form differentially methylated regions (DMR), often also referred as the imprinting control regions (ICR). The ICRs are often associated with enhancers and boundary elements and regulate differential expression of the genes located within the whole cluster (Edwards and Ferguson-Smith, 2007; reviewed in Radford et al., 2011).

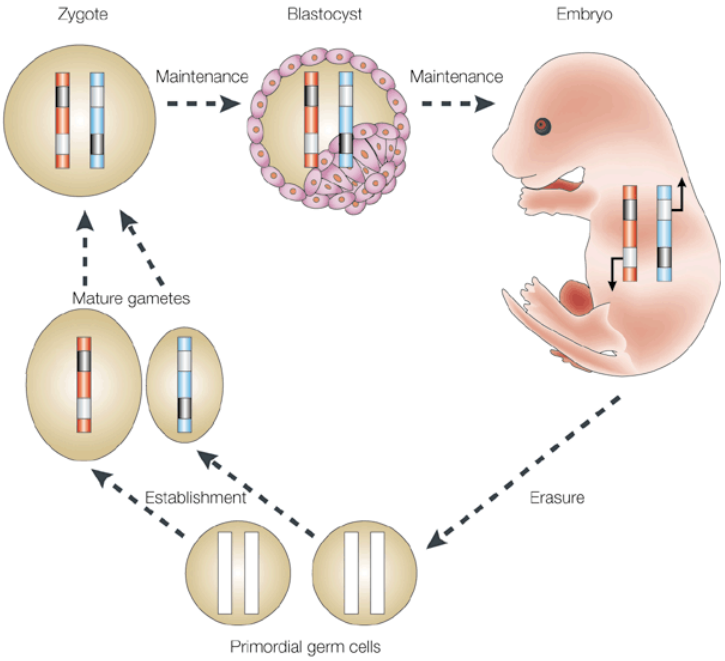


Figure 4 Life cycle of imprinting

The cycle of methylation programming at an ICR begins with the erasure of methylation during the development of primordial germ cells. Subsequently, during oogenesis and spermatogenesis, de novo methylation is differentially reestablished on the maternal and paternal chromosomes in the germline. After fertilization, the preimplantation embryonic genome loses much of its methylation and subsequently de novo methylation is acquired around the time of implantation (Reik and Walter, 2001; reviewed in Radford et al., 2011). However germline imprints appear to be resistant to this postfertilization methylation reprogramming, allowing them to be stably inherited from germline to offspring (Morgan et al., 2005).

2.2. Imprinted genes

The final proof of the existence of genomic imprinting in mammals depended on the identification of genes showing imprinted parental-specific expression. It began with discovery of the insulin-like growth factor 2 gene (*Igf2*) imprinting. The targeted mutation in this gene displayed a heterozygous dwarfing phenotype, but only when the gene was inherited

from fathers, not from mothers. The extent of the growth defect was identical in paternal heterozygotes and homozygotes, suggesting that the father was contributing the entire extent of *Igf2* gene activity. This conclusion was confirmed with molecular methods, and *Igf2* became the first imprinted gene to be identified (DeChiara et al., 1991; Ferguson-Smith et al. 1991). A few months later, *Igf2r* (insulin-like growth factor type 2 receptor) which is a "scavenger" receptor for the growth hormone insulin-like growth factor type 2 (*Igf2*) was identified as a maternally expressed imprinted gene (Barlow et al. 1991). Finally, the *H19* gene (cDNA clone number 19 isolated from a fetal hepatic library) was subsequently shown to be the maternally expressed imprinted gene (Bartolomei et al. 1991).

Since then more than 100 genes have been identified in the primary research model organism – the mouse, with many more predicted to be present (Morison et al., 2005; Brideau et al., 2010). Approximately 50 of them are also imprinted in human. A list of mammalian imprinted genes can be found at www.geneimprint.com or http://igc.otago.ac.nz/home.html.

In addition to mammals and marsupials, imprinted genes have also been identified in the flowering plants. The imprinted chromosomes and chromosomal regions have been reported in insects, while transgenes have identified imprinted chromosomal regions in fish and nematodes (for review see MacDonald, 2012).

A striking feature of imprinted genes is their tendency not to be uniformly distributed as single genes, but rather to cluster together in the genome. These clusters typically contain at least one non-coding RNA and both maternally and paternally expressed imprinted genes (reviewed in Bartolomei and Ferguson, 2011).

2.3. Mammalian *Igf2*/*H19* imprinted cluster

The reciprocal imprinting of the *Igf2* and *H19* genes is mechanistically coupled. The *H19* is maternally expressed and the *Igf2* is paternally expressed. The regulation of imprinting is dependent on the paternal-specific DNA methylation within the differentialy methylated regions (DMRs) to maintain monoallelic expression. In the DNA methyltransferase gene deletion mutants, the *H19* was shown to be expressed in a biallelic manner, whereas the *Igf2* expression was completely lost (Li et al. 1993). The two ICRs exist for the *Igf2* and both are paternally methylated. The DMR2 is located in the exon 6 of the *Igf2* and serves as an enhancer activated by methylation (Constancia et al., 2000; Murrell et al., 2001). In addition, a matrix attachment region (MAR) was found to control proper imprinting of the *Igf2* gene in neonatal liver (Weber et al., 2003). The MARs are conserved regions associating with a

nuclear matrix or scaffold and are implicated in the regulation of chromatin structure (Laemmli et al., 1992; Forrester et al., 1994; Fernandez et al., 2001; Jenuwein et al., 1997; Pemov et al., 1998). The *H19* has one ICR which is located 2-4 kb upstream from the transcriptional start site of the *H19* gene and is also paternally methylated (Bartolomei et al., 1993; Tremblay et al., 1997). Deletions of the *H19* ICR and the *Igf2* DMR1 result in biallelic expression of both the *H19* and the *Igf2* (Arney, 2003).

During the development, both genes share regulatory elements that are located downstream of the *H19*. Two sets of endoderm-specific enhancers are located ~10 kb downstream of the *H19* and these have been shown to regulate the expression of the *H19* as well as the *Igf2* (Figure 5; Leighton et al. 1995b; Kaffer et al. 2000). Recently, these enhancers were shown to be bound by the transcription factor ZAC1, which effects RNA level of these two genes, as well as other imprinted genes (Dlk1 and Cdkn1c; Varrault et al., 2006).

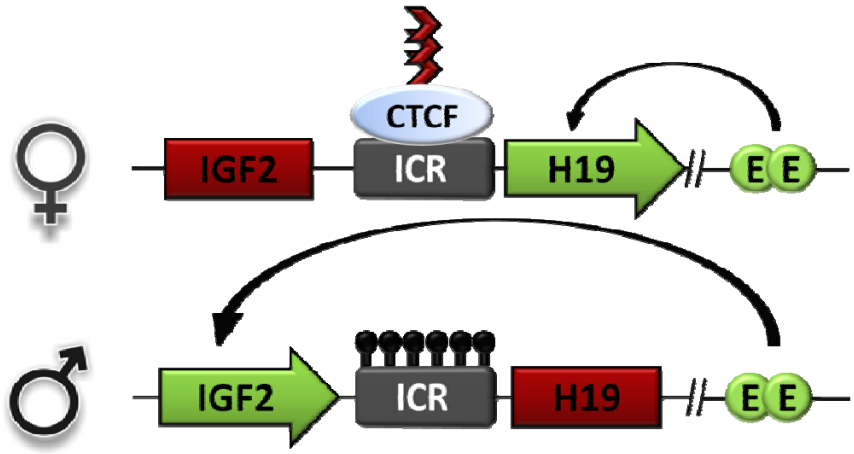


Figure 5 Simplified model of *Igf2/H19* imprinted cluster regulation

The imprinting of this cluster involves the evolutionarily conserved CCCTC-binding factor (CTCF). This zinc-finger insulator protein binds exclusively to the unmethylated maternal ICR (Bell and Felsenfeld, 2000; Hark et al., 2000) and acts as an insulator, blocking access of the shared enhancer to the *Igf2* promoter (Szabó et al., 2004). Paternal methylation of the *H19* ICR inhibits CTCF binding, allowing enhancer to access the *Igf2* promoter on the paternal chromosome (Murrell et al. 2004; Kurukuti et al. 2006); red box – inactive gene, green box – active gene, black lollipops – methylation, dark red arrows – antagonizing barrier.

It was shown that the imprinting mechanism of the *Igf2/H19* locus also involves cohesin-directed intrachromosomal looping. This has produced several models showing each (maternal and paternal) allele to have a particular structure and position in the nucleus due to interactions between different control regions (Weber et al. 2003; Kurukuti et al., 2006,

Murrell et al., 2004, Yoon et al., 2007, Kato and Sasaki, 2005, Han et al., 2008). The Figure 6 shows the latest model of intrachromosomal looping in mammalian cells.

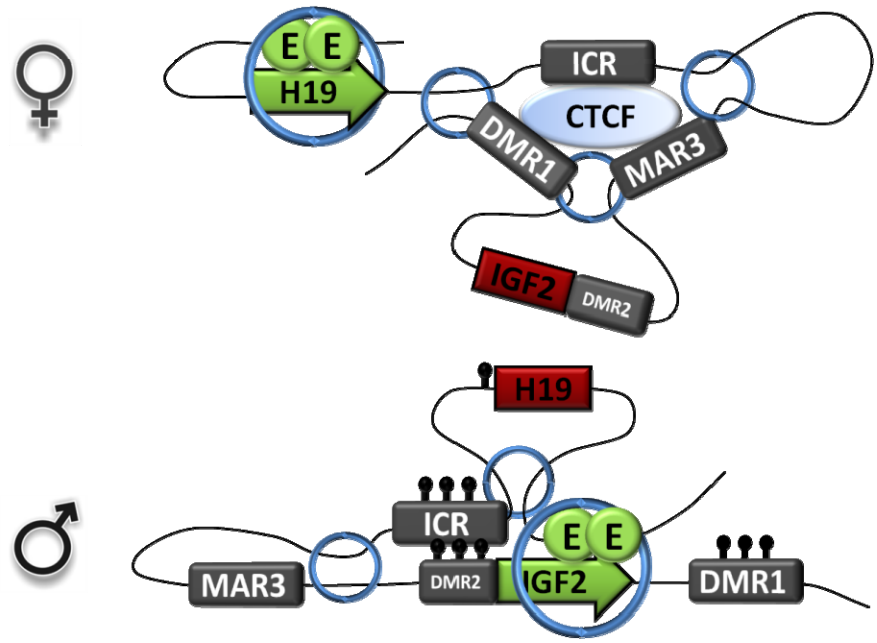


Figure 6 Chromatin looping at mammalian *Igf2/H19* locus

The long-range chromosomal interactions are orchestrated by CTCF and the cohesin complex. On the maternal allele, CTCF and cohesin (blue circles) bring together the unmethylated ICR, DMR1 and MAR3, which sequesters the *Igf2* gene into a silencing loop and prevents interaction between enhancers and the *Igf2* promoter. At the same time, these intra-chromosomal loops prevent DNA methylation of the *H19* ICR and the *Igf2* DMRs (DMR1 and DMR). When CTCF binding at the maternal *H19* ICR is disrupted, *de novo* DNA methylation of the maternal *Igf2* DMR1 and DMR2 have been observed in mouse, suggesting that intrachromosomal looping regulates the entire *Igf2/H19* imprinted region (Kurukuti et al., 2006; Engel et al. 2008; Li et al., 2008a). The enhancers interact with the *H19* promoter and activate its expression. On the paternal allele, the methylated ICR (black lollipops) associates with the methylated DMR2, which moves the *Igf2* into position where its promoter can interact with the enhancers. The methylation spreads to the *H19* promoter that impairs *H19* paternal expression (Nativio et al., 2009; reviewed in MacDonald, 2012). Cohesin binding is only schematic; the red box represents the inactive genes, the green box the active genes; the picture was adopted from Berteaux et al., 2010.

While the *Igf2* locus encodes the insulin-like growth factor-2 (Igf2), which is a growth-promoting peptide hormone, the *H19* locus encodes a non-coding RNA (ncRNA) that gives rise to microRNAs (miRNAs) that have opposite effect on cell proliferation (Gabory et al., 2010). The dual „yin-yang“ function of this locus requires properly balanced expression of *Igf2* and *H19* which is critical primarily for normal fetal development (Reik and Walter, 2001, Pannetier and Feil, 2007, Bartolomei and Ferguson-Smith, 2011).

3. Lipid signalling

The lipid signalling in human cells is an important field of investigation since phosphoinositide signalling has been implicated in the control of nearly all important cellular pathways including metabolism, cell cycle control, membrane trafficking, apoptosis and neuronal conduction. The inositides are now considered the essential co-factors for several nuclear processes, including DNA repair, trancription regulation and RNA dynamics (McCrea and De Camilli, 2009). The phosphatidylinositol 4,5-bisphosphate (PIP2) is utilized by the phospholipase C to generate IP3, a molecule that mobilizes Ca^{2+} stores from the endoplasmic reticulum, and by the phosphatidylinositol 3-kinase (PI3K) to generate PIP3, a critical cell proliferation and transformation signaling molecule (Hokin and Hokin 1953; Streb et al. 1983; Whitman et al. 1988). Later the discovery of direct association of PIP2 with profilactin and profilin provided evidence that PIP2 not only generates the second messengers, but it can also directly modulate the activities of proteins to regulate cellular process (Lassing and Lindberg 1985).

The level of PIPs have been shown to be crucial for the existence and development of many eukaryotes, and deregulation of PIPs and their modifiers may lead to severe pathophysiological disorders and diseases such as cancer, chronic inflammation, myotubular myopathies and Lowe syndrome (Halstead et al., 2005; McCrea and DeCamilli, 2009).

3.1. Phosphoinositides and their structure

The phosphoinositides contain two long hydrophobic fatty acyl tails linked to a glycerol group, which is bound via a phosphodiester bond to the hydrophilic inositol head group. One of the chains is often a saturated fatty acid, while the other is predominantly an unsaturated fatty acid such as arachidonate (Clark et al., 2011; Anderson et al., 2013). How different fatty acyl species affect downstream functions of PIPs is not well understood. The reversible phosphorylation at the 3, 4 or 5 position of the *myo*-inositol ring generates seven different phosphoinositides (Figure 7): PI3P, PI4P, PI5P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (Irvine, 2003). Number of kinases, phosphatases and phospholipases maintain the level of PIPs and the activities of these enzymes may be altered in response to various stimuli, which in turn leads to a change in the profile of PIPs (van den Bout and Divecha, 2009).

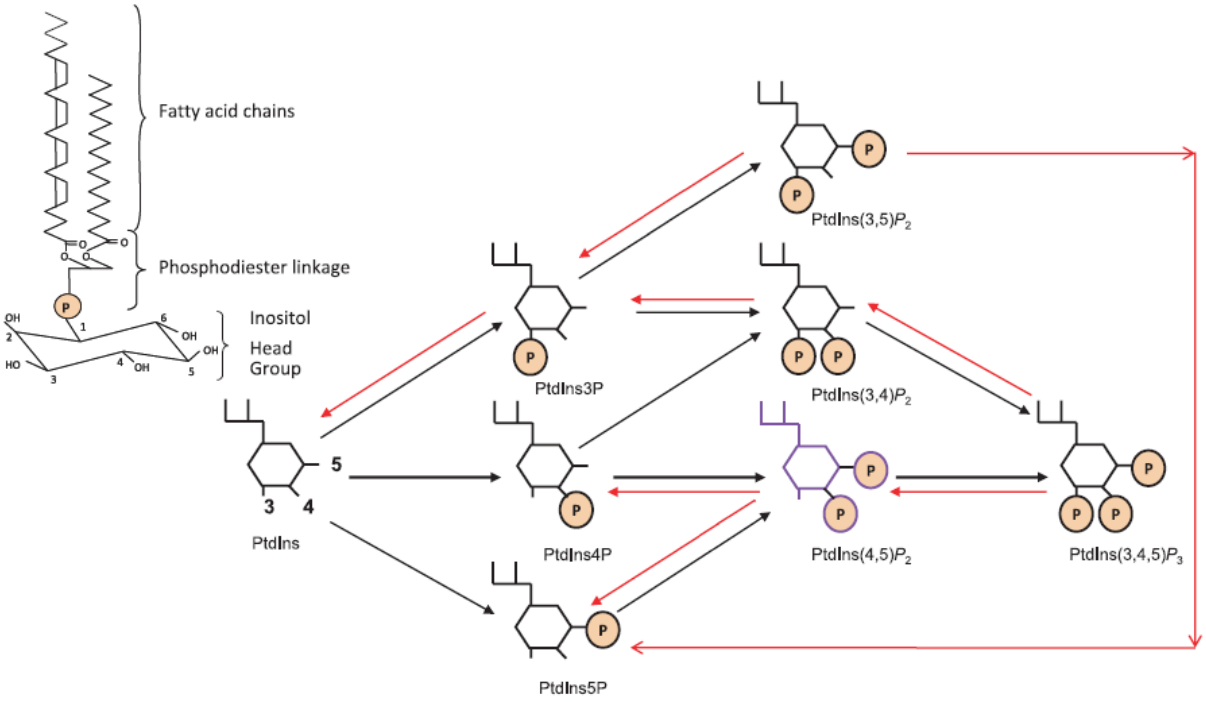


Figure 7 Structure and complex map of nuclear phosphoinositides (PIPs)
The PIPs comprise of two hydrophobic fatty acyl chains and the hydrophilic inositol head group linked to a glycerol group. Number of kinases and phosphatases generate seven different phosphoinositides : PI3P, PI4P, PI5P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3 . The kinase reactions are indicated by black arrows and the phosphatase reactions are indicated by red arrows, for simplicity enzymes are not shown; adopted from Shah et al., 2013.

3.2. Nuclear phosphoinositides (PIPs)

In the cytosol, phosphoinositides (PIPs) are tightly anchored in the membrane, where they act to concentrate the upstream regulators and the downstream targets together leading to faster signalling and specificity (Cocco et al., 1988; McCrea and De Camilli, 2009). The phosphoinositide metabolism also occurs within the nucleus. When the isolated nuclei were incubated with radiolabeled ^{32}P-ATP, radioactivity is incorporated into the phosphatidylinositol phosphates (PIPs; Smith and Wells, 1983a, b, 1984a, b). The physiochemical nature of phosphoinositides within the nucleus is still not clear. In more detailed analysis the nuclei from rat liver were prepared and it was demonstrated that removal of the nuclear envelope correlated with the loss of phospholipids such as phosphatidylcholine, but did not correlate with the loss of phosphoinositides or phosphoinositide kinases, suggesting that the phosphoinositides are present within the nucleus rather than in the nuclear membrane (Divecha et al., 1991 and 1993; Payrastre et al., 1992). Both the nuclear and cytoplasmic PIPs share regulatory enzymes, most of them are not exclusively localized to

cytoplasm or the nucleus (Boronenkov et al., 1998; Doughman et al., 2003; Schill and Anderson, 2009). Thus PIP2 may be synthesized in the nucleus. The probes used for PIPs localization interact with its head group, suggesting that at least some of the PIP2/PIP3 must have the inositol head group localized outwards. It is not clear how the fatty acid tails are constrained, but they may form micelles or may interact with the proteins that hide their hydrophobic characteristic. The interaction of PIP2 with a myristoylated nuclear transcription factor is a possible mechanism for sequestration of the fatty acid tail (Toska et al., 2012).

