The nuclear import mechanism of SRF co-activator MKL1

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Auditorium 1041, Biocenter 2, Viikinkaari 5, Helsinki, on September 25th 2015 at 12 o'clock noon.

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ISBN 978-951-51-1493-8 (paperback)
ISBN 978-951-51-1494-5 (PDF)
ISSN 2342-3161 (paperback)
ISSN 2342-317X (PDF)
Hansaprint, Helsinki 2015

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LIST OF ORIGINAL PUBLICATIONS

This thesis work is based on the following original articles, which are referred in the text by their roman numerals I-III.

I. Pawlowski R, **Rajakylä EK**, Vartiainen MK, Treisman R (2010). An actin-regulated importin α/β-dependent extended bipartite NLS directs nuclear import of MRTF-A. *The EMBO Journal* **29** (20): 3448-3458.

II. **Rajakylä EK**, Viita T, Kyheröinen S, Huet G, Treisman R, Vartiainen MK (2014). RNA export factor Ddx19 is required for nuclear import of the SRF coactivator MKL1. *Nature Communications* **6** (5978).

III. Huet G*, **Rajakylä EK***, Viita T*, Skarp KP, Crivaro M, Dopie J, Vartiainen MK (2012). Actin-regulated feedback loop based on Phactr4, PP1 and cofilin maintains the actin monomer pool. *Journal of Cell Science* **126**: 497-507.*equal contribution

Contributions:

I. Kaisa Rajakylä carried out the experiments for figure 3 (A,B and C).

II. Kaisa Rajakylä performed all the experiments and analyzed the data with valuable help from the co-authors. Kaisa Rajakylä together with Maria Vartiainen wrote the manuscript.

III. Kaisa Rajakylä performed the experiments for figures 6 and 7.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
B1/B2/B3	basic box 1/2/3
CD	cytochalasin D
ChIP	chromatin immunoprecipitation
CRM1	chromosome region maintenance 1
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMT	epithelial mesenchymal transition
F-actin	filamentous actin
FHL2	four and a half LIM domain protein
FISH	fluorescent *in situ* hybridization
FLIM	fluorescence lifetime imaging
FRET	förster resonance energy transfer
G-actin	globular/monomeric actin
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
IP_6	inositolhexakisphosphate
JMY	junction-mediating and regulatory protein
Kap	karyopherin
LatB	latrunculin B
LIMK	LIM domain kinase
LMB	leptomycin B
LPA	lysophosphatidic acid
LZ	leuzine zipper
MAL	megakaryocytic acute leukemia protein
MKL1	megakaryoblastic leukemia protein 1
MRTF	myocardin related transcription factor
mRNA	messenger RNA
NE	nuclear envelope
NES	nuclear export signal
NLS	nuclear localization signal
NTF	nuclear transport factor
Nup	nucleoporin
OTT	one twenty two protein
Phactr	phosphatase and actin regulating protein
pol	polymerase
PPI	protein phosphatase 1

RanGAP	Ran GTPase activating protein
RanGDP	Ran guanosine diphosphate
RanGTP	Ran guanosine triphosphate
RBM15	RNA binding motif protein 15
RCC1	regulator of chromosome condensation 1
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	Rho kinase
SCAI	suppressor of cancer cell invasion
siRNA	small interfering RNA
SMC	smooth muscle cell
SRF	serum response factor
SRE	serum response element
STAR	striated muscle activator of Rho signaling
TAD	transactivation domain
TCF	ternary complex factor
TGF-β1	transforming growth factor β1
Q	glutamine rich stretch

ABSTRACT

An essential transcription factor Serum response factor (SRF) and its co-activators, Myocardin related transcription factors (MRTFs) control the expression of many target genes required for normal growth and actin cytoskeleton regulation. MKL1 (also known as MRTF-A and MAL) is one family member of MRTFs and mediates the signals from the cytoplasm to the nuclear SRF in response to changes in actin dynamics. Although it is well established that actin regulates nucleo-cytoplasmic shuttling of MKL1, the molecular mechanism of this regulation has not been characterized. Therefore the aim of this thesis was to reveal the mechanisms of MKL1 nuclear import.

RNA interference (RNAi) screen identified two proteins as putative proteins mediating MKL1 nuclear localization: Importin-β (Ipoβ), which is the main import receptor in cells, and mRNA export factor Ddx19. The main purpose of this study was to confirm the hits from the RNAi screen and assess their specificity in regulating MKL1 localization. This work revealed that both Ipoβ and Ddx19 are specific and necessary factors for MKL1 nuclear import and thus required for SRF activation. We show that Ipoβ together with its adaptor protein Importin-α (Ipoα), binds to a bipartite nuclear localization signal (NLS) of MKL1, which is located in the actin-binding RPEL repeat and composed of two basic elements. Furthermore, the biochemical assays demonstrate that actin competes with Ipoα/Ipoβ heterodimer for access to the MKL1 NLS, thus explaining the inhibitory effect that actin binding has on MKL1 nuclear localization. By using advanced microscopy techniques, we show that Ddx19 adds an additional regulatory step for MKL1 nuclear import by modulating the conformation of MKL1, which affects its interaction with Ipoβ for efficient nuclear import. The ATPase cycle of Ddx19, which is crucial for its role in mRNA export, is not required in MKL1 nuclear import. In contrast, the RNA-binding activity of Ddx19 seems to be required. My work thus proposes a novel role for Ddx19, a well-known mRNA export factor and regulator of translation, in nuclear import of MKL1.

In addition to MKL1, the conserved actin-binding RPEL repeat is also present in the Phosphatase and actin-regulating proteins (Phactrs). Our work demonstrates that the RPEL repeat of Phactr4 does not determine its localization in cells, but instead facilitates the competitive binding of

monomeric actin and Protein phosphatase 1 (PPI) to Phactr4. This mechanism is required to control the phosphorylation status of cofilin, one of the downstream targets of PPI. Upon decrease in the cellular G-actin levels, Phactr4 activates cofilin through its binding to PPI, which leads to increase in the cellular levels of monomeric actin. Therefore our data points to an important role for Phactr4 in a feedback system, where actin monomers can locally regulate their own abundance. Thus this work highlights the role of RPEL repeat as a universal actin-binding site, which regulates actin homeostasis in cells.

1. INTRODUCTION

1.1 Gene expression and nucleo-cytoplasmic transport

Transcription is the first step of gene expression, where genes are transcribed from DNA into RNAs. Transcription factors control this process by binding to specific DNA sequences, and thereby to either promote or repress transcription. To activate transcription, transcription factors recruit other proteins relevant to transcription initiation, such as general RNA polymerase machinery and chromatin modifying complexes. The latter can modify otherwise tightly packed chromatin to allow the access of the transcription machinery to the specific genes.

Transcription factors themselves are often regulated by cofactors (co-activators or co-repressors). Cofactors provide specificity, but can also increase the regulatory potential of a single transcription factor by mediating different signals to transcription factors (reviewed in Pipes et al, 2006).

Various cytoplasmic and extracellular signals activate signal transduction pathways to initiate gene expression. In order to activate gene expression, these signals must be transmitted to the nucleus. Also, as protein synthesis occurs in the cytoplasm, transcription factors themselves, in addition to the rest of the transcription machinery, must be imported into the nucleus. Conversely, the resulting mRNAs must be exported out of the nucleus for cytoplasmic translation. Therefore, regulated nuclear import and export of macromolecules plays an essential role in the control of gene expression (reviewed in Turpin et al, 1999). To reach their target compartment, these macromolecules must traverse the barrier of the nuclear envelope (NE), which separates the nucleus from the surrounding cytoplasm. This is facilitated by a transport system that allows the selective exchange of substances between nucleus and cytoplasm (reviewed in Raices & D'Angelo, 2012; Wente & Rout, 2010).

1.1.1 Nuclear envelope and nuclear pore complex

The NE consists of two lipid bilayers: the outer and the inner nuclear membrane that are fused at the site, where most of the transport between nucleus and cytoplasm occurs (reviewed in D'Angelo & Hetzer, 2008; Strambio-De-Castillia et al, 2010). These fusion sites contain multiprotein complexes known as nuclear pore complexes (NPCs). NPCs are formed from two functional

domains: the central channel and peripheral structures that comprise the nuclear basket and cytoplasmic filaments (reviewed in Strambio-De-Castillia et al, 2010). The proteins that create the NPC are collectively called as nucleoporins or Nups. Many of the Nups that are located in the central channel of the NPC contain unfolded Phenylalanine-Glycine (FG) domains. By interacting with each other, these FG domains form a sieve-like structure (FG-FG hydrogel) (Frey & Gorlich, 2007; Patel et al, 2007) that prevents the entry of macromolecules, but is freely permeable to ions, water and proteins that are smaller than its pore size [~5 nm in diameter, which corresponds to ~30 kilodaltons (kDa)] (reviewed in Guttler & Gorlich, 2011). Thus, the NPC has two main purposes: to define an upper size limit for passive diffusion and to facilitate the active transport of macromolecules through it (reviewed in Wente & Rout, 2010).

1.1.2 Active nuclear transport of proteins

1.1.2.1 Nuclear transport factors

To overcome the permeability barrier of the NPC, larger macromolecules require the assistance of nuclear transport factors (NTFs) for NPC passage. NTFs facilitate the transport of their cargoes by interacting with the FG Nups that line the central channel of the NPC. They move through the channel by competing with the FG-FG interactions and cause a local disassembly of the network (Frey & Gorlich, 2007). *In vitro* studies have shown that NTFs can accelerate the transport of their cargo molecules over 1000-fold (Ribbeck & Gorlich, 2001). Transport by NTFs also requires energy that is most often provided by the RanGTP gradient. Ran is a member of the Ras family of GTPases that act as molecular switches by binding to either GTP or GDP. Ran in its GTP-bound state is present in the nucleus, whereas cytoplasmic Ran is GDP-bound. This asymmetric distribution across the NE creates the RanGTP gradient, which is maintained by Ran regulatory proteins that control the nucleotide status of Ran (Gorlich et al, 1996b). The main proteins regulating the RanGTPase cycle are the RanGTPase activating protein (RanGAP) and Ran guanine nucleotide exchange factor (RanGEF), also known as regulator of chromosome condensation (RCC1) (Bischoff et al, 1994; Bischoff & Ponstingl, 1991). RanGDP levels are high in the cytoplasm, because RanGAP, which

increases the GTPase activity of Ran, is mainly present at the cytoplasmic side of the NPC (Mahajan et al, 1997). In contrast, RCC1, which mediates the nucleotide exchange from GDP to GTP, is confined to chromatin and is present solely in the nucleus (Bischoff & Ponstingl, 1991; Ohtsubo et al, 1989) (Fig.1).

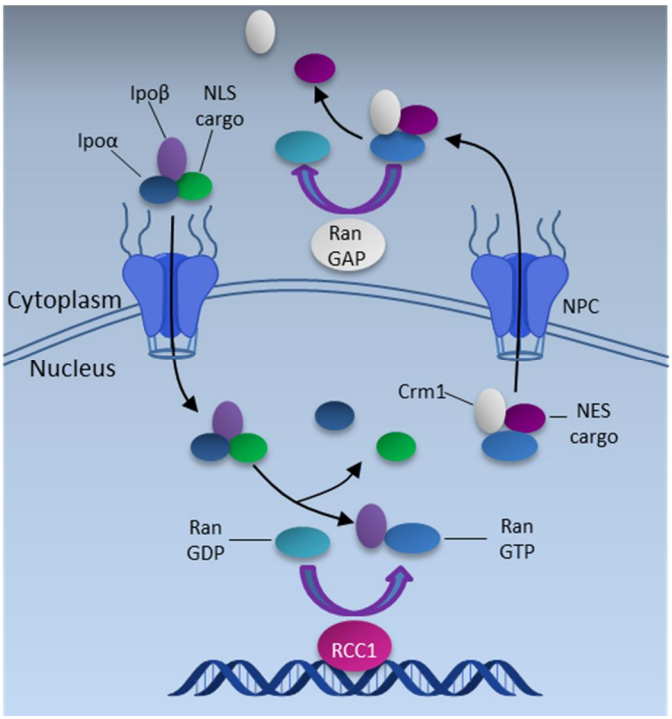

Figure 1. The nuclear transport cycle. Nuclear import and export of macromolecules across the nuclear pore complexes (NPCs) require the assistance of nuclear transport factors (NTFs), as well as energy that is provided by a RanGTP gradient. The RanGTP gradient across the nuclear envelope originates from the distinct localization of Ran-regulatory proteins. Regulator of chromosome condensation 1 (RCC1, pink), which sustain Ran in its GTP-bound form, binds directly to chromatin, whereas RanGTPase activating protein (RanGAP, grey), which promotes the GTPase activity of Ran, is mainly cytoplasmic. The RanGTP gradient also provides directionality to NTF-mediated nuclear transport. In the cytoplasm where the RanGTP levels are low, Importin-β (Ipoβ, purple) together with its adaptor protein Importin-α (Ipoα, dark blue) binds to its cognate nuclear localization signal-containing cargo (NLS-cargo, green). Ipoα/Ipoβ-NLS-cargo complex then travels across the NPC as an entity and dissociates upon RanGTP (blue) binding to Ipoβ in the nucleus. Conversely, nuclear export factor Chromosome region maintenance 1 (Crm1, light grey) binds to its nuclear export signal-containing cargo (NES-cargo, dark purple) in the presence of RanGTP in the nucleus, accompanies it through the NPC and releases it upon RanGTP hydrolysis to RanGDP (cyan) in the cytoplasm (see also below 1.1.2.2, 1.1.2.3, 1.1.2.4).

1.1.2.2 Kap-mediated nuclear transport

The family of karyopherins (Kaps) are the major NTF class and includes 14 members in yeast and 21 members in mammals (Fornerod et al, 1997; Gorlich et al, 1997) (Table 1). They are large HEAT-repeat proteins that have homology to Importin-β and can directly bind to RanGTP (Gorlich et al, 1997). Kaps that mediate the nuclear import are often called importins, whereas those mediating the nuclear export are referred to as exportins (reviewed in Cook et al, 2007).

Table 1. Characterized members of the Kap family. Only examples of cargoes and their references are listed.

NTF	Example of Cargo	Selected reference
Import		
Importin-β	Importin-α, SREBP-2 PTHrP	(Cingolani et al, 2002; Gorlich et al, 1995; Lee et al, 2003)
Transportin or karyopherin β2	Nab2, ribosomal proteins	(Aitchison et al, 1996; Jakel & Gorlich, 1998b)
Importin 4	Transition protein 2	(Pradeepa et al, 2008)
Transportin SR	SR proteins	(Kataoka et al, 1999)
Importin 5/Importin-β3/ RanBP5	ribosomal proteins	(Jakel & Gorlich, 1998a)
Importin 7	Smad, ERK	(Lorenzen et al, 2001; Yao et al, 2008)
Importin 8	Smad	(Yao et al, 2008)
Importin 9	Histones, Actin	(Dopie et al, 2012; Muhlhausser et al, 2001)
Importin 11	UbcM2	(Plafker & Macara, 2000)
NTF2	RanGDP	(Ribbeck et al, 1998)
Export		
Crm1 (Exportin 1)	Leu-rich NES cargoes RanBP1	(Fornerod et al, 1997) (Richards et al, 1996)
Cas (Exportin 2)	Importin-α	(Kutay et al, 1997)
Exp-t (Xpot)	tRNA	(Kutay et al, 1998)
Exportin 5 (Xpo5)	tRNA, eEF1A (via tRNA), miRNA	(Bohnsack et al, 2002; Yi et al, 2003)
Exportin 6	Actin, Profilin	(Stuven et al, 2003)
Exportin 7	p50-RhoGAP	(Mingot et al, 2004)
Import/Export		
Importin 13	UBC9, Mago-Y14 (import) elFIA (export)	(Mingot et al, 2001)
Exportin 4	Sox2, SRY (import) elF5A (export)	(Gontan et al, 2009) (Lipowsky et al, 2000)

Importins and exportins react differently to the RantGTP gradient. Importins bind their cargoes in the cytoplasm in low RanGTP concentrations, and accompany them through the NPC. Solution binding assays have demonstrated that binding of RanGTP to importins leads to the dissociation of cargo protein (Rexach & Blobel, 1995). This dissociation occurs in the nucleus, where the RanGTP levels are high (Gorlich et al, 1996b). For exportins an opposite process occurs: exportins bind to their cargoes in the presence of RanGTP, which is abundant in the nucleus. The exportin-cargo-RanGTP complex then moves across the NPC and dissociates upon hydrolysis of the RanGTP in the cytoplasm (Fornerod et al, 1997; Kutay et al, 1997). Thus, the RanGTP gradient provides the energy and directionality to the Kap mediated nuclear transport (see above Fig.1).

1.1.2.3 Transport signals

Kaps bind to their cognate proteins via transport signals and accompany them through the NPC. At some situations, however, the binding requires an adaptor protein (see below). The best described transport motifs/signals are the nuclear localization signal (NLS) for import and nuclear export signal (NES) for export (reviewed in Cook et al, 2007). Two types of classical NLSs have been described: SV40-type monopartite- and nucleoplasmin-type bipartite NLS. Monopartite NLS consists of a cluster of three to five positively charged lysine/arginine (K/R) stretches (SV40 NLS: KKKRR) (Dingwall et al, 1982). Bipartite NLS consists of two KR-rich regions, which are separated by 10 to 12 amino acid linker sequence (nucleoplasmin: KRxxxxxxxxxxKKKK; x means any amino acid) (Robbins et al, 1991). Cargoes that contain either classical mono- or bipartite NLS are imported into the nucleus by Importin-α/Importin-β (Ipoα/Ipoβ) nuclear import complex (Conti & Kuriyan, 2000).

The best characterized NESs are found from the cargoes of the main cellular export receptor Chromosome region maintenance 1 (Crm1) (Fornerod et al, 1997). They consist of leucine (L)-rich regions or stretches of other hydrophobic residues (reviewed in Kutay & Guttinger, 2005). The typical NESs, LxxxLxxLxL, are found from Crm1 cargoes such as Ran binding protein 1 (Richards et al, 1996) and protein kinase inhibitor A (Wen et al, 1995).

In principle, molecules lacking the NLS/NES cannot bind Kaps and thus are not transported through the NPC. In some situations, however, alternative ways have evolved to overcome this problem. One example is the nuclear import of actin. Actin lacks the classical NLS, but is actively imported into the nucleus as a complex with cofilin that mediates the interaction between actin and its import receptor Importin-9 (Dopie et al, 2012).