The phosphatidylinositol 4,5-bisphosphate (PIP2) is the predominant regulatory molecule not only in the cytoplasm, but also in the nuclear compartment. There are three classes of phosphatidylinositol phosphate kinases (I, II and III). Type I and II kinases both generate PIP2, although by utilizing different substrates, PI4P and PI5P, respectively (Rameh et al., 1997; Anderson et al., 1999; Halstead et al., 2005; Fiume et al., 2012). A metabolic radioisotope labeling in the rat liver nuclei revealed that the predominant way of PIP2 production in the nucleus is phosphorylating PI4P by the type I PIPK as it is also in a whole cell (Vann et al., 1997)

3.3. Diverse functions of nuclear phosphoinositides

The presence of PIPs in the nucleus itself did not draw as much attention as the fact that their levels change in response to stimuli such as differentiation or insulin growth factor receptors such as insulin growth factor I (IGF1; Cocco et al., 1987 and 1988; Divecha et al., 1991 and 1995). The change in nuclear PIPs without apparent changes in the cytoplasmic pool of PIPs indicate a specific signalling role for PIPs within the nucleus (Divecha et al., 1991). Changes in the nuclear PIPs have been documented in response to cell-cycle progression, DNA damage, oxidative stress and UV irradiation, differentiation and growth factor stimulation (Divecha et al., 1993; Divecha and Irvine, 1995; Fiume et al., 2012). The functions of various PIPs are summarized in Table II.

Table II Functions of various nuclear phospholipids

Inositol molecule	Enzyme	Localization	Effectors	Function	Reference
PI3P	PI3-kinase	Nucleolus, NM	?	Cell cycle regulation	Gillooly et al., 2000; Visnjic and Banfic, 2007
PI4P	PI4-kinase	NM	?	Cell cycle, PI4,5P2 precursor	Payrastre et al. 1992

PI5P	PI5-kinase	NM, chromatin	ING, others?	Chromatin organization, apoptosis, DNA damage	Gozani et al., 2003
PI3,4P2	SHIP-2, PIPK IIβ	Membrane, Nuclear speckles	?	Pre-mRNA splicing	Yokogawa et al., 2000; Déléris et al., 2003
PI4,5P2	PIPK Iα, PIPK Iiβ	Membrane, Nuclear speckles, NM, Chromatin	Star-PAP, ING2, ALY, BRG1	3´-end processing, splicing, chromatin organization, precursor IP3	Osborne et al., 2001; Mellman et al., 2008
PI3,4,5P3	PIPK I, PIP3K	NM	PIP3BP, others?	Cell cycle, differentiation, proliferation	Tanaka et al., 1999; Neri et al., 2002
DAG	PI-PLC	NM	?	Cell cycle, differentiation, proliferation	Goto et al., 2006
Ins4,5,P3	PI-PLC	NM	?	Calcium signaling	Echevarría et al., 2003
IPn	IPn kinases	NM, chromatin	?	mRNA export, chromatin structure	York and Majerus, 1994; Steger et al., 2003

3.4. Nuclear functions of PIP2

The phosphoinositol 4,5-bisphosphate (PIP2) as well as other phosphoinositides and PIPKs are localized in the nuclear speckles, which are highly enriched in mRNA splicing factors and were suggested to play a role in pre-mRNA splicing, 3-end processing and export (Spector, 1993; Yu et al., 1998; Boronenkov et al., 1998; Osborne et al., 2001; Gozani et al., 2003; Gonzalez et al., 2008; Mellman et al., 2008). Osborne et al. also showed that PIP2 co-immunoprecipitates with snRNPs, the hyperphosphorylated form of RNA polymerase II and snRNAs (Osborne et al., 2001). The speckle morphology changes with the transcriptional activity, the nuclear speckles become smaller and more numerous when transcriptional activity is increased, and become larger and fewer upon inhibition of transcription (Spector et al., 1983; Davis et al., 1993). Interestingly, PIP2 and PIPKs reorganize with the speckle morphology upon changing transcriptional activity (Boronenkov et al., 1998). In addition, immunodepletion of PIP2 from nuclear extracts attenuates in vitro splicing (Osborne et al., 2001).

The localized synthesis of PIP2 by PIP5K plays a key role in regulation of Star-PAP polyadenylate polymerase, that polyadenylates a subset of stress adaptation-related mRNAs, since PIP2 dramatically and specifically stimulates its activity *in vitro* (Mellman et al., 2008).

Recently, a role for PIP2 has also been suggested in mRNA export. The PIP2 molecule interacts with REF/ALY, a component of TREX complex, and mutation of interaction-mediating residues attenuates mRNA export (Okada et al., 2008).

The level of the nuclear phosphoinositides fluctuates significantly in a cell cycle-dependent manner. The recent reports indicate that nuclear phosholipids and related enzymes are particularly important during G1/S transition and S phase. The contribution of PIP2 in S phase entry has been linked to the cyclin A2 and cyclin-dependent kinases (cdks). In the Swiss 3T3 cells the nuclear PIP2 down-regulation may cause a delay in a phorbol ester-induced S phase entry and this was caused by cyclin A2 downregulation, thus identifying cyclin A2 as a downstream effector of the nuclear PIP2 signalling network (Nelson et al., 2008). The overexpression of PLCβ1 upregulates the expression of cyclin D3 and its kinase cdk4, which enhances progression through G1 phase (Faenza et al., 2003). The overexpression of a lipid phosphatase PTEN leds to decreased nuclear levels of cyclin D1, decreased pRb phosphorylation and growth arrest at the G1/S checkpoint (Radu et al., 2003). Together, these observations point to a modulation of cyclins as one mechanism by which PIP2 and nuclear lipid-related enzymes regulate the cell cycle progression.

There is also an emerging evidence that some actively transcribed genes localize to the nuclear periphery (inner nuclear membrane) and nuclear pore complexes, positioning them for a modulation by phosphoinositides (Akhtar and Gasser, 2007). It was observed that the addition of negatively charged lipids lead to a chromatin decondensation in vitro, whereas addition of positively charged lipids resulted in more condensed chromatin (Capitani et al., 1981; Kuvichkin, 2002). It was further shown that PIP2 can bind to the C-termini of histone H1, a potent inhibitor of RNA polymerase II. The PIP2 binding releases H1 from DNA and thus reverses the inhibition of RNA polymerase II, which may be a mechanism for PIP2 regulation of chromatin remodeling (Yu et al., 1998).

The interaction of PIP2 with RNA polymerase II, specifically with the phosphorylated form, is involved in regulating its transcriptional pausing and elongation (Osborne et al., 2001; Shilatifard et al., 2004). The PIP2 also regulates Polymerase I-mediated transcription of ribosomal RNA, perhaps through direct interaction with Polymerase I (Mortier et al., 2005; Yildirim et al., 2013).

The recent data proposed a different mechanism for involvement of PIP2 in chromatin remodeling. In the T-lymphocytes the SWI/SNF-like chromatin remodelling complex BAF (Brg/Brm-associated factor) is targeted to chromatin and to the nuclear matrix upon activation, in a PIP2-dependent mechanism (Zhao et al., 1998; Rando et al., 2002).

The PIP2 also interacts with myristoylated BASP1, a transcriptional repressor of the Wilms' tumour suppressor protein. A myristoylation-preventing mutation reduces PIP2 binding, inhibits recruitment of histone deacetylase 1 (HDAC1) to BASP1 at the promoter and blocks WT1 repression (Toska et al., 2012).

AIMS

PART I - NUCLEAR FUNCTION OF PAXILLIN

The nuclear localization of paxillin has been linked to the regulation of gene expression, especially the *H19* gene (Dong et al., 2009). However the exact mechanism how paxillin regulates nuclear events remains unclear and its understanding requires answering these questions:

1) **What is the mechanism underlying paxillin role in gene expression?**

2) **What is the paxillin role in respect to the *IGF2/H19* imprinted cluster?**

3) **Which proteins/protein complexes interact with paxillin in the nucleus?**

PART II - NUCLEAR PIP2

Apart from the role of PIP2 in the cytoplasmic signalling, its presence in the nucleus indicates a distinct nuclear functions. However, little is known about its properties and functions in the nucleus. Our lab has recently shown the interaction of nucleolar PIP2 with proteins involved in rRNA transcription and processing, namely RNA polymerase I (Pol I), UBF, and fibrillarin (Yildirim et al., 2013). Here we extended this study by answering these questions:

1) **Does the interaction between PIP2 and RNA Pol I trancription complex depend on the active transcription or it is preserved throughout the cell cycle?**

2) **What is the detailed localization of nuclear PIP2?**

3) **What are the possible functions of PIP2 islets? Do they affect the RNA polymerase II transcription machinery?**

RESULTS

I. NUCLEAR FUNCTION OF PAXILLIN

I.I. Paxillin knockdown promotes gene *H19* expression and slows down proliferation in human HepG2 cells

The overexpression of paxillin in mouse cells was shown to block *H19* expression (Dong et al., 2009). To explore the role of human paxillin in expression of *H19*, we depleted paxillin from HepG2 cells by lentiviral transduction of short-hairpin RNA (shRNA) directed against human paxillin. As expected, paxillin shRNA (shPXN) reduced both paxillin transcripts (Figure 8A) and protein level (Figure 8B) to about 20% of paxillin level in control cells.

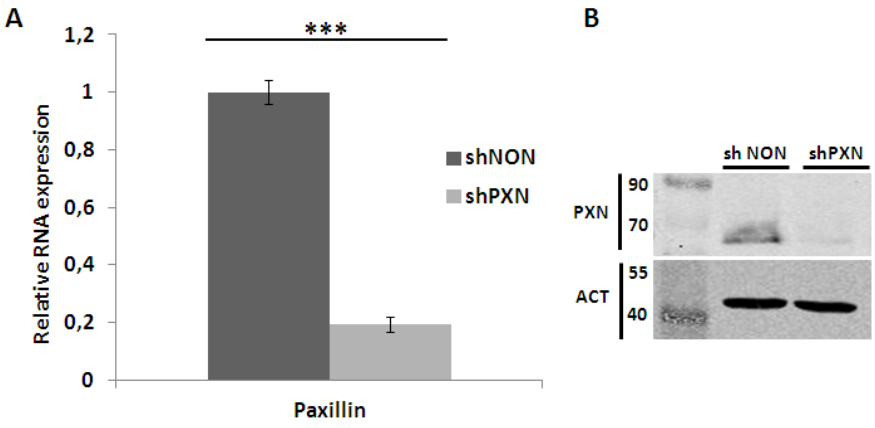


Figure 8 Knockdown of paxillin in HepG2 cells

A) Expression level of paxillin mRNA in paxillin-depleted (shPXN) and control (shNON) HepG2 cells. QPCR data were normalized to GAPDH gene and then to control shNON sample. **B)** Protein level of paxillin in paxillin-depleted and control HepG2 cells. Actin (ACT) was used as protein amount control. Data are presented as means ± standard deviations; n=5; ***$p < 0{,}001$, Student`s t-test.

Importantly, in accordance with published data, the depletion of paxillin resulted in upregulation of *H19* transcription by about 2-fold (Figure 9A) compared to control cells. Three different clones of shPXN were tested with similar results. The clone with the highest knockdown efficacy was selected for further experiments. Genes *H19* and *IGF2* form a gene cluster on human chromosome 11 and share DNA as well as protein regulatory elements (Wendt et al., 2008; Yao et al., 2010). Therefore, we have also tested the effect of paxillin depletion on *IGF2* expression. We observed a slight decrease in *IGF2* expression, however, the difference was not statistically significant ($p > 0{,}05$, Figure 9A).

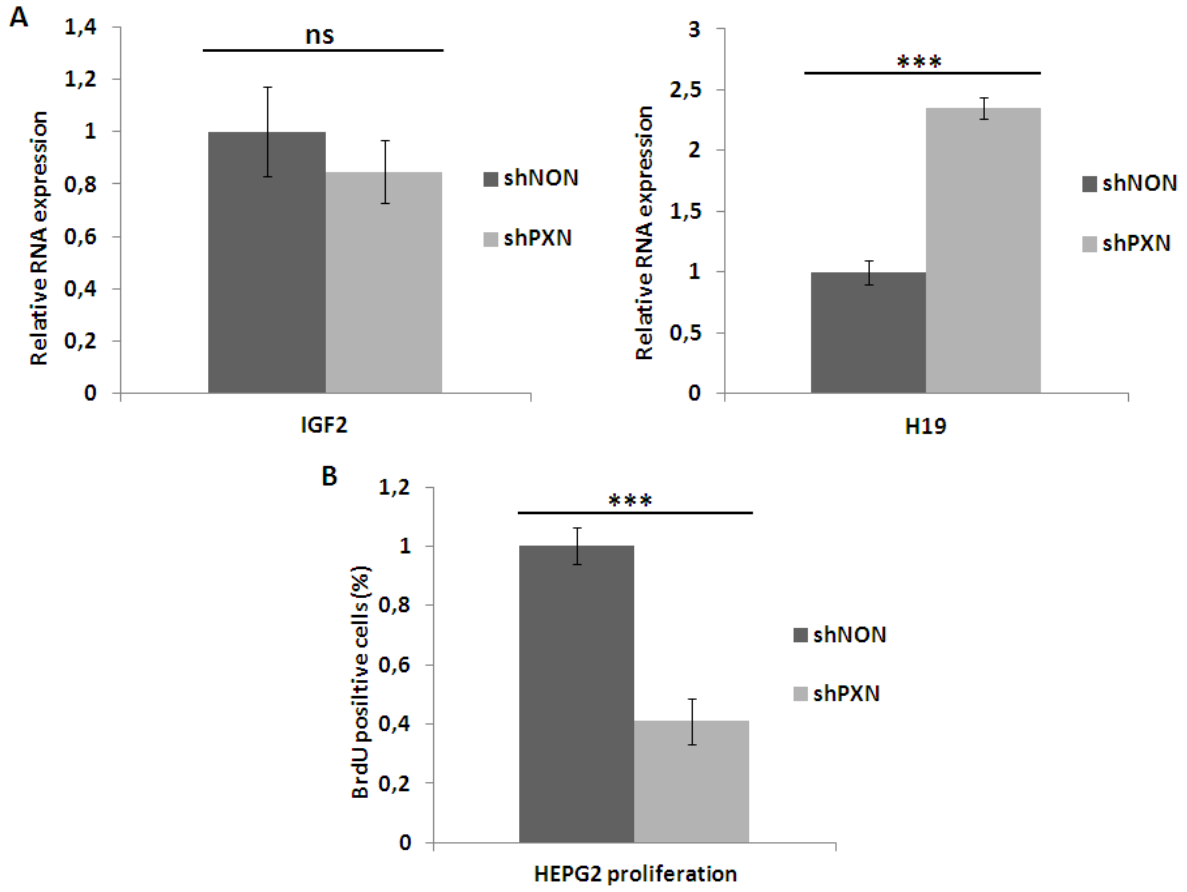


Figure 9 Paxillin affects the expression of *H19* and regulates cell proliferation in HepG2 cell line

A) Quantitative PCR analysis showed that paxillin depletion by shRNA (shPXN) results in upregulation of *H19* compared to control (shNON), but no effect on *IGF2* was observed in HepG2 cells. Data were normalized to GAPDH gene and then to control shNON sample; **B)** Paxillin depletion (shPXN) resulted in decreased number of cells incorporating BrdU, thus undergoing replication. Data are shown as means ± standard deviations from at least 3 independent experiments; ***$p < 0{,}001$; ns: $p > 0{,}05$; Student`s t-test.

H19 and *IGF2* genes have the opposite effect on the cell proliferation. While the *IGF2* encodes a growth-promoting peptide hormone, the *H19* non-coding RNA gives rise to proliferation-repressing microRNAs (Cai and Cullen, 2007; Gabory et al., 2010; Dey et al., 2014). Therefore we asked how paxillin depletion in HepG2 cells affects cell growth and if differences in cell growth reflect the expression changes of proliferation-regulatory genes. Proliferating cells were detected by incubation with synthetic thymidine analog BrdU which is incorporated into newly synthesized DNA during S phase. BrdU assay showed that knockdown of paxillin and subsequent *H19* upregulation results in decreased number of cells

incorporating BrdU within DNA (Figure 9B). In other words, less cells undergo replication and thus, consistently with *H19* growth-repressing function, when paxillin level is decreased the proliferation of HepG2 cells is slowed down.

I.II. Paxillin knockdown does not impair imprinting of *IGF2*/*H19* gene cluster

Next, we tested if the expression change of *H19* following the paxillin knockdown is a consequence of a deregulated imprinting. Thus, we examined allele-specific expression of *H19* and also *IGF2*. To distinguish between alleles, we used single nucleotide polymorphism (SNP; Figure 10A). SAOS2 cell line was selected for these experiments (Figure 10C) since in the SAOS2 cells the *IGF2*/*H19* polymorphisms in genomic DNA are heterozygous (Figure 10B), which is indispensable for discriminating between mono- and bi-allelic expression.