1.1.2.4 Classical nuclear import pathway

Ipoβ is an extensively studied member of the Kap family of NTFs. It consists of 19 tandem HEAT repeats, which form a flexible superhelical structure (reviewed in Conti et al, 2006). Crystal structures of Ipoβ bound to distinct proteins have shown that Ipoβ can adopt variable conformations and provide distinct binding sites for different cargoes. For example, sterol regulatory element-binding protein 2 (SREBP-2) binds HEAT repeats 7 and 17 (Lee et al, 2003), whereas parathyroid hormone-related protein (PTHrP) binds HEAT repeats 2 and 11 (Cingolani et al, 2002). Such structural flexibility allows Ipoβ to bind a broad range of distinct proteins such as adaptor protein Ipoα, Ran, Nups and a variety of cargo proteins. Although Ipoβ can bind directly to some cargo proteins and accompany them into the nucleus, it often utilizes adaptor proteins (reviewed in Marfori et al, 2011). Among them is Ipoα, which has a recognition site for both mono- and bipartite NLSs, as well as an Ipoβ-binding domain (IBB) (Gorlich et al, 1996a; Weis et al, 1996). The recognition site for NLS in Ipoα consists of 10 armadillo motifs, which are organized into three α-helices (Conti et al, 1998). Structural studies have demonstrated that these helices contain two specific NLS binding sites, major and minor binding pockets. The structure of Ipoα in complex with c-Myc shows that Ipoα binds to a monopartite NLS through its major binding pocket, whereas the bipartite NLS of nucleoplasmin engages both major and minor binding pockets (Conti & Kuriyan, 2000). The nuclear import pathway mediated by Ipoα/Ipoβ is considered as the classical nuclear import pathway. Here, the cargo-Ipoα-Ipoβ complex forms in the cytoplasm and translocates as an entity into the nucleus, where the complex is dissociated by RanGTP (Gorlich et al, 1996b). Structural analysis has revealed that RanGTP binding locks Ipoβ into a conformation that cannot bind Ipoα and thus leading to a release of Ipoα-cargo complex (Lee et al,

2005). In the absence of Ipoβ, Ipoα switches into an autoinhibited state, where the IBB domain binds to the NLS recognition site, thus masking it from the cargo (Kobe, 1999). The import adaptor Ipoα is then transported back to the cytoplasm by export receptor CAS (Cse1 in yeast), which specifically recognizes the "NLS-free" form of Ipoα (Kutay et al, 1997).

It has been estimated that 50 % of all nuclear import is mediated by Ipoα/Ipoβ (Lange et al, 2007). Therefore these proteins are indirect mediators of many important cellular processes including gene expression, signal transduction and the cell cycle. The ability of Ipoα/Ipoβ to import such a wide range of cargoes can in part be explained by their flexible solenoid structure, which can be adjusted for the different cargoes (reviewed in Conti et al, 2006). Also, there are as many as seven Ipoα paralogs in mammals, which all can bind to Ipoβ (Pumroy & Cingolani, 2015). This further expands the range of proteins utilizing the classical nuclear import pathway.

1.1.3 Nuclear export of mRNA

Nuclear export of mRNAs differs from the protein transport mechanism presented above. The most notable exception is that mRNA export is not dependent on any Kaps and there is no direct role for the small GTPase Ran (reviewed in Wente & Rout, 2010).

Nuclear export of mRNA has been mainly studied in yeast, but the mechanisms appear to be conserved also in other organisms. Before the mRNAs are ready to be exported, they undergo multiple processing steps, which include 5' capping, splicing, 3' cleavage and addition of the poly(A) tail (reviewed in Stewart, 2010). The primary mRNA export factor in metazoans is Nxf1 (Mex67 in yeast), which forms a heterodimer with Nxt1 (Mtr2 in yeast) (reviewed in Tran et al, 2014). However, it has been shown that Nxf1/Nxt1 complex binds mRNA only weakly and therefore requires adaptor proteins such as ALY (Yra1 in yeast) (Strasser & Hurt, 2000; Zhou et al, 2000), Npl3 (Lee et al, 1996) and Nab2 (Iglesias et al, 2010; Tran et al, 2007). It is thought that these adaptor proteins are added during 3´ processing of nascent transcripts and facilitate the recruitment of Nxf1/Nxt1, thus forming the export competent mRNA-protein complexes (mRNPs). The addition of ALY, which can only associate with the

spliced mRNA, is also a signal of splicing completion (Zhou et al, 2000). Nxf1/Nxt1 heterodimer facilitates the nuclear export of mRNPs across the NPC through interactions with FG-Nups (reviewed in Stewart, 2010). At the cytoplasmic side of the NPC, a RNA helicase Ddx19 (better known as Dbp5 in yeast) mediates the disassembly of Nxf1/Nxt1 and Nab2 from mRNPs. This remodeling prevents mRNA from returning to the nucleus and freeing it to cytoplasmic translation (reviewed in Cole & Scarcelli, 2006). Thus, the function of Ddx19/Dbp5 is to provide directionality to the passage of mRNA across the NPC.

1.1.3.1 Ddx19/Dbp5

Ddx19/Dbp5 belongs to the family of DEAD-box RNA helicases, which are capable of remodeling RNA-RNA and RNA-protein complexes in an ATP-dependent manner (Snay-Hodge et al, 1998). The name of the family refers to amino acids D-E-A-D (aspartic acid D, glutamic acid E, alanine A), which are located in the motif II (see below 1.1.3.2 and Fig.2) and are required for ATP binding and hydrolysis (reviewed in Cordin et al, 2006). Ddx19/Dbp5 is an essential and conserved protein in eukaryotes and functions in mRNA export in all the organisms studied. In yeast it also participates in translation termination (Gross et al, 2007). Ddx19/Dbp5 is primarily localized to the cytoplasm and around the NE, specifically to cytoplasmic filaments of the NPC (Hodge et al, 1999; Schmitt et al, 1999). However, it shuttles between nucleus and cytoplasm in Crm1-dependent manner (Hodge et al, 1999) and has been connected to nuclear functions in fly (Zhao et al, 2002) and in yeast. In yeast Dbp5 associates with the transcription factor II H complex (Estruch & Cole, 2003).

1.1.3.2 Structural domains in Ddx19/Dbp5

Ddx19/Dbp5 contains 9 sequence motifs (I-VI, Ia, Ib, and Q) that contribute to the RNA binding and to the ATP binding and hydrolysis (Fig.2). These motifs are organized within two Rec-A like domains (N-and C-terminal RecA-like domains) that are flexibly connected with a linker region (reviewed in Ledoux & Guthrie, 2011). Rec-A like domains are in a closed conformation when Ddx19/Dbp5 is bound to ATP. The N-terminal region is thought to control the

enzyme activity of Ddx19/Dbp5, because deleting this region significantly increases the ATPase activity of the protein (Collins et al, 2009).

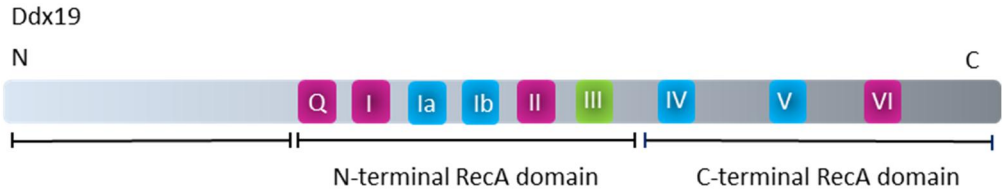

Figure 2. Sequence motifs of Ddx19/Dbp5. Location of sequence motifs, which are required for ATP binding/hydrolysis are colored in magenta, whereas sequence motifs required for RNA binding are colored in cyan. Green color indicates that the motif is required for both ATP- and RNA-binding (adapted from Ledoux & Guthrie, 2011).

1.1.3.3 Ddx19/Dbp5 in mRNA export

Ddx19/Dbp5 is localized to the cytoplasmic filaments of the NPC through an interaction with Nup214 (Nup159 in yeast). There its remodeling activity is controlled by another NPC protein Gle1 and the small molecule inositolhexakisphosphate (IP_6) (Alcazar-Roman et al, 2006; Weirich et al, 2006). Similarly to Ran (see above 1.1.2.1), Ddx19/Dbp5 cycles between the ATP and ADP bound states. Based on studies mainly in yeast (Hodge et al, 2011; Montpetit et al, 2011; Noble et al, 2011; von Moeller et al, 2009), models on how Ddx19/Dbp5 ATPase cycle during mRNA export is regulated have been developed (reviewed in Folkmann et al, 2011; Ledoux & Guthrie, 2011; Tieg & Krebber, 2013). Functional studies favor the model where Gle1-IP_6-binding enhances the ATP loading of Ddx19/Dbp5, which is required for efficient RNA binding (Hodge et al, 2011; Noble et al, 2011). RNA binding in turn stimulates Ddx19/Dbp5 ATP hydrolysis event, thus resulting in release of the remodeled mRNPs (Hodge et al, 2011; Noble et al, 2011). In contrast, structural studies suggest that binding of Gle1-IP_6 to Ddx19/Dbp5 stabilizes an open conformation, which cannot bind RNA and therefore leads to RNA release (Montpetit et al, 2011). Both models agree that the release of the bound RNA allows Nup214 to bind Ddx19/Dbp5, which enhances its ADP release and enzyme recycling to begin the cycle again (Hodge et al, 2011; Montpetit et al, 2011; Noble et al, 2011). As a result, export factors Nxf1-Nxt1 and adaptor protein Nab2, which both are direct remodeling targets of Ddx19/Dbp5, are

released from the exported mRNP and returned to the nucleus, whereas the mRNAs are freed for cytoplasmic translation (Lund & Guthrie, 2005; Tran et al, 2007).

The role of Ddx19/Dbp5 in mRNA export is extensively studied. However, in yeast it also participates in translation termination. Here, Dbp5 is required to enhance the stop codon recognition by release factors (RFs) eRF1 and eRF3. Dbp5 directly interacts with eRF1 and ATP deficient mutants of Dbp5 cannot recruit eRF1 and eRF3 into termination complexes (Gross et al, 2007). Therefore release complex association requires ATPase activity of Dbp5, which is stimulated by Gle1-IP_6 (Alcazar-Roman et al, 2010; Bolger et al, 2008). According to these results, it has been speculated that Dbp5 is required to remodel mRNA/protein structures to allow eRF1 to access the stop codon (Alcazar-Roman et al, 2010; Bolger et al, 2008; Gross et al, 2007). However, the potential contribution of mammalian Ddx19 to translation termination has not been investigated.

1.2 The actin-MKL1-SRF circuit

1.2.1 Serum response factor

Serum response factor (SRF) is a conserved transcription factor that is broadly expressed in many cell types (Shore & Sharrocks, 1995). It was first found by Treisman group in studies concentrated on fibroblast serum response (Norman & Treisman, 1988). SRF controls its target genes by binding to promoter sequence $CC(A/T)_6GG$, which is alternatively named as a CArG box or a serum response element (SRE) (Treisman, 1986). Recent chromatin immunoprecipitation (ChIP) studies combined with deep sequencing (ChIP-seq) have identified over 3100 SRF binding sites in mouse fibroblast (Esnault et al, 2014). One third of them (960) are linked with serum-dependent gene activation (Esnault et al, 2014). SRF target genes are enriched in immediate early, muscle-specific and contractility-related genes (Esnault et al, 2014; Sun et al, 2006). SRF null mice display embryonic lethality (Arsenian et al, 1998). This dramatic phenotype highlights the importance of this transcription factor to the normal development.

The activity of SRF is regulated by two families of co-activators that respond to different signaling events and activate different sets of target genes (Gineitis &

Treisman, 2001; Miralles et al, 2003; Wang et al, 2004; Zaromytidou et al, 2006). The immediate early genes (e.g. *c-fos* and *egr1*) in response to growth factors and mitogen-activated protein (MAP) kinase signaling are activated by the family of ternary complex factors (TCFs) including SAP-1, Elk-1 and NET. These proteins, when phosphorylated, form a ternary complex with SRF on the promoters of immediate early genes to activate their expression (Treisman, 1994). The contractile genes are activated by myocardin related transcription factors (MRTFs), which respond to Rho-induced fluctuations in cellular actin monomer levels (Miralles et al, 2003; Vartiainen et al, 2007) (Fig. 3 and see below).

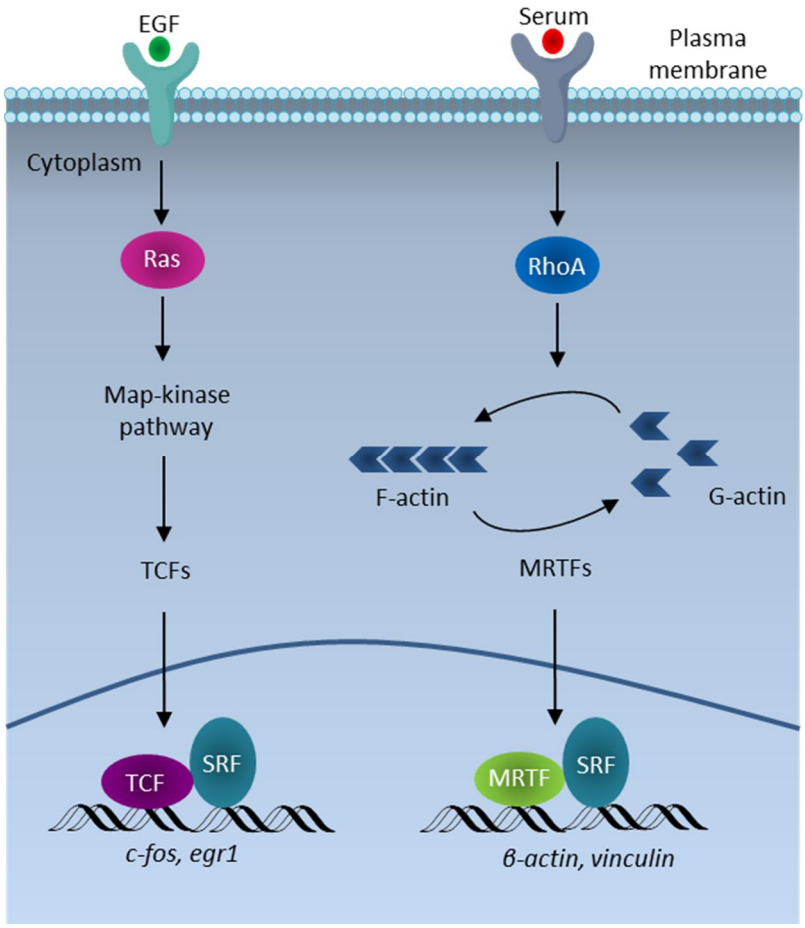

Figure 3. Two types of SRF co-activators respond to different signals and regulate different target genes. Map-kinase pathway is activated by epidermal growth factors (EGF) and small GTPase Ras. As a result, TCFs are phosphorylated, which enables them to form a ternary complex with SRF on the promoters of SRF target genes *c-fos* and *egr1*. In contrast, lysophospatic acid (LPA), a component of the serum, activates SRF target genes *β-actin* and *vinculin* via a signaling pathway that involves the small GTPase RhoA. RhoA induces actin polymerization, which leads to decrease in the actin monomer levels. Decrease in the actin monomer levels activates SRF through MRTFs (adapted from Gineitis & Treisman, 2001).

1.2.1.1 Rho GTPases

Rho family of small GTP-binding proteins (Rho GTPases), including RhoA, Rac1 and Cdc42, are signalling molecules that can transfer signals from membrane-bound receptors to the actin cytoskeleton. Rho GTPases switch between GTP and GDP bound states, and this is controlled by different Rho regulatory proteins. Rho GTPases can interact with and activate their

downstream effectors when they are bound to GTP. These downstream effectors include protein kinases and many actin-binding proteins, which can modulate actin cytoskeleton rearrangements (reviewed in Heasman & Ridley, 2008; Ridley, 2011).

1.2.1.2 Actin in cells

Actin is an essential protein in eukaryotic cells. It is the main constituent of the cytoskeleton and provides both shape and mechanical support to the cells. Actin assembly into filaments is necessary for its motile functions. Filaments (F-actin) are formed by polymerization of actin monomers (G-actin) in their fast-growing barbed end, whereas their disassembly occurs at the opposite pointed end. This dynamic process (polymerization and depolymerization) provides the force that is required in motile cell functions (reviewed in Chen et al, 2000). Different actin structures are regulated by numerous actin-binding proteins, which are direct targets of Rho GTPases. In the nucleus, actin controls many nuclear processes, including chromatin remodeling and RNA polymerase activity (reviewed in Grosse & Vartiainen, 2013).

1.2.2.2 Actin monomers regulate SRF activity

Two pathways downstream of RhoA were shown to stimulate SRF activity via actin polymerization (Geneste et al, 2002; Sotiropoulos et al, 1999). RhoA activates Rho-associated kinases (ROCKs), which promote actin filament stabilization by phosphorylating LIM kinases (LIMKs). These in turn can phosphorylate and thus inactivate the actin filament severing protein cofilin (Geneste et al, 2002; Sotiropoulos et al, 1999). In addition, RhoA promotes actin polymerization through activation of the F-actin assembly protein mDia1 (Geneste et al, 2002; Sotiropoulos et al, 1999), cooperatively with its associating protein VASP (Grosse et al, 2003) (Fig.4). Both pathways lead to increased actin filament formation and hence, the actin monomer levels decrease. Decrease in the actin monomer levels in turn seems to be the activating signal for SRF, because particular actin-binding drugs that stabilize filamentous form of actin also activate SRF, whereas drugs that block actin polymerization inhibit SRF activity (Sotiropoulos et al, 1999). Consistent with this, actin point mutants with different polymerization properties have distinct

effects on SRF activity. Expression of actin mutants that favor F-actin formation activate SRF, whereas expression of actin mutants that cannot polymerize inhibit SRF activation (Posern et al, 2002). Together these observations confirmed that the triggering signal for SRF activation is the decrease in actin monomer levels. These results also lead to the proposal that there must be a cofactor that somehow informs SRF of the cellular G-actin levels.

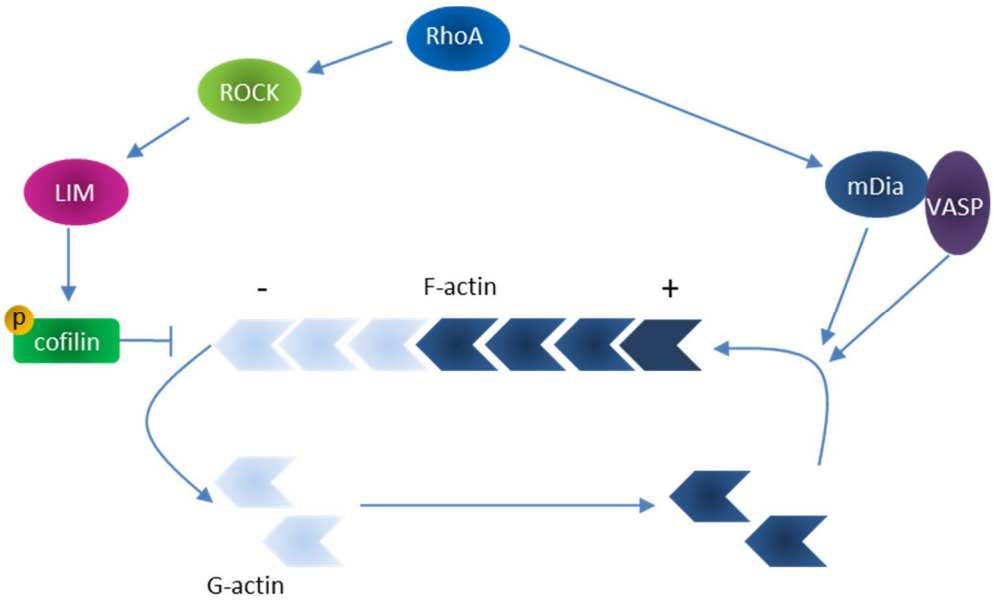

Figure 4. RhoA activates two pathways that control F-actin assembly. In the left pathway, RhoA activates ROCK kinases, which in turn activate LIM kinase to phosphorylate and inactivate cofilin. Inactivation of cofilin leads to stabilization of actin filaments. In the right pathway, RhoA promotes actin polymerization through activation of F-actin assembly proteins mDia1 and VASP. As a result, actin monomer levels decrease, which activates SRF. Light blue actin is ADP-bound and dark blue actin is ATP-bound. –and + represents pointed and barbed ends of actin filament, respectively (adapted from Grosse et al, 2003).