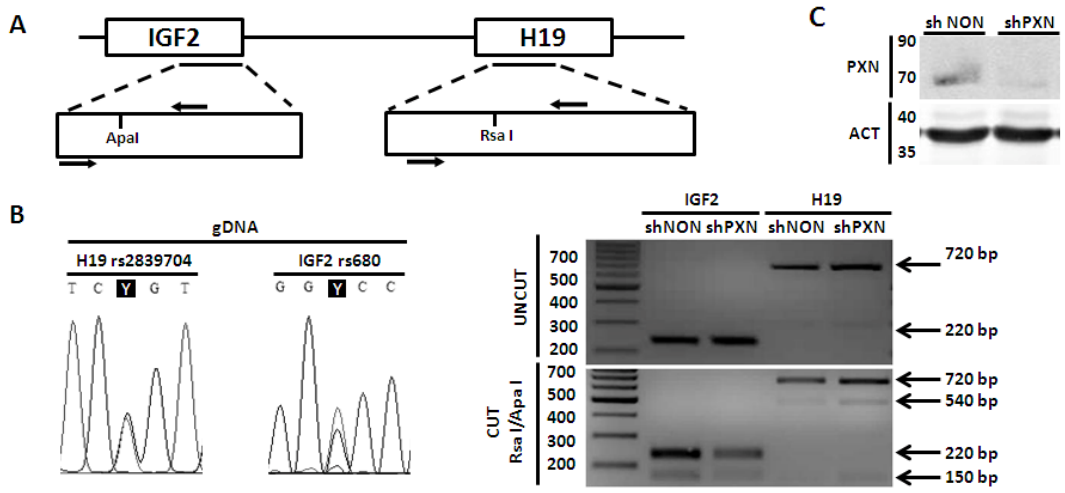


Figure 10 Analysis of single nucleotide polymorphisms in genomic DNA of SAOS2 cells
A) Diagram of *IGF2*/*H19* locus and amplified regions, where arrows show location of primers for analyzed polymorphisms (*IGF2* – ApaI, *H19* – RsaI). **B)** Sequencing as well as restriction analysis showed heterozygosity of *H19* SNP rs2839704 and *IGF2* SNP rs680 in genomic DNA os SAOS2 cells. Polymorphic sites are marked with „Y“ in black square. *IGF2* SNP rs680 (uncut - 220 bp, cut - 150 and 70 bp; 70bp fragment migrated out of the gel) and Rsa I for *H19* SNP rs2839704 (uncut - 720 bp, cut – 540 and 180 bp; 180bp fragment is not marked). Restriction site is present on one allele only. *H19* primers are spanning an intron and, therefore, the PCR fragment amplified from gDNA is bigger than cDNA-amplified fragment. **C)** Protein level of paxillin in paxillin-depleted and control SAOS2 cells. Actin (ACT) was used as protein amount control.

RNA isolated from control and paxillin knockdown cells was reverse transcribed into cDNA, the region covering SNP rs680 for *IGF2* and rs2839704 for *H19* was PCR amplified, and

digested with ApaI or RsaI for *IGF2* SNP or *H19* SNP respectively (Figure 11B). The analysis clearly showed that *H19* retained expressed from maternal allele only, both in control and paxillin knockdown cells, thus, excluding the hypothesis that lack of paxillin affects imprinting of *H19*. *IGF2* showed biallelic expression in both control and paxillin knockdown cell lines. Thus, we deduce that in HepG2 cells *IGF2* imprinting is deregulated regardless of paxillin expression and independent of *H19*.

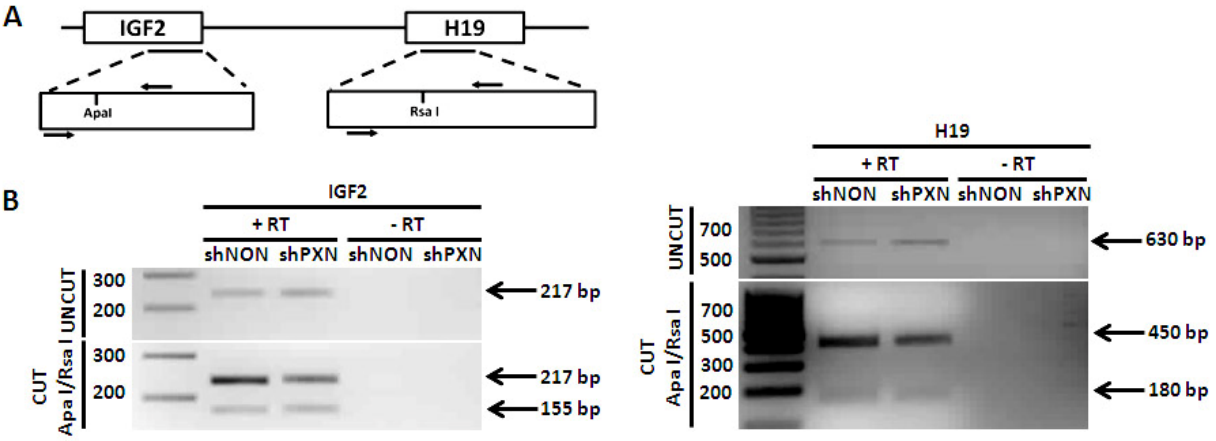


Figure 11 Paxillin depletion does not change allele-specific expression of *IGF2* and *H19*
A) Diagram of *IGF2*/*H19* locus and amplified regions; arrows show location of primers for analyzed polymorphisms (*IGF2* – ApaI, *H19* – RsaI). **B)** *IGF2* showed biallelic expression in paxillin-depleted (shPXN) as well as control cells (shNON): undigested 217bp fragment as well as digested 155bp fragment were present after the restriction digest showing that both alleles are transcribed. *H19* retained monoallelic expression in both shPXN and shNON cells: no undigested 656bp fragment, but only digested 488bp and 168bp fragments were detected after restriction digest suggesting that only one allele is transcribed. *IGF2* SNP rs680, cut with Apa I (uncut - 220 bp, cut - 150 and 70 bp; 70bp fragment migrated out of the gel); *H19* SNP rs2839704, cut with RsaI (uncut - 630 bp, cut – 450 and 180 bp). Restriction site is present in one allele only. + RT reverse transcription included; - RT reverse transcription ommited to control possible contamination with gDNA.

The monoallelic expression of *H19* is further controlled by methylation of imprinting control region (ICR) which lies upstream of *H19*. To test if paxillin plays a role in ICR methylation, we tracked changes in the ICR methylation pattern after paxillin knockdown. Isolated genomic DNA was bisulfite-treated, the region corresponding to ICR covering 13 CpG islands (specified in Figure 12A) was PCR amplified, cloned into a vector and sequenced. The selected region contained polymorphic site rs2071094 enabling us to differentiate between alleles (parent-origin is not known). We analyzed then the methylation status of each allele. Consistently with the observation that paxillin had no effect on the allelic expression of *IGF2*/*H19* (see Figure 11B), the ICR methylation was properly maintained in the control

sample as well as in the paxillin knockdown cells and no gain of methylation on the second allele was observed (Figure 12B). Thus, we conclude that the ICR imprinting barrier of *IGF2*/*H19* cluster is not affected by paxillin knockdown. Therefore, the elevated *H19* expression is not a consequence of expression from both alleles.

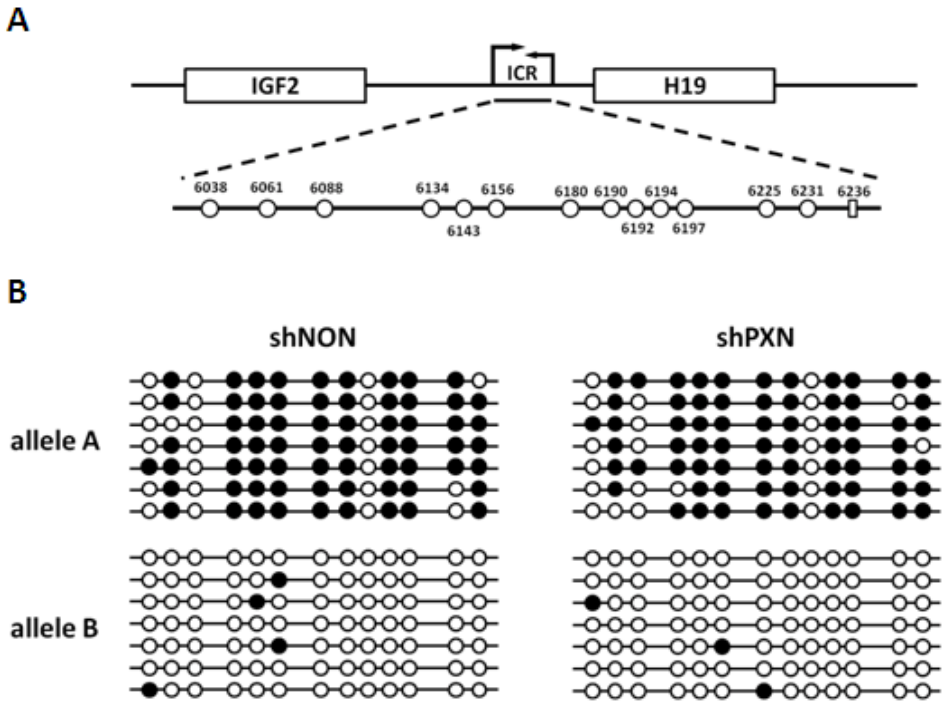


Figure 12 Methylation of ICR is preserved in paxillin-depleted cells
A) A diagram of the analyzed segment of ICR. The enlargement shows particular CpG islands. Rectangle represents single nucleotide polymorphism used to distinguish between the alleles, arrows show position of primers used for amplicifation; base numbering was performed according to GenBank accession number AF087017. **B)** Sequencing of bisulphite-treated genomic DNA from paxillin-depleted (shPXN) or control (shNON) SAOS2 cells showed preserved methylation of one allele. Alleles were distinguished by SNP rs2071094. Each line represents a single sequenced PCR product; black circles – methylated CpGs, white circles – unmethylated CpGs.

I.III. Paxillin regulates *IGF2* and *H19* promoter activity via their shared distant enhancer

To explore the effect of paxillin on *IGF2* and *H19* promoter activity, we constructed luciferase reporter vectors. *IGF2* and *H19* promoters from HepG2 genomic DNA were inserted upstream of Firefly luciferase gene (p*H19*P-luc, p*IGF2*P3-luc; Figure 13A). Whereas *H19* is transcribed from a single promoter, the expression of *IGF2* is controlled by four different promoters (P1, P2, P3, P4; Sussenbach, 1989; Holthuizen et al., 1990; van Dijk et

al., 1991). We observed that 95% of *IGF2* RNA is transcribed from P3 in HepG2 cells (data not shown), thus we used P3 promoter in further experiments. Then, we examined luciferase activity in paxillin knockdown as well as in control cells. Surprisingly, no change in luciferase activity was detected after paxillin knockdown (Figure 13B), suggesting that promoter activity was not affected by reduced paxillin level.

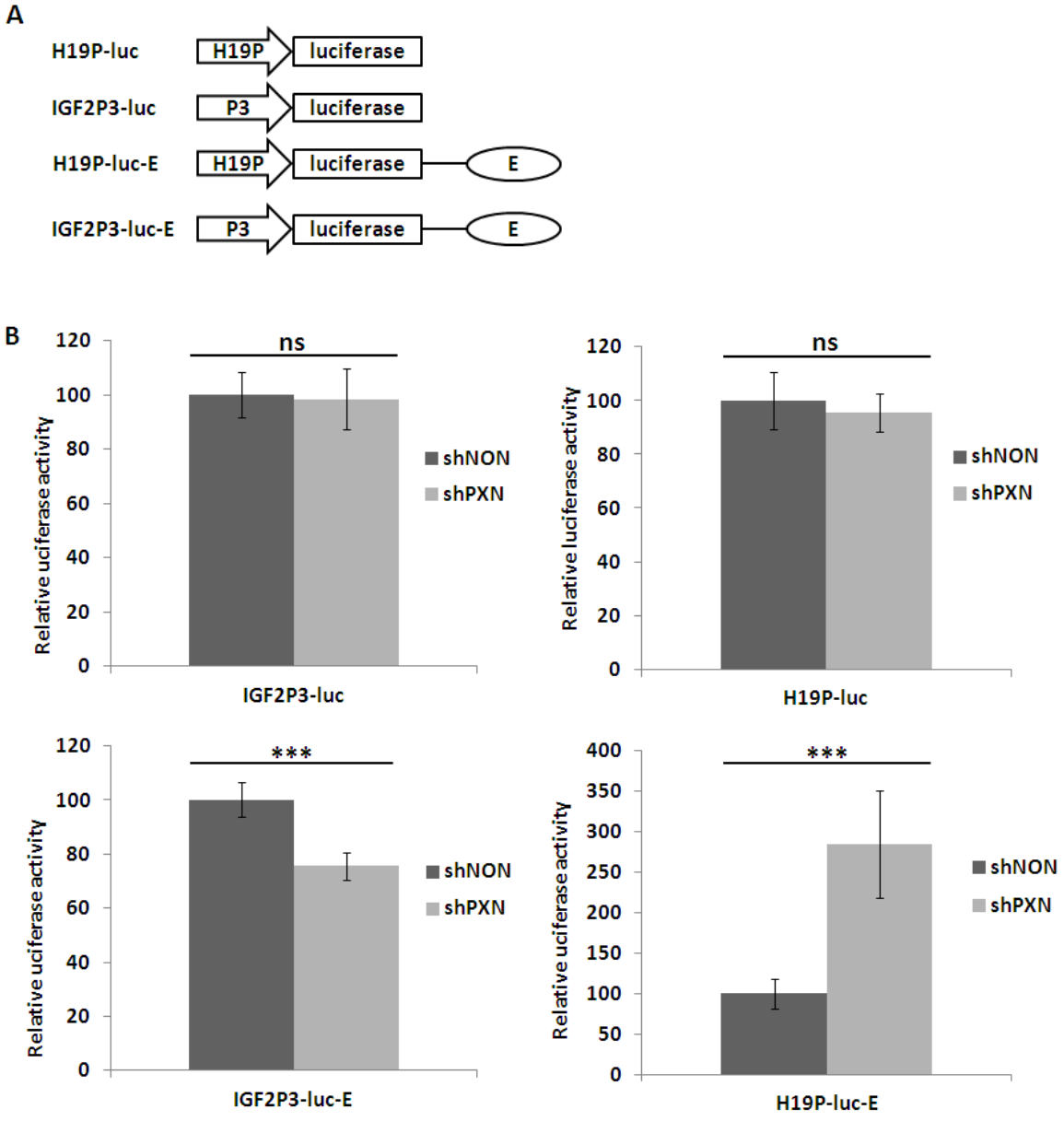


Figure 13 Paxillin regulates *IGF2* P3 and *H19* promoters via their shared enhancer
A) A scheme of luciferase constructs. *H19*P-luc or *IGF2*P3-luc consisted of Firefly luciferase gene (luc) driven by *H19* promoter or *IGF2* promoter 3 (P3), respectively. *H19*P-luc-E or *IGF2*P3-luc-E contained in addition *H19* enhancer sequence located downstream of luc gene. **B)** Paxillin requires enhancer to be able to regulate the *IGF2* P3 and *H19* promoters. Depletion of paxillin (shPXN) increases the activity of *H19* promoter and decreases activity

of the *IGF2* promoter via their shared enhancer, compared to control (shNON). Data were normalized to activity of Renilla luciferase. Results are presented as means ± standard deviations from at least 3 independent experiments; ***p < 0,001; Student`s t-test.

Expression of *IGF2*/*H19* genes is mediated by a distant enhancer, which is located downstream of *H19* coding region. Both genes, *IGF2* and *H19*, compete for this enhancer (Leighton et al., 1995b; Kaffer et al., 2000). We decided to add 5 kb-long region from +6033 to +10972 relative to *H19* transcription start site into the luciferase reporter vectors downstream the luciferase gene, mimicking its position to the *IGF2*/*H19* genes (p*H19*P-luc-E, p*IGF2*P3-luc-E; Figure 13A), and measured luciferase activity in paxillin knockdown as well as in control cells. Strikingly, paxillin knockdown resulted in approximately 3-fold increase in luciferase activity when driven by the *H19* promoter-enhacer, while 0,8-fold decrease when driven by *IGF2*P3-enhancer (Figure 13B). Interestingly, this result is consistent with the observed expression changes of the respective endogenous genes in paxillin knockdown cells (see Figure 9A).

Thus, the lack of paxillin increases the activity of *H19* promoter via its enhancer and decreases activity of the *IGF2* promoter. In other words, normal paxillin levels are likely to have a repressive effect on the *H19* promoter and a positive effect on the *IGF2* promoter. Importantly, paxillin does not regulate these promoters directly, but via the distant shared enhancer.

I.IV. Paxillin interacts with *IGF2*P3/*H19* promoters and their enhancer

As a next question we asked if regulation of *IGF2*/*H19* promoters by paxillin occurs through an interaction of paxillin with the regulatory DNA elements of both genes including *IGF2*/*H19* promoters, shared enhancer or ICR. Therefore, we performed chromatin immunoprecipitation (ChIP) to detect paxillin-DNA complexes in HepG2 cells. ChIP analysis revealed that paxillin interacts predominantly with the *H19* promoter. The interaction of paxillin with *IGF2* P3 promoter and their shared enhancer was weaker while no binding to the ICR region was detected (Figure 14B). For enhancer sequence (+6033 to +10972 bp), we used three different primer sets (A, B, C; Figure 14A) to cover the whole region. Set A amplified the region at the 5‘end (+6189 to +6363) of the enhancer, set B amplified the region approximately in the middle (+7814 to +8038) and set C the region close to the 3‘end (+9978 to +10217). Paxillin was enriched only at the region amplified by set A, which suggests that paxillin interacts with the very begining of the enhancer sequence (Figure 14B). Taken

together, these results indicate that paxillin binds both key transcription elements – promoter and enhancer – and might therefore regulate their mutual interaction.

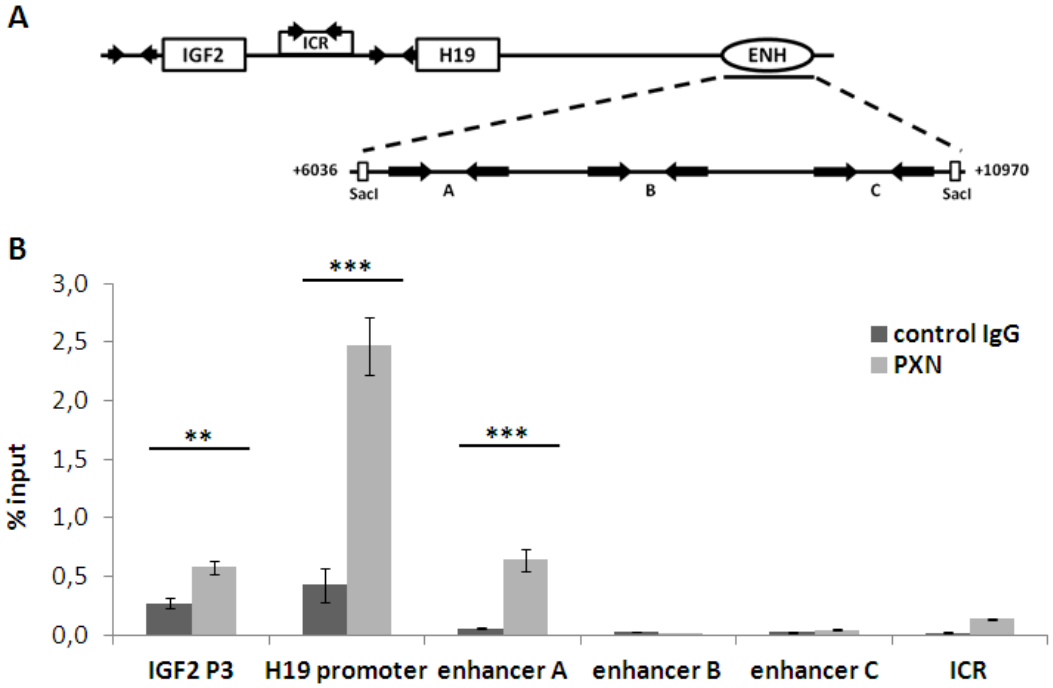


Figure 14 Paxillin interacts with *IGF2*P3/*H19* promoters and their enhancer
A) A scheme of *IGF2*/*H19* cluster, where arrows indicate designed primers. **B)** Chromatin immunoprecipitation (ChIP) using anti-paxillin antibody (PXN) or mouse IgG (control IgG) showed specific enrichment of paxillin at *H19* promoter, *IGF2* promoter 3 (P3) and 5´ end of the enhancer. No binding to the ICR region was detected. For enhancer sequence (+6033 to +10972 bp) we used three different primer sets (A, B, C). Set A amplified the region at the 5'end (+6189 to +6363) of the enhancer, set B amplified the region approximately in the middle (+7814 to +8038) and set C the region close to the 3'end (+9978 to +10217). GAPDH gene was used as a negative control site. Results are presented as means ± standard deviations from at least 3 independent experiments; ***p < 0,001; **p < 0,01; Student`s t-test.