1.2.2 MRTF proteins

MRTFs are G-actin binding proteins that link extracellular signals and actin dynamics to SRF-mediated contractile gene expression programs. In metazoan, three family members have been identified: Myocardin, the founding member of the family (Wang et al, 2001), MKL1 (also known as MRTF-A or MAL) and MKL2 (also known as MRTF-B or MAL16) (Miralles et al, 2003; Selvaraj &

Prywes, 2003; Wang et al, 2002). Myocardin is mainly expressed in cardiovascular system (Wang 2001), whereas MKL1 and MKL2 are broadly expressed in many cell types and enriched in mesenchymal-, muscle- and epithelial cells (Wang et al, 2002). Only one family member, MAL-D, has been identified in fruit flies, and it is most closely related to MKL1 (Somogyi & Rorth, 2004). Knock-out studies of MRTFs hint that these co-activators are necessary for viability in distinct cell types and at distinct developmental phases (reviewed in Olson & Nordheim, 2010). Mice lacking Myocardin show abnormalities in the vascular smooth muscle cell (SMC) differentiation, which leads to embryonic lethality around E10.5 (Li et al, 2003). Also knock-out of MKL2 results in embryonic demise, because of failure in vascular SMC differentiation and deformation of aortic arch (Li et al, 2005; Oh et al, 2005). In contrast, MKL1 deletion does not show any apparent phenotypes before the female mice reproduction. As a consequence, such mice are viable and fertile, but unable to nurse their offspring, because MKL1 is necessary for the differentiation of mammary myoepithelial cells that provide contractility for milk ejection (Li et al, 2006). Consistent with this observation, overexpression of MKL1 in quiescent fibroblasts promotes their transformation into highly motile and contractile myofibroblasts (Small, 2012). In flies, a specific mutation in the *MAL-D* gene, which abolishes MAL-D expression (MAL-D$^{\Delta 7}$), blocks the invasive border cell migration due to a defective actin cytoskeleton (Somogyi & Rorth, 2004). Also, MAL-D loss-of function mutants inhibit mesoderm cell migration and tracheal branching (Han et al, 2004), thus indicating an important role for MRTFs in cell migration during development.

RNA-sequencing (RNA-seq) experiments have shown that majority of the 960 serum-induced SRF target genes are regulated by MRTFs. These genes are mainly involved in actin filament assembly, cell-cell and cell-extracellular matrix (ECM) adhesions, cell motility and microtubule dynamics (Esnault et al, 2014). Also, MAL-D has been described as the main regulator of actin levels in flies. Actin5C is a major target of the MAL-D/SRF pathway, and this is essential for invasive cell migration (Salvany et al, 2014).

1.2.2.1 Functional domains of MRTFs

MRTF family members share sequence homology mainly in their functional domains (Fig. 5), which are the N-terminal RPEL repeat consisting on three RPEL (Arginine-Proline-Glutamine-Leucine) motifs, basic boxes B1 and B2, glutamine rich stretch (Q), SAP domain, leucine zipper motif (LZ) and C-terminal transactivation domain (TAD). Association with SRF is mediated through B1 and Q (Zaromytidou et al, 2006). Both B1 and B2 have been linked to MKL1 nuclear localization (Miralles et al, 2003; Vartiainen et al, 2007), whereas sequences within RPEL repeat have been implicated in Crm1- and actin-mediated nuclear export (Vartiainen et al, 2007). SAP domain has been predicted to be a weak DNA binding motif. Consistently, deletion of this motif inhibits Myocardin from activating a set of SRF target genes (Wang et al, 2001). LZ motif mediates homo- and heterodimerization among family members (Hayashi & Morita, 2013; Miralles et al, 2003). C-terminal TAD region is required for transcriptional activation and is also subject to serum-induced phosphorylation of MKL1 (Miralles et al, 2003; Wang et al, 2001). The N-terminal RPEL domain of MRTFs is an actin-binding element and provides responsiveness to actin signaling (Miralles et al, 2003).

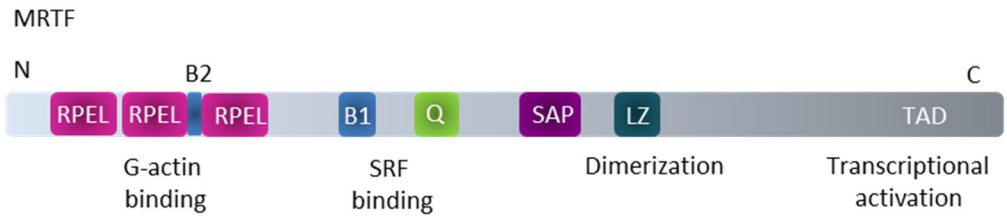

Figure 5. Location of functional domains of MRTFs. MRTFs have three RPEL (Arginine-Proline-Glutamine-Leucine) motifs, which mediate their binding to globular actin (G-actin). SRF-binding is mediated through B1 (basic box 1) and Q (glutamine rich stretch). B1 and B2 have been connected to MKL1 nuclear localization. SAP (SAFA or SAFB, acinus and PIAS domain) domain is a DNA-binding element and LZ (leuzine zipper) facilitates dimerization of MRTFs. TAD (transactivation domain) is required to induce SRF activity (adapted from Guettler et al, 2008).

1.2.2.2 Actin-mediated regulation of MKL1 nucleo-cytoplasmic shuttling

In fibroblasts, the localization and activity of MKL1 is controlled mainly by changes in actin dynamics. In unstimulated cells, where actin monomer levels are high, MKL1-actin interaction retains MKL1 in the cytoplasm. Reduction of

the interaction between MKL1 and actin, which can be a result from Rho-induced depletion of the cellular actin monomer pool for example upon serum stimulation, enhances nuclear accumulation of MKL1. Also, experimental treatments with actin-binding drugs that interfere with MKL1-actin interaction induce MKL1 nuclear accumulation (Miralles et al, 2003; Vartiainen et al, 2007).

Treatment of fibroblasts with leptomycin B (LMB), which blocks the nuclear export receptor Crm1 (Fornerod et al, 1997), also induces MKL1 nuclear accumulation without any additional signals. This indicates that MKL1 utilizes Crm1 in its nuclear export and constantly shuttles between nucleus and cytoplasm in unstimulated cells. However, the steady-state cytoplasmic localization of MKL1 is achieved by effective Crm1-mediated nuclear export, which also requires the interaction with actin, because disrupting the MKL1-actin complex either with actin-binding drugs or with mutations in the MKL1 RPEL repeat prevents MKL1 nuclear export. Interestingly, LMB-induced nuclear accumulation of MKL1 can be opposed by treatment of cells with latrunculin B, which is a G-actin-sequestering drug or by overexpressing actin mutant that cannot polymerize. As both of these scenarios lead to increased actin monomer levels in the cells, this indicates that in addition to export, actin also controls the nuclear import of MKL1 (Vartiainen et al, 2007). Nuclear import studies have shown that in LMB-treated cells the basal nuclear import rate of MKL1 is very high, and does not increase upon serum stimulation. Therefore the serum-induced nuclear accumulation of MKL1 is not due to increased nuclear import. Indeed, nuclear export studies have demonstrated that serum stimulation reduces nuclear export rates of MKL1, indicating that nuclear export rather than import regulates the serum-induced nuclear accumulation of MKL1 (Fig. 6) (Vartiainen et al, 2007). Interestingly, Myocardin, which shows entirely nuclear localization, does not respond to actin signaling. This can be explained by its decreased affinity to actin due to sequence variations within the RPEL repeat (Guettler et al, 2008). Instead, Myocardin, the other family member of MRTFs, seems to interact with actin-related protein 5 in the nucleus, which regulates its transcriptional activity (Morita & Hayashi, 2014).

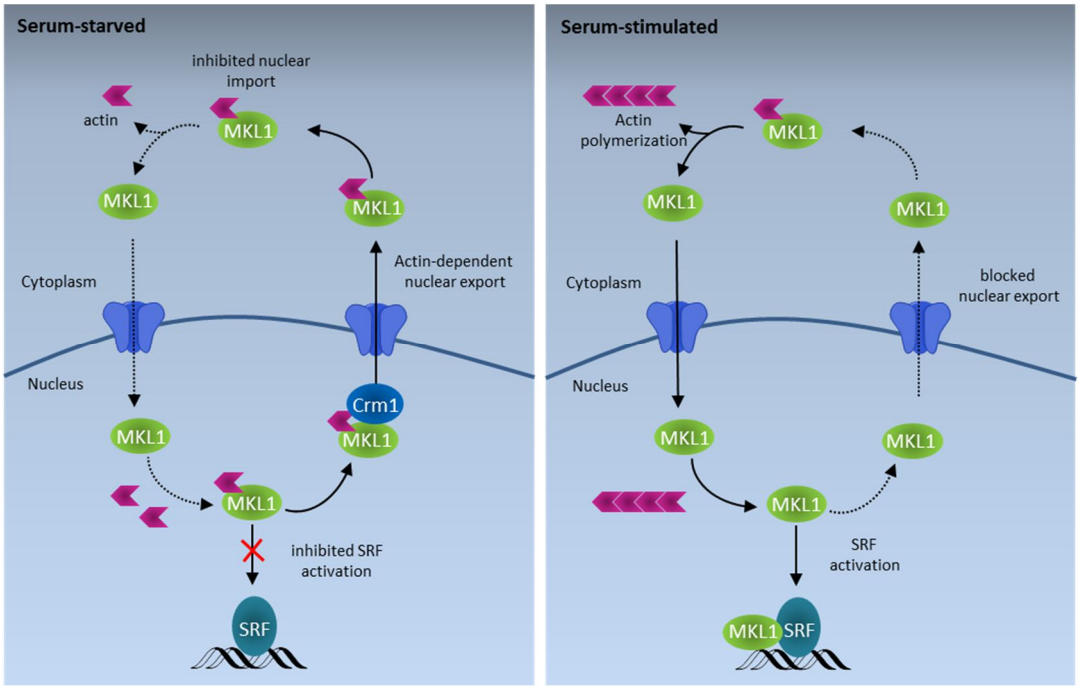

Figure 6. Actin regulates MKL1 nucleo-cytoplasmic shuttling and nuclear activity. In serum-starved cells, MKL1 is mainly cytoplasmic, because it is bound to G-actin, which promotes its Crm1-mediated nuclear export and inhibits nuclear import. In the nucleus, binding to actin prevents MKL1 from activating SRF-mediated transcription. Upon stimulus that induces actin polymerization, actin monomer levels decrease and actin-free MKL1 accumulates in to the nucleus, where it can activate SRF (adapted from Vartiainen et al, 2007).

Also other mechanisms affecting the localization of MKL1 have been reported. Thymosin-β4, a protein sequestering G-actin, has been shown to regulate MKL1 localization by competing with MKL1 for G-actin binding. This competition interferes with G-actin-MKL1 complex formation and hence induces MKL1 nuclear accumulation (Morita & Hayashi, 2013). Also phosphorylation seems to control the subcellular localization of MKL1. Serum induction activates extracellular kinase 1/2 that phosphorylates MKL1 at serine 454. This has been suggested to enhance actin-mediated nuclear export of MKL1 (Muehlich et al, 2008).

The regulatory mechanisms presented above are applicable to many cell types (e.g. muscle cells and fibroblasts). However, in neurons MKL1 is constantly nuclear despite the stimulus that induces actin polymerization (Kalita et al, 2006; Stern et al, 2009). This might be due to the fact that most of the neuronal

actin is concentrated to growth cones that are far away from the nucleus (reviewed in Knoll, 2010). Therefore it seems that in neurons, nuclear actin controls MKL1 transcriptional activity rather than its localization (Stern et al, 2009).

1.2.2.3 Nuclear activity of MKL1

MKL1-actin complexes are also present in the nucleus and this prevents MKL1 from activating SRF-mediated transcription until the nuclear pool of G-actin is sufficiently depleted. Interestingly, the same signaling events that lead to actin polymerization in the cytoplasm also apply in the nucleus (Vartiainen et al, 2007), where the serum-induced actin polymerization is dependent on mDia proteins (Baarlink et al, 2013). Also emerin, an actin-binding protein localized to the inner nuclear membrane, has been shown to be required for MKL1 nuclear activity most probably because it enables nuclear actin polymerization (Ho et al, 2013). Active MKL1, which is not bound to actin, seems to be required to facilitate the recruitment of RNA pol II to SRF target genes (Esnault et al, 2014). However, the role of actin in this mechanism is not known. Once in the nucleus, transcriptional activity of MKL1 can also be regulated by Smad3 transcription factor, which can have either a positive or negative effect on MKL1 transcriptional activity (Masszi et al, 2010; Morita et al, 2007). During epithelial-mesenchymal transition (EMT), induced by transforming growth factor β1 (TGF-β1), both MKL1 and Smad3 are located in the nucleus, where they can form a complex. This complex then binds to the Smad-responsive element on the promoter of the *Slug* gene and activates it. Activation of *Slug* transcription promotes the disassembly of cell-cell contacts, which is required for EMT (Morita et al, 2007). On the promoter of *α-smooth muscle actin* (*SMA*) gene, Smad3 can inhibit the complex formation between MKL1 and SRF, thereby inhibiting *SMA* transcription. This has been shown to be critical for fate-determining mechanisms in epithelial myofibroblast-transition (Masszi et al, 2010). Four and a half LIM domain protein 2 (FHL2) can also inhibit MKL1-SRF activity on the promoters of smooth muscle genes, by competing with MKL1 for SRF binding. As FHL2 is one of MKL1-SRF targets, it may create a negative feedback system on MKL1-SRF pathway (Philippar et al, 2004). Interestingly, FHL2 may also contribute to MKL1 activity by affecting

its protein stability. Expression of FHL2 has been shown to increase MKL1 protein levels, indicating that FHL2 may protect MKL1 from proteosomal degradation (Hinson et al, 2008).
Together these observations show that actin is the main regulator of MKL1 localization and activity in cells. As actin is the key target of MKL1-SRF pathway (Salvany et al, 2014), actin controls its own expression. This feedback system therefore maintains the appropriate actin levels and hence, provides a basis for cellular actin dynamics. Since Rho-dependent actin dynamics is critical for MKL1-SRF activity, many upstream signals that induce Rho activity can also regulate MKL1-SRF circuit.

1.2.2.3 Signaling upstream of Rho GTPases

Many signals that promote RhoA-dependent actin polymerization have been shown to activate MKL1-SRF pathway. Soluble signals, such as lysophosphatic acid (Hill & Treisman, 1995), sphingosine-1-phosphate (Lockman et al, 2004), endothelial growth factor (Chai et al, 2004) and neurotrophin (Wickramasinghe et al, 2008) can activate the pathway. Also androgens (Schmidt et al, 2012) and other hormones that line up peripheral and central clock cycling (Gerber et al, 2013), can activate MKL1-SRF pathway. Actin-binding protein Striated muscle activator of Rho signaling (STARS) has been shown to induce MKL1 nuclear localization and SRF activity by its ability to promote actin polymerization through Rho (Kuwahara & Olson, 2005). Additionally, TGF-β1 can also stimulate MKL1-SRF activation via Rho-actin signaling (Gupta et al, 2013; Morita et al, 2007; O'Connor & Gomez, 2013).
Several studies have reported a link between mechanical signals and MKL1-SRF activation. Mechanical signals are sensed by integrins that attach cells to ECM and therefore can link ECM to actin cytoskeleton. Activation of integrins facilitates their maturation into focal adhesions that leads to actin cytoskeleton remodeling through Rho GTPases (reviewed in Butcher et al, 2009). This, in turn, potentiates MKL1 nuclear accumulation and SRF activation (reviewed in Janmey et al, 2013). Mechanical signals such as matrix stiffness (Huang et al, 2012), tension (McGee et al, 2011; Somogyi & Rorth, 2004) and even cell shape (Connelly et al, 2010; O'Connor & Gomez, 2013) have all been shown to promote MKL1-SRF activity. Also, the loss of cellular contacts has been shown

to induce Rho GTPase signaling and MKL1 nuclear accumulation (Busche et al, 2008). Here, E-cadherin containing adherens junctions, but not tight junctions, are important components of the pathway that leads to MKL1-SRF activity upon disassembly of epithelial junctions (Busche et al, 2010). The first phases of EMT include the disassembly of epithelial junctions. Therefore it is not surprising that MKL1-SRF has been linked to TGF-β1 induced EMT, where MKL1-SRF activity is specifically required to increase the expression levels of cytoskeletal proteins (Morita et al, 2007; O'Connor & Gomez, 2013). As MKL1 is also required to control the expression of *slug* gene together with Smad3 (see above), these observations suggest a dual role for MKL1 in EMT (Morita et al, 2007). These data, and the fact that many MKL1-SRF target genes are involved in mechanosensing, argues convincingly that MKL1-SRF pathway is a critical nuclear component in mechanotransduction (Esnault et al, 2014).

1.2.2.4 MKL1 in cancer

If cells lose their ability to sense mechanical cues from their environment, it can contribute to pathological processes, such as cancer (reviewed in Butcher et al, 2009). Indeed, MKL1-SRF pathway has been linked to cancer. In fact, MKL1 was first found from patients with acute megakaryoblastic leukemia fused with the one twenty two (OTT) protein, also known as RNA binding motif protein 15 (RBM15) (Ma et al, 2001; Mercher et al, 2001; Sasazuki et al, 2002; Wang et al, 2002). The OTT-MKL1 fusion is not responsive to upstream signaling and it constitutively activates SRF-mediated transcription, thus creating a deregulated oncogenic protein (Descot et al, 2008), whose expression promotes abnormal megakaryopoiesis (Mercher et al, 2009). Interestingly, the activity of OTT-MKL1 can be blocked by expressing suppressor of cancer cell invasion (SCAI) (Brandt et al, 2009b), which seems to be down-regulated in many human tumors (Brandt et al, 2009a). SCAI has been linked to cancer cell invasion, because it can regulate the expression of β1-integrin. SCAI forms a repressive complex with MKL1-SRF, thus inhibiting MKL1-SRF activity on the β1-integrin gene (Brandt et al, 2009a). Also, another study has identified MKL1 in tumor cell invasion and metastasis. Knock-down of MKL1 by small interfering RNAs (siRNAs) reduced cancer cell motility and lung metastases formation in breast cancer cells (Medjkane et al, 2009). In breast cancer, MKL1

activation has been linked to cancer progression (Gurbuz et al, 2014) and it seems to induce hormonal escape in estrogen-responsive MCF-7 breast cancer cells (Kerdivel et al, 2014). Collectively, these data support an important regulatory role for MKL1 in cancer. Therefore MKL1-SRF pathway could represent a novel target for anticancer therapies.