I.V. Paxillin regulates the *IGF2*P3/*H19* promoter-enhancer interaction

Since we have shown that paxillin regulates activation of both promoters via the shared enhancer and it also occupies these regulatory elements, we speculated that paxillin might stimulate forming of long-range chromatin loops between *IGF2* P3/*H19* promoter and enhancer. To test this hypothesis, we used Chromatin Conformation Capture (3C) technology that allows for studying physical interactions between chromosomal regions (Dekker et al., 2002). Instead of mapping all the interactions at the *IGF2*/*H19* locus, we focused on comparing *IGF2*P3/*H19* promoter-enhancer interaction frequency between control and

paxillin knockdown cells. Interaction frequency was examined using promoter as an anchor and enhancer as a test region (schematically shown in Figure 15A).

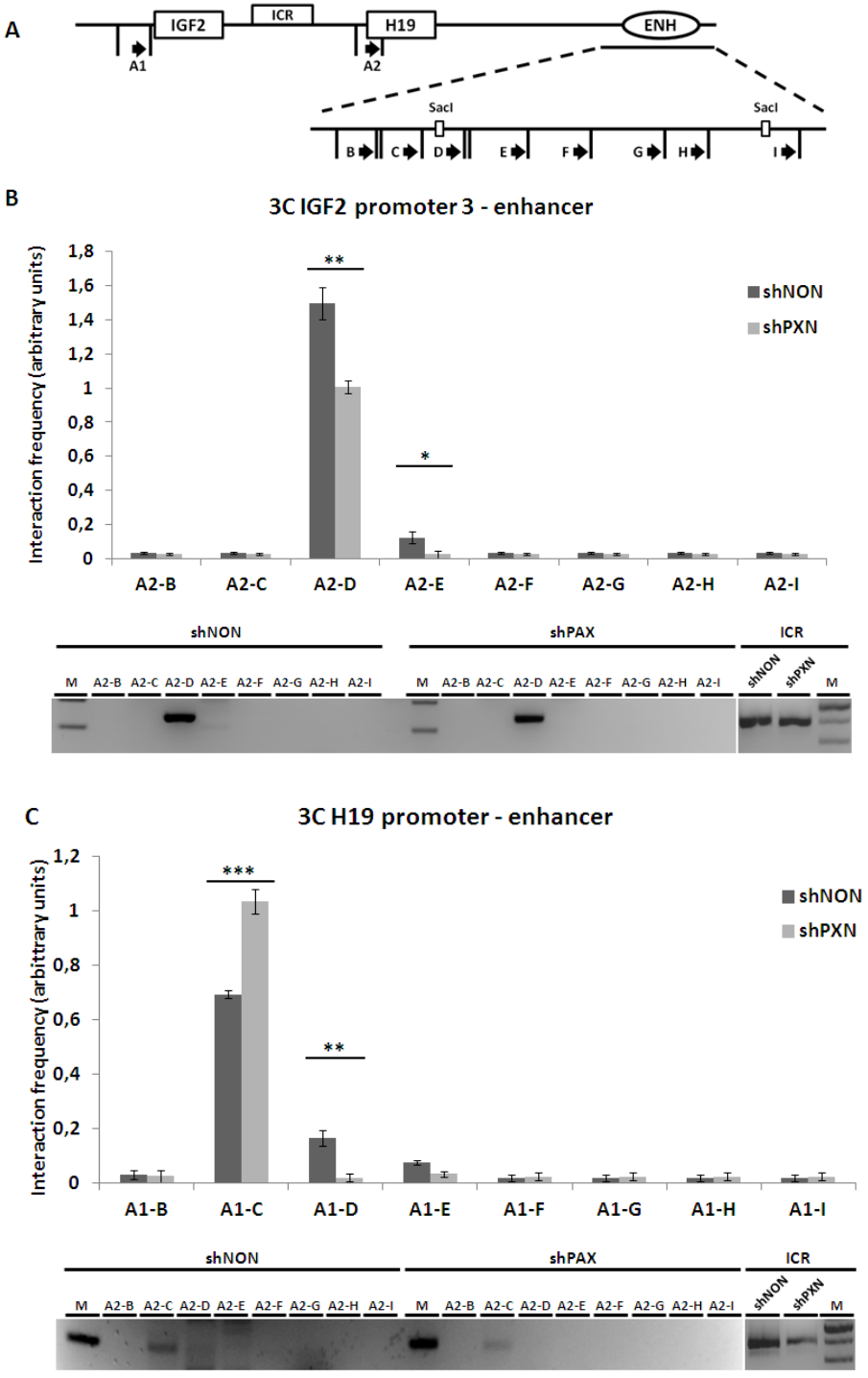


Figure 15 Paxillin regulates *IGF2*P3/*H19* promoter–enhancer interactions

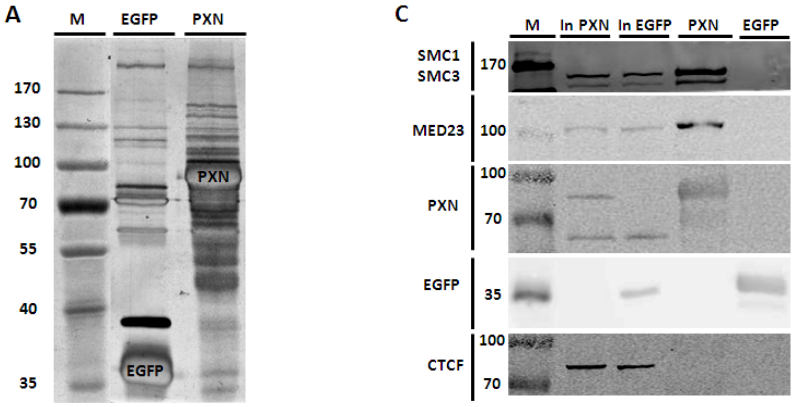
A) Design of 3C analysis. Vertical lines show PstI restriction sites and black arrows represent designed primers. **B)** The interaction frequency between *IGF2* P3 and the enhancer was significantly lower after paxillin knockdown (shPXN) compared to control (shNON). Chromatin Conformation Capture (3C) assay was performed using *IGF2* P3 (primer A1) as an

anchor. **C)** Paxillin depletion increased the interaction frequency between *H19* promoter and the enhancer. 3C assay was performed in the same way, but using *H19* promoter (primer A2) as an anchor. Amplified PCR bands were resolved on 3% agarose gel and semiquantitatively analysed. Background was subtracted and data normalized to loading control (ICR region). Results are presented as means ± standard deviations from at least 3 independent experiments; ***p < 0,001; **p < 0,01; Student`s t-test.

The strongest interaction was detected at the 5'end of enhancer sequence. In agreement with previous data, the interaction frequency between the *H19* promoter and the enhancer was increased in paxillin knockdown cells (Figure 15B). Correspondingly, the interaction frequency between *IGF2* P3 and the enhancer was significantly lower after paxillin knockdown (Figure 15C). This result clearly confirms that paxillin regulates the activation of the *IGF2* P3 and *H19* promoters via the same shared enhancer, and that it does so in an opposite manner. In other words, paxillin promotes the contact of the *IGF2* P3 promoter and the enhancer, while simultaneously blocking the activation of the *H19* promoter.

I.VI. Paxillin binds SMC1, SMC3 and MED in the nucleus

Finally, we searched for protein complexes involved in regulatory activity of paxillin on *IGF2*/*H19* promoters. We established a HEK293 cell line stably expressing EGFP-STrEP-paxillin (PXN) and identified proteins pulled-down by the tagged protein using LC-MS-MS (Figure 16A). Proteins identified in the experiment are sumarized in Figure 16B.



B

Protein	Symbol	MW (kDa)	Peptides	SC (%)
Structural maintenance of chromosomes 1A	SMC1A	141	13	10
Structural maintenance of chromosomes 3	SMC3	143	12	9
Mediator of RNA polymerase II subunit 15	MED15	86	1	2
Mediator of RNA polymerase II subunit 23	MED23	156	1	2
Mediator of RNA polymerase II subunit 24	MED24	110	1	2
Focal adhesion kinase 1	FAK1	120	22	28
ARF GTP-ase activating protein 1	GIT1	84	39	51
ARF GTP-ase activating protein 2	GIT2	85	24	38
Serine/threonine protein kinase 1	PAK1	60	18	38
Serine/threonine protein kinase 2	PAK2	58	13	34
Rho guanine nucleotide exchange factor 7	ARHGEF7	90	31	31
Rho guanine nucleotide exchange factor 6	ARHGEF6	87	15	18
Tubulin alpha	TUBA	50	9	29
Tubulin beta	TUBB	50	18	44

Figure 16 Paxillin binds SMC1, SMC3 and MED in the nucleus

A) A representative gel of paxillin pull-down stained with silver. Cells expressing only EGFP with affinity tag were used as a control. **B)** Paxillin-binding proteins identified by mass spectrometry analysis (LC-MS-MS). Cohesin proteins SMC1, SMC3 and Mediator subunits have not been reported to be associated with paxillin so far. The table shows protein molecular weight (MW), number of analyzed peptides and sequence coverage of these peptides (SC). **C)** The specificity of the identified interactions was confirmed by Western blot. Cohesin proteins SMC1, SMC3 and MED23 were bound to PXN, but not to the control EGFP. No interaction of paxillin and CTCF was observed. Pull-down fractions from HEK293 cell stably expressing STrEP-EGFP-PXN (PXN) or STrEP-EGFP (EGFP, used as a background control) were immunobloted with marked antibodies. Anti-paxillin antibody (PXN) labels both endogenous paxillin (lower bands) as well as recombinant paxillin (upper band). In – input; M – marker.

In addition to many known paxillin-binding proteins, our search identified also some new nuclear gene expression regulatory proteins interacting with paxillin. Two proteins of cohesin family were repeatedly identified in paxillin pull-down experiments, namely structural maintenance of chromosomes 1A (SMC1A) and Structural maintenance of chromosomes 3

(SMC3). Further, we also identified 3 subunits of MED complex, specifically MED15, MED24 and MED23. Although the number of peptides found was very low, no peptides, however, were found in the control which indicates that the interaction is specific.

The specificity of the identified interactions was confirmed by Western blotting (Figure 16C). Both SMC1 and SMC3 proteins, as well as MED23 were found in paxillin pull-down fraction, but not in the control, which corresponds with our MS data. Low incidence of other Mediator subunits in pull-down disabled their identification on Western blots. On the other hand, no CTCF was found in the pull-down fractions showing that paxillin is not part of cohesin-CTCF complexes. Thus, we present the evidence of the interaction between paxillin and gene expression regulatory proteins SMC1A, SMC3 and Mediator complex.

I.VII. Paxillin binds directly to both cohesin subunits SMC1 and SMC3

We further examined which part of the cohesin complex directly interacts with paxillin. In order to test this, the glutathione S-transferase (GST)-tagged recombinant proteins SMC1, SMC3 and paxillin (GST-SMC1, GST-SMC3, GST-PXN, respectively; Figure 2B) were expressed in E. coli and purified. After the purification, GST-PXN was cleaved with a protease Xa to remove GST-tag and pull-down assay was performed.

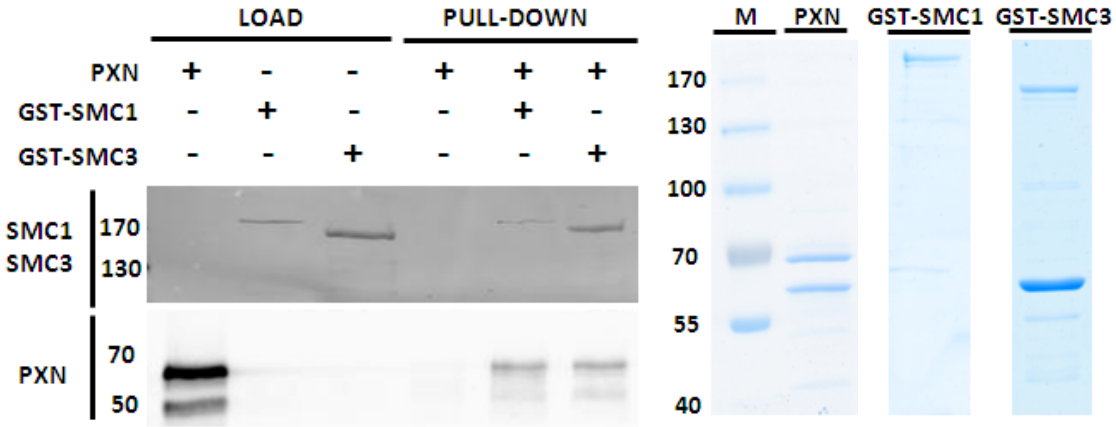


Figure 17 Paxillin binds directly to both cohesin subunits SMC1 and SMC3
A) The recombinant proteins GST-SMC1 or GST-SMC3 were bound to glutathione-agarose and subsequently recombinant paxillin (PXN) was added. Paxillin was pulled-down by GST-SMC1 as well as GST-SMC3, showing that paxillin directly binds both cohesin subunits. The GST-tag was cleaved from paxillin by protease Xa. The empty glutathione-beads were used as a control. **B)** The purified recombinant proteins used in GST-pull-down assays stained with Commassie blue. GST-SMC3 co-purified with a 60-kDa bacterial protein.

As shown in Figure 2A, paxillin was pulled-down by both GST-SMC1 and GST-SMC3, but not glutathione-agarose alone (control). The paxillin antibody detected two bands on Western blot, which might correspond to shorter, improperly translated paxillin form. These data clearly show that paxillin binds directly to both cohesin subunits SMC1 and SMC3.

II. PIP2 IN RNA POLYMERASE I TRANSCRIPTION

II.I. PIP2 associates with RNA Polymerase I pre-initiation complex in mitotic cells

We showed recently that PIP2 associates with RNA Polymerase I (Pol I) transcription foci in the interphase nucleoli (Yildirim et al., 2013). Nevertheless, it is not known whether the interaction occurs also in mitotic cells. This would indicate, that PIP2 localization to the sites of Pol I transcription is independent of ranscriptional status of the cell. We therefore tested the interaction between PIP2 and components of Pol I complex by immunoprecipitating PIP2 from mitotic cell extract. We specifically detected UBF and two subunits of Pol I, PAF53 and RPA116, co-immunoprecipitating with PIP2 (Figure 18). This clearly shows that PIP2 interacts with Pol I transcription complex also during mitosis.

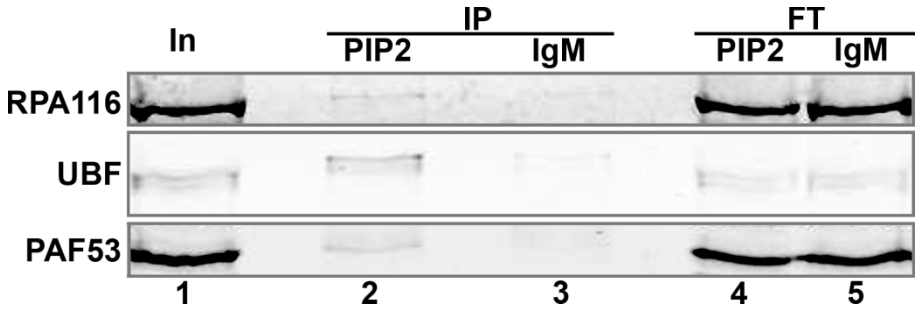


Figure 18 PIP2 binds to the proteins of Pol I transcription complex in mitotic cells
The immunoprecipitation assay using anti-PIP2 antibody detected UBF and the Pol I subunits, PAF53 and RPA116, in the mitotic PIP2-protein complex. Lane 1: input, lane 2: protein immunoprecipitated with anti-PIP2 antibody, lane 3: protein immunoprecipitated with isotype control; lane 4: protein unbound to anti-PIP2 antibody (flow-through), lane 5: protein unbound to isotype control (flow-through), In: input, IP: immunoprecipitate, FT: flow-through.

II.II. PIP2 is a stable component of the Pol I pre-inititation complex throughout all mitotic phases

Having identified the association of PIP2 with Pol I subunits and UBF in mitotic cells, we asked whether this association is retained in NORs during all mitotic stages. The NORs are characterized by less compacted structure as compared to the bulk of condensed chromatin and UBF serves as a marker of the mitotic NORs due to its persistent presence on the extended fibers of rDNA during the entire cell cycle (Gébrane-Younès et al., 1997).