1.3 Phactr family of proteins

RPEL motifs are also found from another family of proteins, the phosphatase and actin regulating proteins (Phactrs), consisting on four members (Phactr1, Phactr2, Phactr3/Scapinin and Phactr4) (Allen et al, 2004). They have a conserved C-terminal G-actin-binding RPEL repeat as well as a C-terminal protein phosphatase 1 (PPI) binding tail, which allows them to control the phosphatase activity of PPI (Allen et al, 2004; Kim et al, 2007; Sagara et al, 2003). Phactr family members show distinct expression patterns in the developing nervous system of mice, suggesting that individual family members are required in different developmental stages (Kim et al, 2012). Consistently, in adult rat brain, Phactr family members are expressed in different regions. Phactr1 is enriched in thalamus, whereas Phactr2 is highly expressed in cerebellum. Phactr3 is expressed throughout the brain and Phactr4 is enriched in periventricular regions (Allen et al, 2004). In cultured cells, the subcellular localization of Phactrs seems to vary and cell membrane, cytoplasmic and nuclear localizations have been reported (Favot et al, 2005; Sagara et al, 2003; Zhang & Niswander, 2012). However, neither the nucleo-cytoplasmic shuttling properties of Phactrs nor the role of the RPEL repeat in this have been explored. PPI is a widely expressed multifunctional dephosphorylating enzyme, which regulates many cellular processes such as cytoskeleton organization, cell division, muscle contraction and neuronal development (reviewed in Aggen et al, 2000). As the Phactr proteins can regulate PPI activity, they may participate to these processes as well. In mice, a mutation named *humdy* disrupts the interaction between Phactr4 and PPI. This has been shown to cause defects in closure of the neural tube, development of the eye (Kim et al, 2007) and directional migration of enteric neural crest cells (Zhang et al, 2012). These defects result from an abnormal phosphorylation of the retinoblastoma protein

(Kim et al, 2007) and cofilin (Zhang et al, 2012) due to misregulation of PPI activity.

Phactr proteins can bind actin via their C-terminal RPEL repeat (Allen et al, 2004) and are therefore linked to many actin-dependent processes. For example, expression of Phactr3 in Hela cells stimulates cell spreading and motility and this activity is dependent on its ability to bind actin (Sagara et al, 2009). Phactr3 has also been shown to inhibit axon elongation via regulation of actin dynamics in rat cortical neurons (Farghaian et al, 2011). Phactr3, while binding to actin monomers, inhibits actin polymerization (Sagara et al, 2009), and this seems to hamper axon elongation in the distal axon shafts (Farghaian et al, 2011). Phactr1 is also implicated in angiogenesis. SiRNA-mediated depletion of Phactr1 disrupts actin polymerization, which, in turn, disrupts blood vessel formation. Moreover, the defect in actin polymerization, which is caused by the Phactr1 depletion, leads to alterations in lamellipodial dynamics (Allain et al, 2012). Overexpression of any Phactr family member changes the morphology of myoblasts: cells form hair-like protrusions (Favot et al, 2005), further highlighting the important role of Phactr-family proteins in regulating actin cytoskeletal dynamics.

Many functions of Phactr family members are linked to their ability to bind either actin or PPI. Interestingly, the sequences required for PPI binding are located near the actin-binding RPEL repeat and in Phactr3 overlap with RPEL motif 3 (Sagara et al, 2003). This suggests that there is interplay between actin and PPI binding to Phactr proteins. However, this has not been investigated.

2. AIMS OF THE STUDY

MKL1 is an important co-activator of the essential transcription factor SRF. In order to activate SRF-mediated transcription, MKL1 must be transported from the cytoplasm, where it is located in unstimulated cells, to the nucleus. However, it is not known how MKL1 is transported into the nucleus. The main aim of this study was to understand the molecular mechanism of MKL1 nuclear import.

As a basis for this study, I used RNA interference (RNAi) screen that identified two proteins, Importin-β and Ddx19 as candidates for regulating MKL1 nuclear import. Specific research aims were:

- To confirm the RNAi screen hits and assess their specificity in regulating MKL1 localization
- To assess the requirement of Importin-β and Ddx19 for SRF activation
- To study the binding interactions between the prospective import factors and MKL1
- To investigate the mechanisms involved

In addition, the aim was to explore, whether Phactr proteins, which share a similar actin-binding RPEL repeat with MRTFs, are also regulated by this motif.

3. MATERIALS AND METHODS

The methods that I personally utilized in this study are listed in the table 2. Details can be found from the publications, which are marked with roman numerals.

Table 2. The methods used in this study. The roman numeral refers to the original publication.

Method	**Publication**
Co-immunoprecipitation assay	II
Fluorescent *in situ* hybridization	II
Fluorescence microscopy	I, II, III
Förster Resonance Energy Transfer measured by Fluorescence Lifetime Imaging	II
GST pull-down assay	II
Immunofluorescence	I, II, III
Luciferase assay	I, II
Mammalian cell culture and transfection	I, II, III
Plasmid construction	I, II
Real-Time quantitative PCR	II
RNA interference	I, II, III

4. RESULTS AND DISCUSSION

4.1 Nuclear import mechanism of MKL1 (I, II)

To activate SRF, MKL1 residing predominantly in the cytoplasm, must be relocalized to the nucleus. Therefore, regulated nuclear import of MKL1 is critical for its ability to control SRF-mediated gene expression. Although the role of actin in this regulation is well established (see above) the mechanism and the prospective proteins controlling the nuclear import of MKL1 have remained unclear. Molecular weight of MKL1 is ~100 kDa and therefore it has to be transported through the NPC by an active transport mechanism. To find proteins, which might regulate MKL1 nuclear import, an RNAi screen was performed with proteins involved in nucleo-cytoplasmic shuttling. This screen identified two proteins, Ipoβ and Ddx19, as putative nuclear import factors for MKL1. Depleting either one of these proteins inhibited serum-induced nuclear accumulation of MKL1 in human cells (Vartiainen, unpublished). The main aim of this study was to confirm the involvement of these proteins in MKL1 nuclear import and investigate the mechanisms involved.

4.1.1 Importin-β and Ddx19 are both necessary for MKL1 nuclear localization and SRF activation (I, II)

In this study, we decided to use NIH 3T3 cells (mouse embryonic fibroblasts), because previous studies had shown that MKL1 localization in these cells is very sensitive to changes in actin dynamics (Miralles et al, 2003; Vartiainen et al, 2007). In serum starved conditions (0.3% serum), where actin monomer levels are high, MKL1 is mainly cytoplasmic, but rapidly accumulates in the nucleus upon serum stimulation that induces actin polymerization. Also, treatments with drugs such as cytochalasin D (CD), which inhibits actin polymerization and disrupts MKL1-actin interaction (Vartiainen et al, 2007), or Leptomycin B (LMB), which blocks MKL1 nuclear export by inactivating the export receptor Crm1/Exportin-1 (Fornerod et al, 1997; Vartiainen et al, 2007), induce MKL1 nuclear accumulation.

To confirm the RNAi screen results in NIH 3T3 cells, we monitored the localization of MKL1-GFP and endogenous MKL1 in cells depleted of Ipoβ and Ddx19. The results showed that in both conditions, MKL1 is mainly

cytoplasmic regardless of the stimulations that induce MLK1 nuclear accumulation in control cells (I, Fig.3a and II, Fig.1a,b,c). The efficiency of Ipoβ and Ddx19 depletion was confirmed by Western blotting (I, Fig.3a; II, Fig.1a) and in the case of Ddx19, also by RNA fluorescence *in situ* hybridization (FISH). FISH detected nuclear accumulation of mRNAs upon Ddx19 depletion (II, Supplementary Fig. 1b,c), as shown before (Hodge et al, 1999; Snay-Hodge et al, 1998). Importantly, the defect in nuclear import of MKL1 caused by the depletion of Ipoβ or Ddx19 was also reproduced in human cell lines (I, Fig. 3d; II, Supplementary Fig. 1d,e).

Next, we performed a rescue experiment, which showed that the siRNA resistant versions of Ipoβ and Ddx19 were able to restore the nuclear localization of MKL1 in their respective knock-down phenotypes (I, Fig. 3b; II, Fig. 3b,c). This experiment confirmed that the effects were specific and not caused by off-target effects of RNAi that could lead to incorrect interpretations of target gene function (Jackson & Linsley, 2004). Control experiments, such as rescue experiment performed here, are therefore important to confirm the specificity of an RNAi phenotype (Kittler et al, 2005). Together, these experiments validated the RNAi screen results and demonstrated the involvement of both Ipoβ and Ddx19 in MKL1 nuclear localization.

Interestingly, depletion of Ipoβ or Ddx19 also impaired nuclear localization of Myocardin, another MRTF family member, which localizes to the nucleus in unstimulated cells (Wang et al, 2001). Nuclear localization of Myocardin was clearly reduced in the cells depleted of Ipoβ or Ddx19 (II, Fig. 1d). This indicates that proteins of the MRTF family share the same nuclear import mechanism. This conclusion is further supported by a study, which shows that also Myocardin utilizes the same classical nuclear import pathway as MKL1 does (see below) (Nakamura et al, 2010).

Next we asked whether Ipoβ and Ddx19 are also required for SRF activation, as the nuclear localization of MKL1 has previously been shown to be necessary for SRF-mediated transcription (Miralles et al, 2003). As expected, depletion of either protein reduced the serum and CD-induced SRF reporter activity (I, Fig. 3c; II, Fig. 2e). In addition, depletion of Ddx19 significantly decreased the expression levels of endogenous SRF target genes, *vinculin* and *Acta2* (II, Fig.

2f). Hence, these results confirmed the importance of Ipoβ and Ddx19 for MKL1 localization, and thus for SRF activation.

Ipoβ is the main nuclear import receptor, and almost all known nuclear import and export events of proteins are mediated by the Ipoβ family members (reviewed in Harel & Forbes, 2004). In contrast, Ddx19 is a well-established regulator of mRNA export (Hodge et al, 2011; Noble et al, 2011; Schmitt et al, 1999; Snay-Hodge et al, 1998), and therefore its specific effect on MKL1 localization was surprising. Although Ddx19 is also implicated in other cellular processes, such as transcription and translation termination (Estruch & Cole, 2003; Gross et al, 2007), our data provided the first evidence that Ddx19 can contribute to the nuclear import of a protein. Therefore we wanted to confirm its specificity in MKL1 nuclear localization.