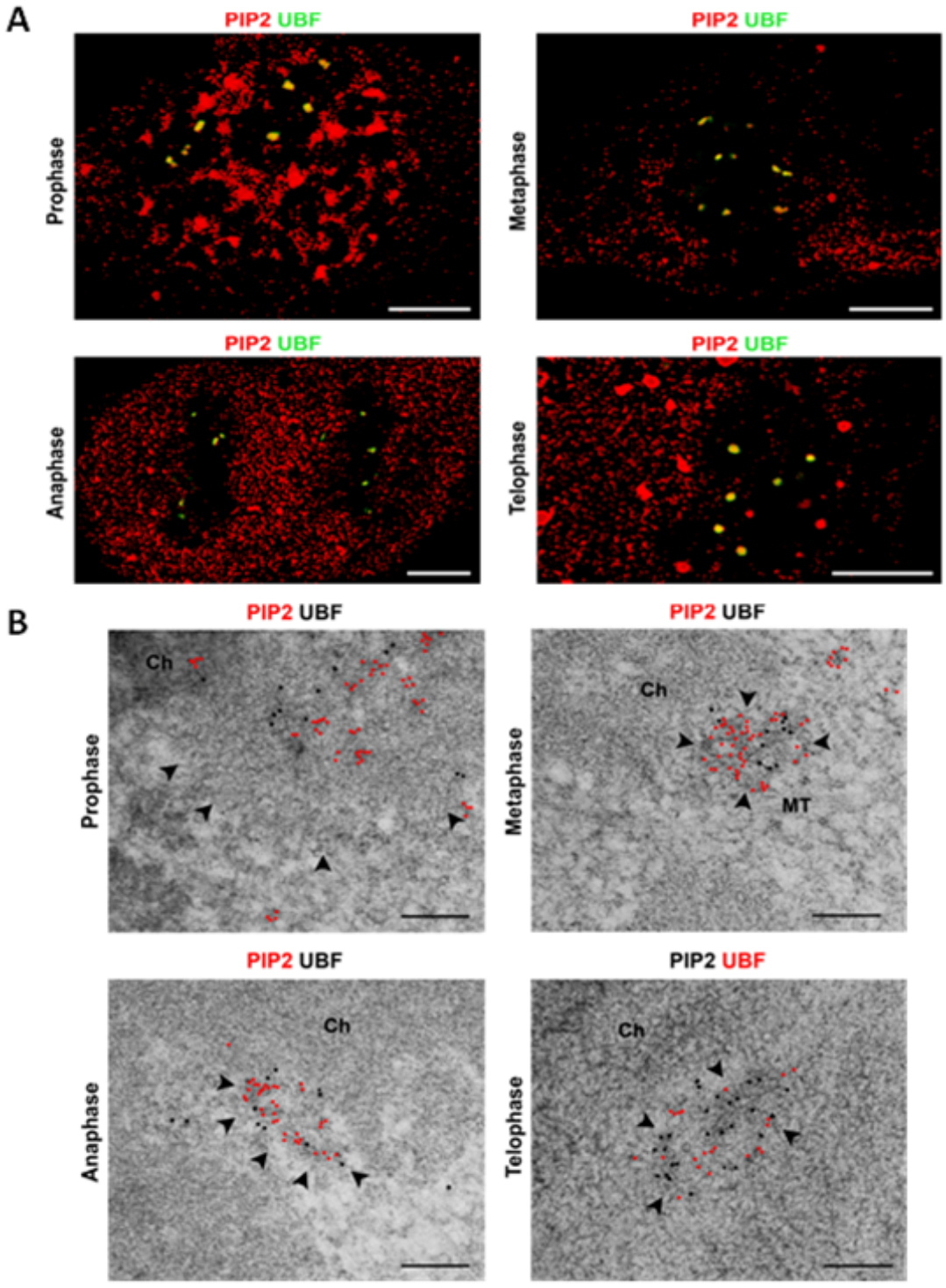


Figure 19 PIP2 retains its colocalization with UBF during all mitotic phases

A) Super-resolution structured illumination microscopy combined with multi-immunolabeling showed that PIP2 colocalizes with UBF in NORs, which are the mitotic counterparts of the interphase FCs, in prophase and metaphase. This colocalization pattern persists in anaphase and telophase in "daughter" NORs. Scale bar: 5 μm. **B)** Immunogold electron microscopy of the cryo-immobilized and freeze-substituted mitotic cells demonstrated in ultrastructural detail that PIP2 colocalizes with UBF in NORs (marked by arrowheads). PIP2 occupies the whole NOR volume rather than being sequestered in distinct sub-domains. Ch: chromatin, MT: microtubules. Scale bar: 200 nm.

To test this, we studied the localization of PIP2 by super-resolution structured illumination microscopy (SIM) and the electron microscopy (IEM) combined with high-pressure freezing and freeze-substitution (HPF/FS). We showed previously that HPF/FS preserves the near-native state of nuclear compartments and molecular complexes (Sobol et al., 2010). We observed co-localization of PIP2 and UBF during all mitotic phases. SIM showed colocalization between PIP2 and UBF in NORs throughout prophase and metaphase, continuing in "daughter" NORs in the anaphase and telophase (Figure 19A). Electron microscopy confirmed colocalization of PIP2 with UBF in clearly distinguishable NORs from prophase to telophase (Figure 19B).

II.III. Inhibition of transcription abolishes association of PIP2 with fibrillarin but not UBF and Pol I

In mitosis the transcription is physiologically inhibited. We therefore presumed that association of PIP2 with Pol I pre-initiation complex might be independent of transcription. Thus we investigated the effect of chemically provoked inhibition of Pol I transcription on PIP2 localization. We first showed that in transcriptionally active interphase nucleoli, PIP2 colocalizes with Pol I, UBF and fibrillarin, as seen by signal intensity profiles (Figure 20A). In agreement with this, IEM also showed PIP2 localization in a close proximity to Pol I, UBF and fibrillarin in the FC and DFC region of nucleoli, respectively (Figure 20A). Pol I inhibition by AMD treatment resulted in nucleolar segregation and formation of a central body associated with caps (Puvion-Dutilleul et al., 1997). When Pol I transcription was blocked, PIP2 still colocalized with Pol I and UBF (Figure 20B). IEM further demonstrated intermingling clusters of PIP2 with Pol I and UBF in the light part of the caps as seen in Figure 20B. However, upon inhibition of Pol I transcription, PIP2/fibrillarin colocalization was lost as seen in the signal intensity profile (Figure 20B). Fibrillarin is concentrated in the dense part of the caps, and in most cases does not colocalize with PIP2 after AMD treatment (Figure 20B). These results show that the association of PIP2 with the Pol I and UBF is not dependent on active Pol I transcription, while fibrillarin associates with PIP2 only when transctiption of Pol I is active.

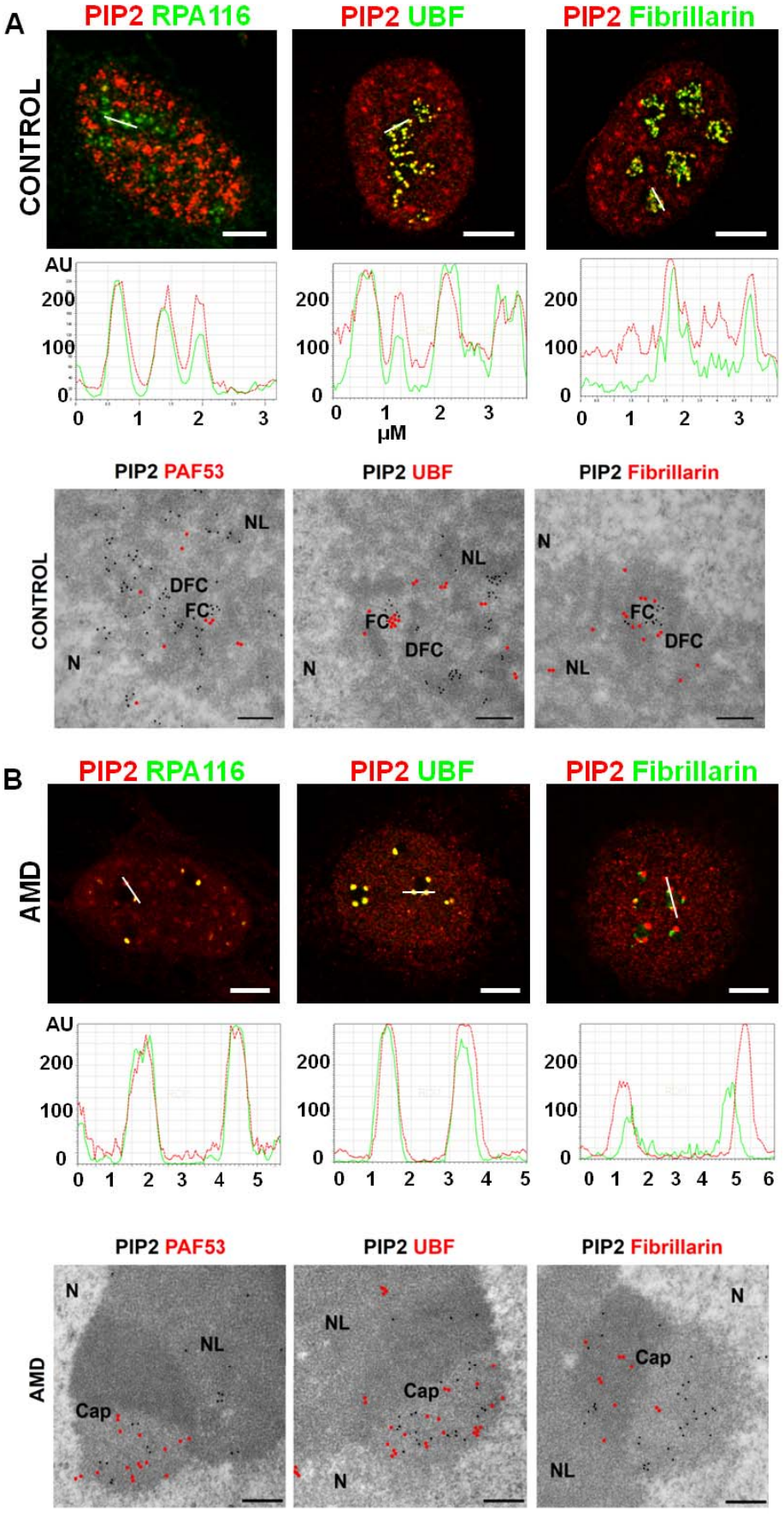

A
PIP2 RPA116
PIP2 UBF
PIP2 Fibrillarin
CONTROL
AU
200
100
0
0 1 2 3
μM
PIP2 PAF53
PIP2 UBF
PIP2 Fibrillarin
NL
DFC
FC
N
B
AMD
0 1 2 3 4 5
0 1 2 3 4 5 6
Cap

Figure 20 Inhibition of transcription abolishes association of PIP2 with fibrillarin but not UBF and Pol I

(**A**) Immunolocalization studies show co-localization of PIP2 with Pol I, UBF and fibrillarin in control cells as seen in the intensity profiles. (**B**) Pol I transcription inhibition by AMD 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. 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 fluorescent as well as electron microscopy. In graph: x-axis is in μm, y-axis is in arbitrary units. Scale bar for fluorescent microscopy: 5 μm. N: nucleus, NL: nucleolus, FC: fibrillar center, DFC: dense fibrillar component. Scale bar for electron microscopy: 200 nm.

III. PIP2 ISLETS

III.I. The distribution of PIP2 in the nucleus

The study of PIP2 was previously focused on nuclear speckles and nucleoli (Osborne et al., 2001; Mortier et al., 2005; Sobol et al., 2013; Yildirim et al., 2013). Instead, we examined the detailed localization in the other parts of the nucleus. We mapped PIP2-containing structures using 3D electron tomography combined with pre-embedding immunolabeling (Figure 21A), and super-resolution structured illumination microscopy (SIM; Figure 21B).

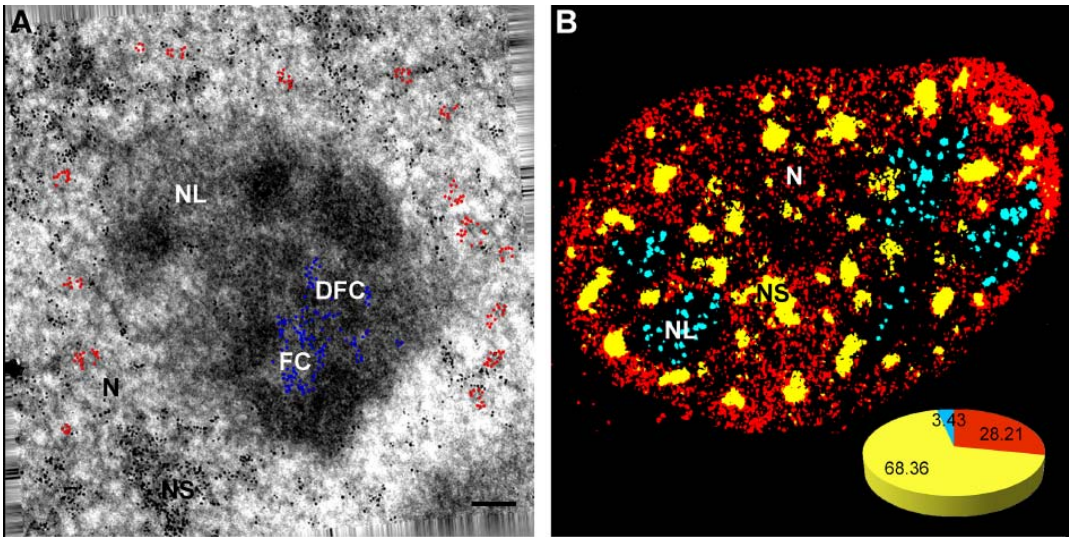


Figure 21 The nuclear pool of PIP2 is distributed between nuclear speckles, nucleoli and PIP2 islets.

We revealed that substantial portion of PIP2 nuclear pool is localized in the previously uncharacterized structures, which are clearly distinguishable from the nuclear speckles by the size and roundish shape, and we refer to them as PIP2 islets. **A)** 3D electron tomography combined with pre-embedding immunolabeling using anti-PIP2 antibody. Ultrastructural tilt image shows PIP2 labeling in nucleolar subcomponents (FC - fibrillar center, DFC - dense fibrillar component; individual PIP2 molecules are marked in blue), nuclear speckles (NS) and PIP2 islets (individual PIP2 molecules are marked in red). N – nucleus, NL - nucleolus. Bar is 200 nm. **B)** Super-resolution structured illumination microscopy (SIM) combined with immunofluorescent labeling using anti-PIP2 antibody. 2D maximum intensity projection image from 3D z-stack represents the PIP2 labeling in U2OS cell nucleus reconstructed and colour-coded using the software NIS-Elements and MATLAB. N – nucleus, NS – nuclear speckles, NL – nucleolus. Nuclear speckles are distinguished from PIP2 islets based on the co-labeling with a speckle-specific marker - anti-Son antibody. The insertion in the down-right corner represents the percentage distribution of PIP2 in the nuclear pool calculated using the reconstruction data. Nuclear speckles are in yellow, PIP2 islets are in red, nucleolar PIP2 is in blue.

We revealed that nuclear PIP2 occupies three different regions: 68.36±4.43% of nuclear PIP2 localizes to speckles, 3.43±1.10% localizes to nucleoli and 28.21±4.92% of the nuclear pool of PIP2 is concentrated in previously uncharacterized structures. These structures are clearly distinguished from the nuclear speckles by the size and roundish shape. Here we refer to them as PIP2 islets.

III.II. PIP2 islets are enriched in carbon-rich compounds and surrounded by nucleic acids and proteins

To characterize the composition of PIP2 islets, we determined the elemental composition of PIP2 islets. Using the electron energy loss spectroscopy (EELS), we mapped phosphorus (P), nitrogen (N), and carbon (C) in relation to PIP2 islets (Figure 22B, E). We showed that PIP2 islets are enclosed by nucleic acids and proteins, while the inner space of PIP2 islets seems to lack them both (Figure 22C). We revealed that the interior of PIP2 islets is mainly composed of carbon-rich compounds (Figure 22E).

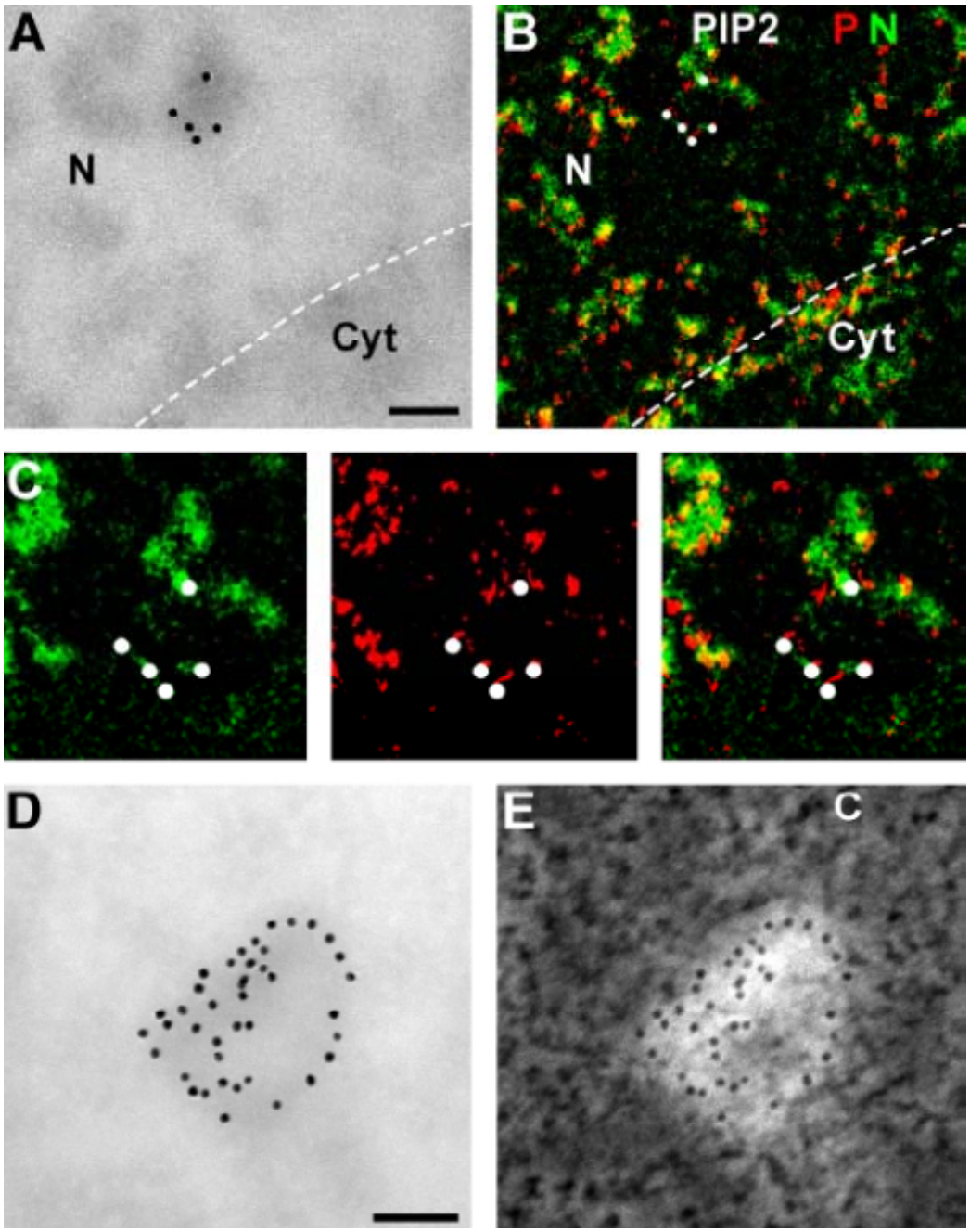


Figure 22 PIP2 islets are enriched with carbon-rich compounds and surrounded by chromatin and proteins

We performed the electron energy loss spectroscopy (EELS) analysis mapping phosphorus (P), nitrogen (N) and carbon (C) in relation to PIP2 islets. **A), B), C)** The ultrathin sections (80 nm) were immunolabeled with anti-PIP2 antibody and secondary antibody conjugated with 12 nm gold particles. Magnified part clearly showed that PIP2 islet is surrounded by chromatin and proteins, while the inner part of PIP2 islet appears to be devoid of both chromatin and proteins. **D), E)** Carbon mapping showed that the inner space of PIP2 islet enriched with carbon-rich compounds. N – nucleus outlined by a dashed line; Cyt – cytoplasm; bar is 100 nm.

III.III. PIP2 islets are evolutionary conserved nuclear structures

To prove that PIP2 islets are not a unique feature of human immortal cell lines, we studied the distribution of PIP2 in the nuclei of evolutionary different organisms using the immunogold electron microscopy (IEM). We demonstrated (Figure 3A-H) that 40-100 nm large PIP2 islets are present in nuclei of yeast (*Saccharomyces cerevisiae*), protozoa (*Giardia lamblia*, *Tetrahymena thermophila*), beans (*Vicia*; germinated bean meristem cells), mice (*Mus musculus*; primary skin fibroblasts), and humans (*Homo sapiens:* mesenchymal stem cells, stimulated lymphocytes, cervical carcinoma cells). Ultrastructural analysis showed that PIP2 islets are excluded from both the heterochromatin regions as well as the nuclear envelope.

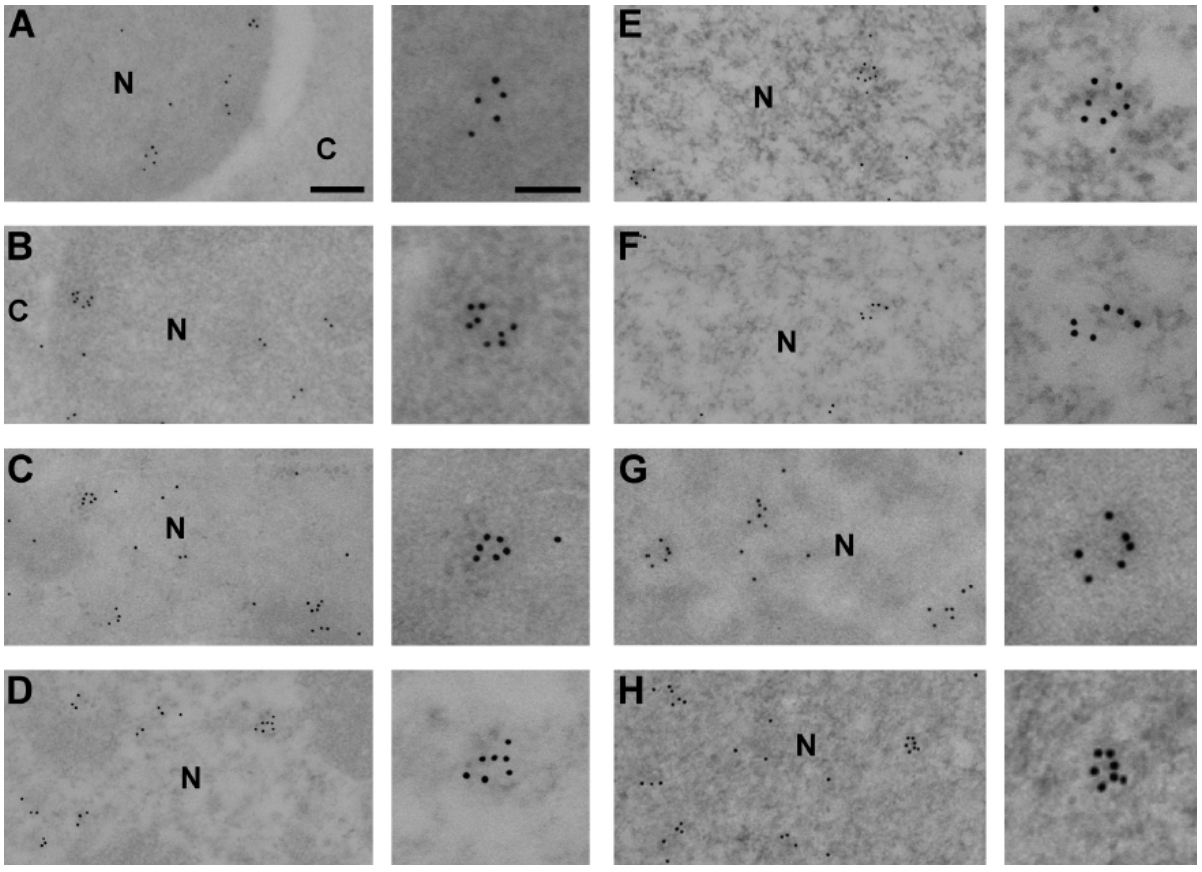


Figure 23 PIP2 islets are evolutionary conserved nuclear structures

The roundish PIP2-structures of 40-100 nm (PIP2 islets) were visualized using anti-PIP2 antibody and immunogold electron microscopy (IEM) in nuclei of yeast (*Saccharomyces cerevisiae* - **A**), protozoa (*Giardia lamblia* – **B**, *Tetrahymena thermophila* - **C**), beans (*Vicia*; germinated bean meristem cells - **D**), mice (*Mus musculus*; primary skin fibroblasts - **E**), humans (*Homo sapiens:* mesenchymal stem cells – **F**, stimulated lymphocytes – **G**, cervical carcinoma cells – **H**). Secondary antibody was conjugated to 12 nm gold particles. PIP2 islets are not associated with either the heterochromatin or the nuclear envelope. N – nucleus, C – cytoplasm. General view: bar is 200 nm, magnified view: bar is 100 nm.

III.IV. PIP2 islets are involved in RNA polymerase II transcription

Our lab has recently shown that nucleolar-localized PIP2 is involved in RNA polymerase I transcription (Yildirim et al., 2013). We hypothesized that nucleoplasmic PIP2 islets might play a role in RNA polymerase II transcription.

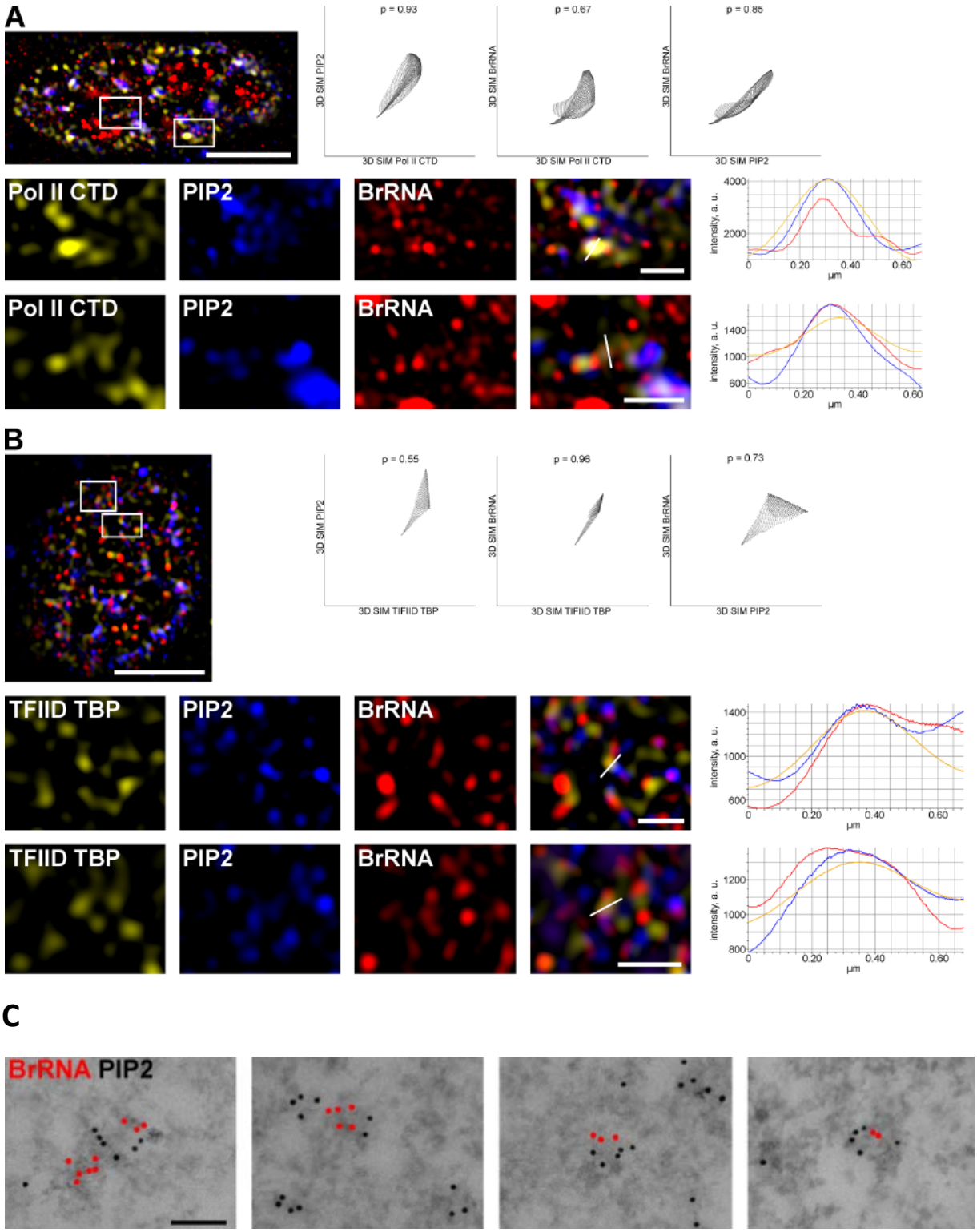


Figure 24 PIP2 islets co-localize with RNA polymerase II transcription complex and nascent RNA transcripts
A) Super-resolution SIM combined with multi-immunolabeling demonstrated that PIP2 islets, C-terminal domain of Pol II and nascent RNA transcripts co-localize in the nuclues of HeLa cells. **B)** PIP2 islets colocalized with TATA-box binding protein (TBP) and nascent RNA transcripts. Colocalization plots with Pearson's correlation coefficient are shown. In the graphs, x-axis represents the distance in μm, y-axis represents the fluorescence intensity in arbitrary units. General view – bar: 5 μm; magnified view corresponds to the area outlined in white, bar: 1 μm. **C)** Immunoelectron microscopy clearly shows that PIP2 molecules form the roundish islets colocalized at their periphery with nascent RNA transcripts. Hela cells were incubated with BrUTP, a labeled precursor for the RNA synthesis, and processed for electron microscopy. We confirmed that colocalization between PIP2 molecules and nascent RNA transcripts is significant at the distance of 25 - 75 nm. Bar: 100 nm.

To test this, we first co-localized PIP2 islets with RNA Polymerase II, transcription factor TATA-box binding protein (TBP) and nascent RNA transcripts. Cells were pulse-chase labeled with precursor of RNA synthesis BrUTP and this analog was visualized by antibody in newly transcribed RNAs. We showed that PIP2 islets co-localize with C-terminal domain of Pol II, TATA-box binding protein (TBP) as well as nascent RNA transcripts (Figure 24). Immunogold electron microscopy (IEM) clearly demonstrated that nascent RNA transcripts colocalize with the periphery of PIP2 islets (Figure 24). We confirmed that the colocalization between PIP2 molecules and nascent RNA transcripts is significant at 25 - 75 nm.

We further examined whether PIP2 islets are necessary for RNA polymerase II transcription. We hydrolyzed PIP2 by incubating the cells with phospholipase C enzyme. This resulted in reduction of the overall PIP2 level by 18.3% compared to control. Then we examined the level of transcription, which corresponded to the intensity of florouridine (FU) incorporated into newly transcribed RNA molecules. We showed that total nuclear fluorescence intensity of *in vivo* labeled FU-RNA transcripts, was decreased by 36.5% upon PIP2 hydrolysis (Figure 25A). Remarkably, the same effect was observed in PIP2 islets only, here the reduction of the PIP2 level by 18.1% resulted in decrease of the transcription level by 36.1% (Figure 25B). This shows that PIP2 islets are important for RNA polymerase II transcription.

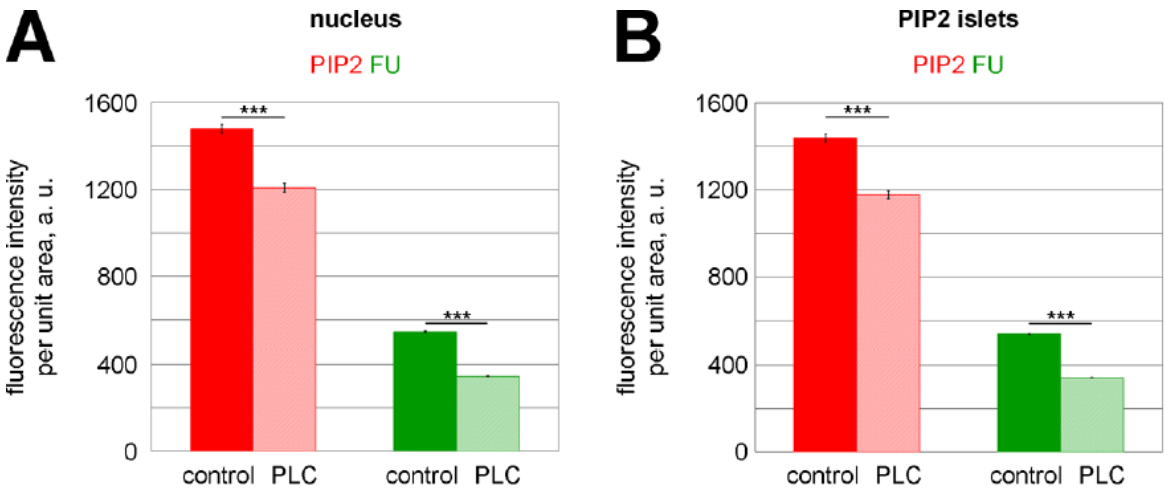


Figure 25 Hydrolysis of PIP2 islets reduces the level of RNA polymerase II transcription U2OS cells were *in vivo* treated with PLC enzyme for PIP2 hydrolysis and incubated with FU for the monitoring of the RNA synthesis. A) The PLC treatment causes a decrease in the level of transcription corresponding to the nuclear fluorescence intensity of labeled FU-RNA transcripts. B) Hydrolysis of PIP2 islets decreased the level of RNA polymerase II transcription. *** p value ≤ 0.001.

DISCUSSION

I. Nuclear functions of paxillin

Multicellular organisms are composed of many different cell types that become organized during development into distinct tissues and organs. One important process required for these cell rearrangements is cell adhesion, both to the extracellular matrix and to adjacent cells. Cell adhesion as well as spreading or motility is coordinated by important structural elements - focal adhesions. They mediate transmission of information regarding cell environment and participate in the response to such information, thereby playing an important role in the control of many cellular processes. The assembly of focal adhesion complexes is often associated with changes in cell proliferation, survival and differentiation. This connection has raised the question: what is downstream of cell adhesion that is responsible for signalling to the nucleus and causing changes in gene expression?

The first indication of the link between focal adhesions and the regulation of gene expression has come from studies of mammary epithelial cell cultures. Interaction of integrin and extracellular matrix caused upregulation of casein gene leading to differentiated phenotype of these cells (Schmidhauser et al., 1992). It was later shown that focal adhesion proteins can directly influence nuclear functions and zyxin, protein of paxillin family, was the first focal adhesion protein which was shown to shuttle between focal adhesions and the nucleus (Nix and Beckerle, 1997). Paxillin was also previously thought to occupy exclusively focal adhesions, but recent studies have revealed that it accumulates in the nucleus upon the blockage of the CRM1-dependent nuclear export pathway with leptomycin B, indicating that it undergoes nucleocytoplasmic shuttling (Wang and Gilmore, 2003; Dong et al., 2009).

We showed that depletion of paxillin upregulates *H19* gene expression in human HepG2 cells consistently with previous findings (Dong et al., 2009). Surprisingly, we did not observe a significant change in the *IGF2* expression. This was unexpected because *H19*/*IGF2* genes usually react reciprocally towards the regulating signals including the depletion of CTCF or cohesin (citace). Furthermore, depletion of these proteins also abrogates ICR methylation, which disrupts monoallelic expression (Constancia et al., 2000; Wendt et al. 2008; Nativio et al., 2009). In case of paxillin depletion, however, the ICR methylation was preserved and the allelic expression unaffected. Interestingly, whereas *H19* was transcribed from a single allele, *IGF2* was biallelically transcribed both, in the control as well as in paxillin depleted cells, respectively. Since ICR insulation barrier responsible for *IGF2* imprinting was intact, there

has to be another regulatory mechanism of this process, or alternativelly, the imprinting mechanism for *IGF2* is disfunctional in HepG2 cells. Indeed, reactivation of *IGF2* expression on maternal allele has been previously reported in many human tumors and tumor cell lines (Li et al., 1995; Singer et al., 1995; Takeda et al., 1996; Zhang et al., 1997; Cui et al., 1998; Hofmann et al., 2002). Similarly, regulation of this element could be abrogated in our hepatocellular carcinoma cell line (HepG2) resulting in uncontroled *IGF2* expression. This deregulation could prevail over paxillin effect on *IGF2* expression explaining why we did not observe any significant difference in *IGF2* expression after paxillin depletion.

Further, we show that paxillin occupies promoters of *IGF2* and *H19* genes and their shared endodermal enhancer. Two different enhancer sequences for *IGF2*/*H19* have been identified so far, an endodermal and a mesodermal. Whereas the endodermal enhancer has been described in both mice and humans (Leighton et al., 1995b; Kopf et al., 1998; Ohana et al., 1999), the mesodermal enhancer is more elusive and a putative mouse mesodermal enhancer region 22-28 kb downstream of *H19* (Ishihara et al., 2000) has not been studied in human cells yet. In our experiments, we used endodermal enhancer (+6033 to +10972 bp relative to *H19*; delineated by Sac I restriction sites). This sequence was originally identified in mouse (Yoo-Warren et al., 1988; Arney, 2003) and later reported to stimulate *H19* promoter in human endodermal cell lines, such as HepG2 (Kopf et al., 1998; Ohana et al., 1999; Long and Spear, 2004; Varrault et al., 2006). We further expand this finding by identifying the role of paxillin in endodermal enhancer regulation. Further examination is needed to decide whether the mechanism is general or specific for the endodermal enhancer only.

Further, we show that paxillin regulates the formation of long-range interactions between promoters of *H19* and *IGF2* genes and their shared enhancer. Finally, we identified the interaction of paxillin with cohesin complex (subunit SMC1, SMC3). Interestingly, cohesion complex has been established as the main regulator of long-range chromatin interactions at *IGF2*/*H19* cluster (Wendt et al. 2008; Nativio et al., 2009). Moreover, cohesin has been found to colocalize with Mediator (Kagey et al., 2010). These proteins have been shown to mediate long-range interactions between distal enhancers and promoters of key pluripotency transcription factors in mouse embryonic stem cells (Conaway and Conaway, 2011). There, the ring-shaped structure of cohesin is employed to lock together the enhancer and the promoter regions of a single chromatid once they are brought into proximity by their simultaneous binding to the activator (Mediator) of RNA polymerase II (Hadjur et al., 2009). We indeed found subunits of Mediator, specifically its tail module (Malik and Roeder, 2010), among paxillin associated proteins in pull-down experiments. Therefore, we propose a model

(Figure 26) where paxillin assembles with cohesin and Mediator and mediates long-range chromosomal interactions between *IGF2* or *H19* promoter and the shared distal enhacer, and thus regulates their transcription. Specifically, paxillin enhances the interaction between the enhancer and the *IGF2* promoter, but blocks the *H19* promoter–enhancer interaction, resulting in stimulation of *IGF2* and repression of *H19* expression. Our model also explains significant decrease in cell proliferation after paxillin depletion, since it causes upregulation of *H19* non-coding RNA giving rise to proliferation-repressing microRNAs (miRNAs; Gabory et al., 2010). Both *IGF2* and *H19* genes are widely expressed during embryonic development, after which they are downregulated (except in skeletal muscle; Brunkow and Tilgham, 1991; Delaval and Feil, 2004). Their proper expression is therefore crucial especially during fetal development (Delaval and Feil, 2004; Pannetier and Feil, 2007; Gabory et al., 2010) and even though their regulatory mechanisms are preserved in cultured somatic cells, we presume that the real significance of paxillin regulatory model lies in the developmental stages.

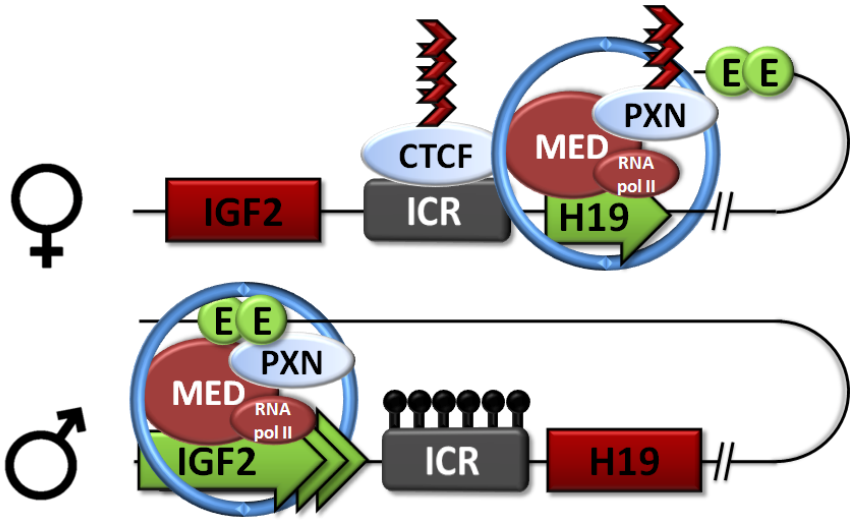


Figure 26 Model of *IGF2*/*H19* regulation by paxillin
The model of *IGF2*/*H19* locus regulation. Paxillin assembles with cohesin and Mediator and mediates long-range chromosomal interactions between *IGF2* or *H19* promoter and the shared distal enhacer, and thus regulates their transcription. Specifically, paxillin enhances the interaction between the enhancer and the *IGF2* promoter, but blocks the *H19* promoter–enhancer interaction, resulting in stimulation of *IGF2* and repression of *H19* expression. The model shows paternal and maternal allele of the genes; red box – inactive gene, green box – active gene, black lollipops – methylation, blue circle – cohesin complex, dark red arrows – ICR barrier.

Based on our findings, we propose a novel regulatory mechanism for *IGF2*/*H19* locus employing paxilllin in a complex with cohesin and Mediator, which mediate promoter-

enhacer interactions. Taking into consideration that paxillin is a focal adhesion protein, this shows that cells regulate gene expression by taking advantage of the physical barrier formed by the nuclear envelope and thus controlling the access of regulators to their target genes.

II. Localization and function of nuclear phopholipids

The eukaryotic nucleus was believed to be composed mainly of proteins and nucleic acids. However, the nuclear interior was shown to retain significant amount of lipids, including phospholipids (Rose and Frenster, 1965). The existence of phosphatidylinositol 4,5-bisphosphate (PIP2) nuclear pool as well as the enzymes and the substrates involved in its metabolism was confirmed shorty after that (Cocco et al., 1987; Vann et al., 1997), remarkably, also using membrane-depleted nuclei, which disproved the hypothesis, that nuclear membrane is the main source (Payrastre et al., 1992). Later experiments with the antibody against PIP2 and PIP2-binding domains as probes, revealed its localization in distinct nuclear compartments such as the interchromatin granule clusters (speckles) and the nucleolus (Mazzotti et al., 1995; Osborne et al., 2001; Watt et al., 2002).

The general architecture of the nucleolus comprises three sub-compartments which have been well defined by electron microscopy: fibrillar centers (FCs), dense fibrillar component (DFC), and granular component (GC). Transcription of ribosomal DNA (rDNA) by RNA polymerase I (Pol I) takes place mostly at the FC/DFC border (Grummt, 2003; Grummt and Pikaard, 2003; Raska et al., 2004). We found that PIP2 forms clusters in FC and DFC, i. e. regions responsible for transcription and processing of rRNA, but it does not localize to the GC region, where the assembly and maturation of pre-ribosome particles occur. Similarly, we have recently shown that PIP2 binds to the transcription factor UBF and makes a complex with Pol I on the promoter of ribosomal genes. Moreover, PIP2 colocalized with nascent transcripts in the nucleolus and the depletion of PIP2 resulted in reduction of Pol I transcription showing that PIP2 stimulates Pol I processing (Yildirim et al., 2013). This indicates that PIP2 might be involved in both transcription and early processing of rRNA.

However, we found that upon either physiological (mitosis) or chemically induced (AMD) inhibition of Pol I transcription, PIP2 maintains its association with UBF and Pol I subunits, the components of Pol I pre-initiation complex, but not with fibrillarin. It has been shown that Pol I subunit PAF53 contacts UBF directly, and both PAF53 and RPA116 subunits remain associated with Pol I transcription machinery independent of the rRNA synthesis (Hanada et

al., 1996; Seither et al., 1997). These data reinforce the view that PIP2 interacts with fibrillarin only upon active pre-rRNA transcription while its binding to UBF in Pol I complex is not dependent on the synthesis of rRNA. UBF associates with the extended fibers of rDNA during the entire cell cycle (Gebrane-Younes et al., 1997) and has been reported to have a role in the formation of nucleoli (Fomproix et al., 1998; Mais et al., 2005). We showed that association of PIP2 and UBF is preserved throughout the whole cell cycle, indicating a structural role of PIP2 in the formation and maintenance of nucleolar architecture. We suggest that PIP2 in complex with UBF is engaged in the formation of the core part of rDNA helix structure thus maintaining the open chromatin state of NORs independent of the Pol I transcription.

We used electron microscopy and 3D electron tomography to examine detailed PIP2 localization in the nucleus and revealed for the first time that PIP2 forms structures, different from nuclear speckles. These spherical structures, which form about one third of total nuclear pool, are 40-100 nm in diameter and we called them "PIP2 islets". The elemental mapping showed that PIP2 islets are characterized by carbon-rich interior, which is surrounded by nucleic acids and proteins. Thus, we suggest that organization of PIP2 islets reflects vesicle arrangement: hydrophobic interior surrounded by a hydrophilic outward part. Remarkably, we showed that PIP2 islets are chomatin-independent which is consistent with earlier observations that sphingomyelin is localized in the perichromatin region, where DNA replication and transcription occur, and is excluded from the condensed chromatin region (Jaunin and Fakan, 2002; Fakan, 2004; Scassellati et al., 2010). Osborne et al (2001) showed the association of PIP2 with hyperphosphorylated Pol II and snRNAs, providing an evidence of the involvement of PIP2 in mRNA splicing. We showed that PIP2 islets co-localize with nascent RNA transcripts and both Pol II CTD and TFIID TBP, which form the core of transcription complex and initiate transcription. Moreover, PIP2 hydrolysis upon PLC treatment resulted in the significant reduction of the transcription level in the whole nucleus as well as in PIP2 islets. We propose a model of the mutual arrangement of PIP2 molecules and PIP2 islet-associated proteins in the nuclear space (Figure 27). We suggest that PIP2 islets provide surface for the Pol II transcription, serving as the docking stations for the formation of the Pol II transcription complexes.

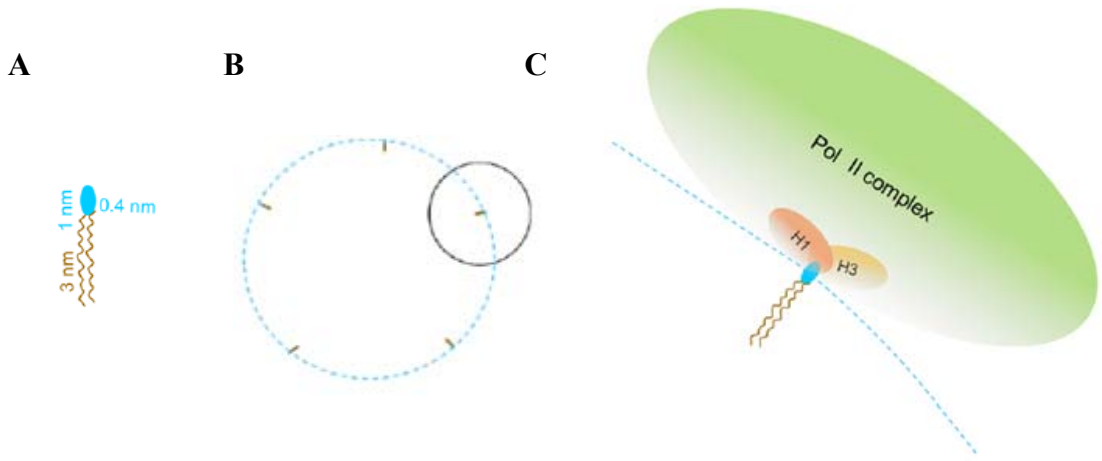


Figure 27 The proposed model of PIP2 islet associated with RNA polymerase II transcription complex
A) The size of PIP2 molecule. B) PIP2 islet (blue dashed circle) is schematically drawn in proportion to the size of PIP2 molecule. C) Magnified view of the surface of PIP2 islet, corresponding to the area outlined in black. We propose that the PIP2 islet serves as a docking station for the Pol II transcription complex.

The nuclear phospholipids were found also in other species. The nucleus of *Saccharomyces cerevisiae* was shown to contain PI4K which phosphorylates position 4 of the inositol ring producing PI4P (Garcia-Bustos et al., 1994). In *Tetrahymena*, biochemical studies revealed the presence of PIP2 as a substrate for PI3K, which is implicated in the macronuclear division mediated by microtubules (Smith et al, 2004). Regarding plants, heat stress caused PIP2 accumulation in the nuclei of *Nicotiana tabacum*, *Arabidopsis thaliana*, and Rice plants, which is considered as a part of the complex response implicating transcriptional and post-transcriptional changes (Mishkind et al., 2009). Remarkably, we showed that PIP2 islets are evolutionary conserved nuclear structures occuring in yeast, protozoan, plant, and animal cells and they localize out of the heterochromatin regions or the nuclear envelope.

Perhaps the most fundamental question that remains to be answered concerns the identity of PIP2 subnuclear fractions. While we commonly think of phosphoinositides within the context of a membrane, it is clear that nuclear PIP2 is present within non-membrane structures. It has been suggested that other hydrophobic molecules maintain a particular environment for phospholipids (reviewed in Irvine, 2003 and 2006). In this respect, nucleolar proteins are rich in basic residues (Hernandez-Verdun, 2011) and might harbor acidic phospholipids. The nucleus retains 40% of PIP2 under extraction with highly concentrated detergents, which supports notion of coverage with proteins (Vann et al., 1997; Gonzales and Anderson, 2006).

The crystal structure of the yeast phosphoinositide transfer protein (PITP) Sec14p revealed a large hydrophobic pocket into which PI is inserted upon extraction from phospholipid membranes (Sha et al., 1998). Interestingly, there are around 500 proteins containing SEC14 domain in their molecule, which might harbour nonmembranous PIP2. Furthermore, these SEC14 domain proteins differ primarily in the region predicted to bind the phospholipid head group, suggesting a mechanism of substrate specificity (Bunce et al., 2006).

The presence of phospholipids, particularly PIP2, in the nucleus has been extensively documented and it is now clear that PIP2 metabolism plays essential roles in a number of nuclear processes ranging from chromatin remodeling, gene expression, DNA repair and pre-mRNA splicing to stress response, growth and proliferation (reviewed in D´Santos et al., 1998; Bunce et al., 2006; Irvine, 2003). In recent years, significant progress has been made in understanding PIP2 nuclear events. Despite the accumulating data, there are still more questions raised than answered and much work remains to be done before we completely understand nuclear phosphoinositides.

CONCLUSIONS

PART I – NUCLEAR FUNCTION OF PAXILLIN

Paxillin regulates expression of growth-related genes *IGF2* and *H19* in human cells
We present a novel proliferation-regulatory pathway where paxillin enhances the transcription of *IGF2* growth-promoting peptide, while it suppresses the expression of growth-repressing gene *H19*.

Paxillin controls expression of *H19* and *IGF2* by regulating promoter-enhancer interactions
We show that paxillin regulates long-range chromosomal interactions formed between promoters of the *IGF2*/*H19* genes and the enhancer. In detail, paxillin stimulates the interaction between the enhnacer and the *IGF2* promoter and thus enhances the transcription of *IGF2*. Concurrently, it restrains the interaction between enhancer and the *H19* promoter, suppressing the expression of *H19*.

Paxillin mediates nuclear functions via binding to cohesin and Mediator complex
We show that paxillin interacts with cohesin and Mediator which have been shown to mediate long-range chromosomal looping. We propose that these interactions occur at the *IGF2*/*H19* gene cluster, regulating the formation of loops between the *IGF2*/*H19* promoters and the enhacer and thus their expression.

PART II - NUCLEAR PIP2

PIP2 is a stable component of RNA pol I transcription complex throughout the whole cell cycle
PIP2 is associated with Pol I subunits and UBF in a transcription-independent manner throughouth the whole cell cycle indicating that PIP2 plays wider role in nucleolar organization.

PIP2 forms spherical structures in nucleoplasm – „PIP2 islets“
We show that substantial part of nuclear PIP2 is located in previously uncharacterized structures, which are distinct from nuclear speckles and we refer to them as PIP2 islets. They are surrounded by nucleic acids and proteins, while the inner space seems to be mainly composed of carbon-rich compounds. We showed that PIP2 islets are evolutionary conserved from protozoa to human.

PIP2 islets are involved in RNA Pol II transcription
We show that PIP2 islets colocalize with C-terminal domain of Pol II, TATA-box binding protein (TBP) as well as nascent RNA transcripts. Moreover, PIP2 hydrolysis with PLC enzyme reduces the level of RNA pol II transcription. We propose that PIP2 islets are involved in RNA pol II transcription.

FUTURE PROSPECTS

PART I – NUCLEAR FUNCTION OF PAXILLIN

We proposed a novel regulatory mechanism for the IGF2/H19 locus mediated by paxillin in the complex with cohesin and Mediator, employing promoter-enhacer interactions. The cohesin and Mediator have a widespread role in the formation of promoter-enhancer chromatin loops in mammalian genome. However, is paxillin a constitutive part of this complex or does it provide gene selectivity? The interactions between the promoter and the enhancer have also been observed at the androgen receptor-dependent genes (Wang et al., 2005). Considering that AR-paxillin complex occupies promoters of AR-responsive genes, such as PSA or NKX3-1, in prostate cancer cell line (Sen et al., 2012), it will be interesting to find if paxillin is employed here in a similar way as we observed in the case of IGF2/H19 genes.

As we pointed out in the discussion, the proper expression of the H19 and IGF2 gene is crucial for normal development of a fetus. Since paxillin-knockout mice have embryonic-lethal phenotype, where paxillin-null embryos are significantly smaller than their wilde-type littermates and have impaired development of multiple organs (Hagel et al., 2002), our results might help to explain these developmental disorders and thus, this will be the way of our further research.

PART II - NUCLEAR PIP2

It is evident from our and numerous current studies that nuclear PIPs, in particular PIP2, are present in the nucleus. One of the key objectives which we want to address now is to describe the nuclear cycle of PIPs and the mechanisms involved in the regulation of their production. Is there a crosstalk between nuclear and extranuclear phosphoinositides? Do they share enzymes and substrates? What are the transport pathways crossing the nuclear membrane? Moreover, there are different pools of nuclear PIP2. It localizes to nucleoli, speckles and we also revealed PIP2 islets in the nucleoplasm, but how are these pools maintained? Are they isolated or do they share PIP2 molecules?

Although we have suggested how PIP2 islets are involved in transcription, serving as a platform for the formation of the Pol II transcription complexes, it is not clear how the process

is coordinated. Do gene transcriptional units move to the PIP2 islets or move the islets to relevant genomic areas? In the former case, how are the genes brought to the PIP2 islets?

We further plan to inspect the localization of other nuclear phosphoinositides and their possible crosstalk in nuclear processes. Are the different PIPs there to perform different functions?

METHODS

Cell lines

SAOS2 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), HepG2 cells in RPMI-1640 medium, both supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were incubated at 37 °C in a humidified 5% CO2/air atmosphere. Plasmids transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer´s protocol. Stable cell lines were established by selection with geneticin G-418 (800 μg/ml, Gibco, Life Technologies) or puromycin (2 μg/ml, Gibco, Life Technologies). Cells were treated with AMD (0.02 μg/ml) for 2 h or with DRB (50 μg/ml) for 1 h.

Antibodies

Following antibodies were used in this study: N-term rabbit monoclonal anti-paxillin (Merck Millipore); rabbit polyclonal anti-DRIP130 (MED23, Abcam); rabbit polyclonal anti-SMC1 (ChIP Grade, Abcam); goat polyclonal anti-SMC3 (Santa Cruz); rabbit polyclonal anti-CTCF (ChIP Grade, Abcam); rabbit polyclonal anti-H3K4me2 (Merck Millipore); rabbit polyclonal anti-GFP (Molecular Probes, Life Technologies); mouse monoclonal anti-actin (Sigma Aldrich); mouse monoclonal IgM anti-PIP2 (Abcam), rabbit polyclonal anti-GST (gift from Dr Igor Shevelev), rabbit polyclonal anti-UBF (Santa Cruz Biotechnology), rabbit polyclonal anti-PAF53 (gift from prof. Ingrid Grummt), mouse monoclonal anti-PAF53 (BC Transduction Laboratories), rabbit polyclonal anti-RPA116 (gift from Prof Ingrid Grummt), rabbit monoclonal anti-fibrillarin (Cell Signaling Technology Inc.), mouse monoclonal anti-fibrillarin (Abcam).

Cloning

Vector pSTrEP-GFP-PXN was prepared by ligation of full length human paxillin (U14588.1; kind gift from Ravi Salgia, M.D., Ph.D., The University of Chicago Medicine) into pSTrEP-EGFP-C3, which was previously prepared by insertion of the One-StrEP-tag sequence (IBA) into pEGFP-C3 (Dzijak et al., 2012). Firefly luciferase reporter vectors p*H19*P-luc and *IGF2*P3-luc were constructed by insertion of *H19* promoter (-819 to +13 bp relative to *H19* transcription start site) or *IGF2* promoter 3 (-499 to +13 bp relative to *IGF2* transcript 3 transcription start site) into pGL4.10 (Promega) using KpnI/HindIII sites. *H19* endodermal

enhancer sequence (+6033 to +10972 bp) was inserted into SalI site of p*H19*P-luc or p*IGF2*P3-luc to create p*H19*P-luc-E or p*IGF2*P3-luc-E, respectively.

GST-tagged PLCδ1PH (1–140) was received from Dr Hitoshi Yagisawa (Schneider et al., 2007). Recombinant PLCδ1PH domain is a commonly used PIP2 probe due to its high affinity to the head domain of PIP2 molecule (Schneider et al., 2007; Yildirim et al., 2013). The purification of For GST-tagged PLCδ1PH was performed on glutathione-agarose column (G4510, Sigma Aldrich).

Lentiviral transduction

The lentiviral particles containing shRNAs were generated in HEK293T cells using Addgene two-plasmid system (pMD2G, psPAX2) and pLKO.1 vectors containing anti-paxillin shRNA (shPXN) or non-target shRNA (shNON; Sigma, Non-Target shRNA Control), according to the manufacturer´s protocol. Five clones of shPXN were purchased and screened for knockdown efficiency, clone TRCN0000123136 was selected for further experiments. The viral supernatants were spun down, concentrated by PEG precipitation (PEG 6000, Fluka), aliquoted and stored in -80 °C. HepG2 or SAOS2 cells were seeded on a 24-well plate 24 h before transduction. Virus-containing supernatant was added to the medium, incubated overnight and after 24 h replaced with fresh medium containing puromycin. Protein, RNA or DNA content was analysed 5 days post transduction, unless otherwise stated.

BrdU incorporation assay

Cells were seeded on glass coverslips overnight, BrdU was added to the medium to final concentration of 1 μM and cultured for 1h. Subsequently, cells were fixed in 3% paraformaldehyde, DNA was denatured by incubation in 2 M HCl for 30 min and immediately neutralized in 0,1 M borate buffer, pH 9.0. After permeabilization with 0,1% Triton (w/v) and several washes in PBS, cells were incubated with primary anti-BrdU antibody for 1h, followed by anti-rabbit secondary antibody for 30 min and mounted with Mowiol (Sigma) containing 0.1 μg/ml DAPI (Sigma). Images were acquired with confocal microscope Leica TCS SP and rations BrdU-positive/total cells were counted using ImageJ.

Western blotting

Cells were homogenized in SDS lysis buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS) and briefly sonicated. Resulting lysates were cleared by centrifugation and total protein content was measured using Bradford assay. Equal amounts of protein were loaded on 10%

polyacrylamide gel, proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The blots were incubated with appropriate primary and fluorescently conjugated secondary antibodies and scanned on a Li-cor Odyssey imager.

Mass spectrometry analysis

Samples were prepared as folows: proteins were eluted with 50mM Tris pH 8.0, 2,5 mM desthiobiotin, eluates were digested with 1 μg of trypsin (Trypsin Gold, Promega) overnight at 30 °C. Peptides were desalted using microtrap column (MichromBioresources, CA), dried down and dissolved for LC-MS analysis. The liquid chromatography/mass spectrometric analysis was performed as previously described (Stodulkova et al., 2008) with minor modifications. The mass spectrometric analysis (type MALDI-TOF-TOF) was performed using Mascot 2.0 search engine (Matrix Science, USA) with the search parameters: SwissProt database, taxonomy human, trypsin specificity, no fixed modifications and oxidized methionine as variable modification, MS-tolerance 50 ppm, MS/MS tolerance 0.5 Da. Only proteins identified on three peptides with Mascot score above 30 were considered as positive hits.

RNA isolation and QPCR

Total RNA was isolated using GenElute Miniprep Kit (Sigma) according to manufacturer's protocol. RNA was treated with Rnase-free Dnase I for 30 min at room temperature. Concentration of RNA was measured by spectrophotometry and the integrity of RNA was checked on denaturing agarose gel. A total of 100 ng of RNA was reverse-transcribed with random hexamer primers using TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time PCR was performed on ABI Prism 7300 instrument using SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers. Data were evaluated with ΔΔCt method and transcript levels were normalized to GAPDH gene. Primers used for RT-PCR are shown in Supplementary Table SI.

Pull-down assays

Cells were harvested by trypsinization, spun down, washed twice in ice-cold PBS and resuspended in a lysis buffer (20 mM HEPES/KOH pH 7.4; 5 mM CH3COOK; 150 mM NaCl; 0,5 mM MgCl2; 0,5 mM DTT; 0.5% (w/v) Triton-X100) containing EDTA-free protease inhibitor coctail (cOmplete, Roche) and phosphatase inhibitor cocktail (Phos STOP, Roche). The lysate was centrifuged at 16 000 g for 20 min to pellet insoluble proteins. STrEP-

T-actin coupled sepharose beads (IBA) were washed several times with lysis buffer, added to the lysate and the mixture was incubated for 2h. Then the beads were spun down, washed 5 x 10 min with the lysis buffer and finally bound proteins were eluted in native form using lysis buffer or 50 mM Tris pH 8.0 (for MS analysis) containing 2,5 mM desthiobiotin. The eluate (called „pull-down“) was analyzed by SDS-PAGE or MS and proteins were visualized by staining polyacrylamide gel with SilverQuest kit according to manufacturer´s protocol (Invitrogen). If needed, eluates were concentrated by ultrafiltration (Ultracel 10K, Milipore).

Genotyping and allele-specific expression assay

Region covering *IGF2* SNP rs680 and *H19* rs2839704 was amplifed from SAOS2 gDNA with appropriate primers (see Table AI in Appendix) and sequenced. RNA isolated from SAOS2 shNON or SAOS2 shPXN cells was reverse transcribed (+RT), amplified with the same primers and digested with RsaI or ApaI for *H19* SNP or *IGF2* SNP, respectively. Fragments were resolved on 3% agarose gel. Absence of DNA contamination in the RNA samples was checked by parallel controls without reverse transctiption (-RT).

Methylation analysis

Genomic DNA isolated from SAOS2 cells was subjected to bisulfite conversion using Methylamp DNA Modification Kit (Epigentek) according to manufacturer´s protocol. Bisulfite treatment efficiently converts unmethylated cytosines to uracil, whereas 5-methylcytosine remains unchanged (Hayatsu et al., 1970). Treated DNA was purified and amplified by PCR with the primers corresponding to the ICR region. Primers specific for bisulfite-treated DNA were designed in Bisulfite Primer Seeker program (Zymo Research, see Table AI in Appendix). The amplified PCR product was inserted into pJET vector and transformed into competent cells. Plasmid DNA isolated from at least 30 bacterial colonies (for each variant shNON and shPXN) was sequenced.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using MAGnify Chromatin Immunoprecipitation System (Invitrogen, Life Technologies) according to manufacturer´s protocol. Around 300 K cells were used as a starting material and DNA was sheared with 15 sonication cycles (30 s ON, 30 s OFF; intensity HIGH) using Bioruptor Next Gen sonicator (Diagenode). Antibodies against paxillin, CTCF, control IgG (negative control) or H3K4me2 (positive control) were used. Input and immunoprecipitated DNA was quantified by QPCR. Enrichment was calculated as

IP over input and further normalised to a region where paxillin does not bind (GAPDH coding region). ChIP primers are shown in Table AI in Appendix.

Luciferase reporter assay

The Firefly luciferase reporter vectors p*H19*P-luc, p*IGF2*P3-luc, p*H19*-luc-E, p*IGF2*P3-luc-E (see „Cloning" for details) were co-transfected with Renilla luciferase reporter vector pGL4.74 into shPXN or shNON HepG2 cells, 4 days after lentiviral transduction. All cells were lysed after 24h and luciferase activity of both species was measured following Dual-Glo®Luciferase Assay System protocol (Promega), using ModulusTM II Microplate Multimode Reader (Turner Biosystems). Data were normalized to Renilla luciferase activity and all assays were done in triplicate and repeated at least three times.

Chromatin Conformation Capture Assay (3C)

Technique was adopted from Nature Protocols (Hagege et al., 2007), except from final semiquantitative analyses (Naumova et al., 2012). Briefly, cells expressing shNON or shPXN (5 days after transduction) were crosslinked with 1% formaldehyde, lysed and digested with PstI restriction enzyme. After ligation, samples were reverse-crosslinked overnight at 65 °C. Isolated DNA was amplified with corresponding primers (schematicly shown in Figure 15A; see Table AI in Appendix for sequences) and PCR products were resolved on 3% agarose gel. Amount of DNA input was first titrated, bands were analysed semiquantitatively using ImageJ program, background was subtracted and data normalized to loading control not affected by restriction digest (ICR region). Three biological replicates were prepared and analysed in three technical repeats.

Light microscopy, super-resolution structured illumination microscopy (SIM)

Cells grown on high performance cover glasses 18x18 mm² with restricted thickness-related tolerance D=0.17 mm +/- 0.005 mm and refractive index = 1.5255 +/- 0.0015 were fixed with either 3% formaldehyde and permeabilized with 0.1% Triton-X-100. Unspecific targets were blocked with either 0.25% bovine serum albumin (BSA) and 0.25% gelatin. All solutions were diluted in PBS. Then, cells were incubated with primary antibodies diluted in PBS, secondary antibodies diluted in PBS or PBST, stained with DAPI (only for SIM) and mounted in Mowiol. Importantly for both super-resolution microscopy techniques, extensive washes, up to 5 times for 5 or 10 min, were done in between all steps.

Images were acquired with either a confocal microscope (Leica TCS SP5 AOBS TANDEM) with 100 × (NA 1.4) oil immersion objective lens or microscope Nikon ECLIPSE Ti-E equipped with Andor iXon3 897 EMCCD camera by objective CFI SR Apochromat TIRF 100x/1.49 oil. Software NIS-Elements AR 4.20.01 and NIS Elements AR 4.30 was used for capturing and analysing the images. In each experiment, the images of all samples were acquired and reconstructed with the same parameters of acquisition and reconstruction respectively to ensure their comparability.

Immunoelectron microscopy

Interphase HeLa cells were fixed in the mixture of 3% formaldehyde and 0.1% glutaraldehyde, and embedded into LR White resin by standard procedure.55 Mitotic HeLa cells were high-pressure frozen, freeze-substituted and embedded into LR White resin according to a previously published protocol.56 Thin sections (70 nm) were examined in a FEI Morgagni 268 transmission electron microscope at 80 kV and in a Tecnai G2 20 LaB6 electron microscope (FEI) at 200 kV. The images were captured with Mega View III CCD camera (pixel size 6.45 × 6.45 μm) and with Gatan Model 894 UltraScan 1000 camera (pixel size 14 × 14 μm). Multiple sections of at least three independent immunogold labeling experiments were analyzed. Adobe Photoshop CS3 Version 10.0 was used to identify the geometrical centers of 6 nm gold nanoparticles and then cover co-centrically with red dots to facilitate the visualization of these small nanoparticles in images.

Electron energy loss spectroscopy (EELS) and elemental mapping

Elemental mapping was performed on ultrathin sections embedded in LR White or Quetol 651 resin. The 80-nm sections were immunolabelled with anti-PIP2 antibody and secondary antibody conjugated with 12 nm gold particles. Contrasting by uranyl acetate was omitted. EELS mapping of specific elements distribution was performed using a TECNAI G2 20 LaB6 transmission electron microscope (FEI) operated at 200 kV, equipped with GATAN imaging filter. Two pre-edge and one post-edge energy filtered images were collected at 100 eV, 120 eV, and 152 eV ($L_{II,\ III}$ edge) for phosphorus; at 353 eV, 383 eV, and 416 eV (K edge) for nitrogen; at 484 eV, 514 eV, and 532 eV (K edge) for oxygen; at 130 eV, 150 eV, and 165 eV ($L_{II,\ III}$ edge) for sulphur; at 252 eV, 272 eV, and 294 eV (K edge) for carbon. Drift correction was performed using an automated statistically determined spatial drift (SDSD) correction script for Digital Micrograph (Schafer et al., 2004), and elemental maps were calculated by

three-windows method using Digital Micrograph (Gatan). The images were processed using Digital Micrograph and Adobe Photoshop.

Detection of transcription sites

HeLa cells were washed in PB buffer on ice (100 mM CH_3COOK, 30 mM KCl, 10 mM Na_2HPO4, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 10 U/ml human placental RNase inhibitor (HPRI), 1 mM Na_2ATP; pH 7.2), permeabilized with 0.2% Triton-X-100 for 6 min on ice and transcription reactions were started by incubation with 100 mM BrUTP, 100 mM CTP, 100 mM GTP, and 0.3 mM $MgCl_2$ in PB. After 15 min at +35 ^oC, reactions were stopped by rinsing in ice-cold PB. Cells were either fixed with 4% formaldehyde for 40 min on ice, additionally permeabilized with 0.5% Triton-X-100 for 20 min, and further processed for SIM as described above or directly processed for IEM (Sobol et al., 2010).

5-Fluorouridine (FU) incorporation

U2OS cells grown on the coverslips were treated with home-made PLC (29 µg/ml in the buffer consisting of 50 mM HEPES, pH7.4 and 100 mM KCl) for 1 h at +37 ^oC. After 30 min of treatment, 5-fluorouridine (FU) was added to the final concentration of 2 mM and the cells were incubated for 30 min. Then the cells were washed, fixed, permeabilized and FU incorporated into the nascent RNA transcripts was detected with anti-BrdU antibody as described (Boisvert et al., 2000; Casafont et al., 2006; Kalendová et al., 2014; So et al., 2010). Images were acquired using Olympus IX81 high-throughput wide-field microscope with objective UPLSAPO 40x/0.90 oil. SCAN-R automated image and data analysis software was used to capture and analyse the images.

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APPENDIX

Research papers

Paxillin interacts with cohesin and regulates the expression of *IGF2*/*H19* gene cluster

Marášek P., Dzijak R., Studenyak I., Fišerová J., Uličná L., Novák P., Hozák P.

Submitted manuscript

Marasek P. performed the experiments and wrote the manuscript.

UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity.

Sobol M., Yildirim S., Philimonenko V. V., **Marášek P.**, Castano E., Hozak P.

Nucleus 2014, 4:6, 478–486. IF: 3.148

Marasek P. designed and performed cell fractionation and immunoprecipitation experiments.

PIP2 islets in the cell nucleus as a novel nuclear compartment

Sobol M., Philimonenko V., **Marášek P.**, Kalendová A., Kalasová I., Uličná L., Pastorek L., Hozák P.

Submitted manuscript

Marasek P. optimized conditions for PIP2 hydrolysis, designed and performed transcription-related experiments.

Table AI Primers used in this study

QPCR	
QPXN_FW	AAAGTTGCGGGGCATAGAC
QPXN_REV	AAGAACACAGGCCGTTTGGA
QGAPDH_FW	AAGGTGAAGGTCGGAGTCAA
QGAPDH_REV	AATGAAGGGTCATTGATG
QIGF2_FW	GTGGCATCGTTGAGGAGTG
QIGF2_REV	CACGTCCCTCTCGGACTTG
Q*H19*_FW	CTTTACAACCACTGCACTACCTGAC
Q*H19*_REV	GATGGTGTCTTTGATGTTGGGCTGA
SNPs genotyping, allele-specific expression	
SNP_IGF2_FW	CAACAACCCCTCTAAAACTAATTGGC
SNP_IGF2_REV	CCTCCTTTGGTCTTACTGGG
SNP_H19_FW	TACAACCACTGCACTACCTG
SNP_H19_REV	TGGAATGCTTGAAGGCTGCT
Bisulphite treatment	
ICR_BT_5962 FW	TGTTGAAGGTTGGGGAGATGGGA
ICR_BT_6413 REV	CCCAAACCATAACACTAAAACCCTC
Chromatin immunoprecipitation	
ChIP_GAPDH_FW	ACATCAAGAAGGTGGTGAAG
ChIP_GAPDH_REV	AGCTTGACAAAGTGGTCGTTG
ChIP_ENH A_FW	CCCAGGAAGATAAATGATTTCCTCCTC
ChIP_ENH A_REV	TGGGTCTCAGGGAATGGTCTC
ChIP_ ENH B_FW	CAAAGACATTTAGAAAAACCGGTTTAG
ChIP_ ENH B_REV	TGCAGACATCACTGTTGACACAC
ChIP_ ENH C_FW	ATGGGGGAGATGGACAACAG
ChIP_ ENH C_REV	GGGGGTCCATTTCTAGGCTCT
ChIP_ IGF2 P3_FW	AAATTTTGGGGAACGCAAGG
ChIP_ IGF2 P3_REV	CCCAAACCTGTAATCTATTTTTCTGGA

ChIP_ CTCF_FW	CTCCTTCGGTCTCACCGCCTGGAT
ChIP_ CTCF_REV	CCTTAGACGGAGTCGGAGCTG
Chromatin Conformation Capture (3C)	
3C_A1	AGAGCGGGAAGACAGGCAGT
3C_A2	GAAAACATCAACACAGCCAGGG
3C_B	ACAGGCCAATTTGACTTACCCAAG
3C_C	ATCTCCCAGCACTGCCCAT
3C_D	CTCTACCCCGCTCCCTGGC
3C_E	CAACACCTGGCTGGACACAG
3C_F	CACGTTGCAGAAAACTGGCTG
3C_G	AGCACAGAGAGGCACACGTAGG
3C_H	GTTCACAGGAAGCATTCGAGATAAG
3C_I	GAGACCCCATCTCTACCAAATATCA
IGF2 promoter-specific expression	
IGF2_T1_FW	GCCCCAACTGCGAGGCAGAG
IGF2_T2_FW	CGTAGAGCAACTCGGATTTGG
IGF2_T3_FW	CCAGAGCGGCGCTGGCAG
IGF2_T4_FW	TTCCTCCTCCTCCTCCTGCCCCAG
IGF2_T_REV	CTGAAGTAGAAGCCGCGG