4.1.2 Ddx19 is a specific regulator of MKL1 nuclear import (II)

We observed that in Ddx19-depleted cells mRNAs accumulated in the nucleus (II, Supplementary Fig. 1b,c) consistent with the previous studies (Hodge et al, 1999; Snay-Hodge et al, 1998). We performed control experiments to exclude the possibility that Ddx19 depletion would cause unspecific effects for NPC function or that MKL1 nuclear import defect is due to misregulated mRNA export.

First, we did not detect any changes in the localization of known nuclear proteins, such as SRF and hnRNPU, in control or Ddx19 depleted cells (II, Fig. 1d,e and Supplementary Fig. 2). This indicates that depletion of Ddx19 does not block the Kap-mediated nuclear transport in general.

Second, we studied whether the defect in MKL1 import, caused by Ddx19 depletion, could be an indirect consequence of mRNA nuclear accumulation. Here we utilized depletion of Gle1, which is necessary for Ddx19-mediated mRNA nuclear export (Alcazar-Roman et al, 2006; Weirich et al, 2006). Depletion of Gle1 did not affect MKL1 localization (II, Fig. 2a,b), but clearly inhibited mRNA export (II, Supplementary Fig. 1b,c). Therefore we concluded that a block in general mRNA export pathway does not prevent MKL1 nuclear import. These results also revealed that the activity of Gle1 is not required in Ddx19-mediated nuclear import of MKL1, suggesting that MKL1 nuclear import and mRNA export are mechanistically distinct processes. Moreover,

since the activity of Gle1 is linked to Ddx19-mediated translation termination (Bolger et al, 2008; Gross et al, 2007), the defect in MKL1 import cannot be a consequence of deregulated protein synthesis. Together these experiments suggest a specific role for Ddx19 in nuclear import of MKL1.

4.1.3 Nuclear import of MKL1 is regulated by a long bipartite nuclear localization signal (I)

The N-terminal RPEL repeat of MKL1 is an actin-binding element and has been previously shown to be necessary and sufficient for MKL1 nucleo-cytoplasmic shuttling (Guettler et al, 2008). It includes a short basic element, B2, between RPEL motifs 2 and 3 that was earlier shown to be the putative nuclear localization signal (NLS) (Miralles et al, 2003; Vartiainen et al, 2007). In our study, closer examination of the RPEL repeat sequences identified an additional NLS embedded in RPEL motif 2 (I, Fig.1a), and referred as B3. Mutational analysis revealed that both basic elements, B2 and B3, are required for efficient nuclear accumulation of MKL1 (I, Fig.1b,c,d) and can even mediate the nuclear import of normally cytoplasmic pyruvate kinase protein (I, Fig.2a,b). Together these results identified B2 and B3 as parts of a long bipartite NLS. These two elements are separated by an unusually long 30-residue linker. Such long linkers, nearly three times the size of traditional linker sequences (Robbins et al, 1991), have been identified before (Lange et al, 2010), and are hypothesized to affect the flexibility of conformation-dependent NLSs (Kosugi et al, 2009). We speculated that since the NLSs are embedded in the actin-binding RPEL repeat of MKL1, perhaps the longer linker allows NLSs to be regulated through binding of actin monomers. Indeed, further structural studies have shown that the extended linker region between NLSs is optimal for the stoichiometry of actin-binding and, as such, actin-mediated regulation of the protein (Hirano & Matsuura, 2011; Mouilleron et al, 2011).

4.1.4 Ipoβ functions together with Ipoα in MKL1 nuclear import (I)

The results above confirmed that Ipoβ is a specific regulator of MKL1 nuclear localization. However, Ipoβ cargos are often recognized and bound by an import adapter Ipoα, which has a recognition site for mono- and bipartite NLSs, as well as Ipoβ-binding domain (IBB) (Gorlich et al, 1996a; Weis et al, 1996).

Although Ipoα did not score as a hit in the RNAi screen (Vartiainen unpublished), its possible contribution was studied by an *in vitro* nuclear import assay (I, Fig. 4). In this assay GFP-tagged N-terminal RPEL repeat of MKL1 (MRTF-A(2-204)-2GFP), which displays similar regulation as the full-length protein (Guettler et al, 2008), was imported into the nucleus of semi-permeabilized cells. We found that the nuclear import of RPEL repeat was inhibited by peptides that either block Ipoα binding to its cargoes or otherwise disrupt the interaction between Ipoα/Ipoβ (I, Fig. 4b). Furthermore, only the combination of both Ipoα and Ipoβ promoted MKL1 nuclear import, as neither protein worked alone (I, Fig.4c). Biochemical assays demonstrated that the interaction between Ipoα3 and MKL1 bipartite NLS was dependent on both the major and minor binding pockets of Ipoα3 (I, Fig.5c). This finding is consistent with a structural study, which showed that Ipoα engages both of its binding pockets when bound to bipartite NLS (Conti & Kuriyan, 2000). This observation was later confirmed by a study where the RPEL repeat of MKL1 was crystallized with Ipoα (Hirano & Matsuura, 2011). The crystal structure showed that the NLS residues of MKL1 interact with the major and minor binding pockets of Ipoα in an extended conformation (Hirano & Matsuura, 2011). These results confirmed that Ipoα is required for efficient nuclear import of MKL1. Although all the individual family members of Ipoα were tested in the RNAi screen, the possible reason why neither of them scored as a hit could be due to functional redundancy among the different Ipoα paralogs (Goldfarb et al, 2004). Therefore we think it is likely that MKL1, as well as other proteins, can utilize different Ipoα paralogs depending on their availability.

Together these results revealed that MKL1 utilizes the classical nuclear import pathway mediated by Ipoα/Ipoβ heterodimer. As mentioned before, also Myocardin utilizes the same pathway (Nakamura et al, 2010). Although these proteins have sequence variations in their RPEL motifs 1 and 2, which lead to different actin-binding affinities, the B2 region is highly conserved (Guettler et al, 2008). This may explain their shared mechanism of nuclear import regulation.

4.1.5 Nuclear import factors of MKL1 interact with its RPEL repeat (I, II)

The results above showed that MKL1 bipartite NLS is within the actin-regulated N-terminal RPEL repeat. Therefore we next asked whether the MKL1 nuclear import factors can bind to this repeat. Indeed, our GST pull-down assays revealed that both Ipoβ and Ddx19 can associate with the MKL1 RPEL repeat and that these interactions were inhibited by RanGTP (II, Fig.4a). This is a common feature for importins, which release their cargo at high RanGTP concentrations in the nucleus (Gorlich et al, 1996b; Rexach & Blobel, 1995). Thus, the results suggest that such interaction take place in the cytoplasm. Moreover, both interactions were direct, as both the purified Ipoβ (I, Fig. 5d) and Ddx19 (II, Fig. 5b) bound efficiently to the recombinant RPEL repeat. Closer mapping of the interactions between RPEL repeat and Ipoβ revealed that the interaction was dependent on B3 element but not on B2 (I, Fig. 5d). The efficient interaction of Ipoβ with both basic elements required its adaptor protein Ipoα3 (I, Fig. 5a,b). These results were in agreement with our earlier observations that both basic elements are required for MKL1 nuclear localization (I, Fig.1b,c,d) and that only a combination of both Ipoα3 and Ipoβ was sufficient for MKL1 import *in vitro* (I, Fig. 4c). The exact interaction site for Ddx19 in the RPEL repeat, however, warrants further studies.

The results above clearly argued that MKL1 indeed utilizes the classical nuclear import pathway mediated by Ipoα/Ipoβ heterodimer. However, they did not explain the involvement of actin in this process. Previous *in vivo* studies have shown that monomeric actin inhibits MKL1 nuclear localization (Miralles et al, 2003; Vartiainen et al, 2007). Our data further demonstrated that the importin-associating bipartite NLS is embedded in the actin-binding RPEL motifs (I, Fig. 1a) and according to structural studies (Mouilleron et al, 2008), directly overlaps with actin-binding sites. Therefore we next asked whether there is an interplay between actin and the MKL1 nuclear import factors.

4.1.6 Actin competes with Importin-α/β binding to RPEL repeat of MKL1, but not with Ddx19 (I, II)

To study the effect of actin on MKL1 interaction with the Ipoα/Ipoβ heterodimer, we performed a pull-down assay with increasing amounts of nonpolymerizable Latrunculin B (LatB)-actin added in to the Ipoα3/Ipoβ-GST-

RPEL mix. Interestingly, the increase in actin amount correspondingly decreased the interaction between GST-RPEL and Ipoα3/Ipoβ heterodimer (I, Fig.6; II, Fig.4b). Thus, monomeric actin directly competes with the Ipoα/Ipoβ complex for interaction with the MKL1 RPEL repeat. These results are not only in full agreement with previous observations on the inhibitory effect of actin-binding in MKL1 nuclear import (Miralles et al, 2003; Vartiainen et al, 2007), but also provided a mechanistic explanation to why actin-binding inhibits the nuclear import of MKL1. Based on these data and previous observations (Vartiainen et al, 2007) we propose a model where in conditions of high G-actin concentration, actin-binding masks the NLSs embedded in the RPEL repeat, thus preventing its recognition by Ipoα/Ipoβ complex. Therefore under these conditions MKL1 is mainly cytoplasmic. Conversely, when G-actin levels are low, the actin-free NLSs are exposed to the Ipoα/Ipoβ complex, which leads to active import of MKL1 into the nucleus. Indeed, structural studies showed that MKL1 RPEL repeat can bind altogether five actin monomers, three actins to the RPEL motifs and two actins to the intervening spacer elements (Mouilleron et al, 2011). The α-helical conformation of MKL1 RPEL repeat in complex with five actins (pentamer complex) is different from the extended conformation of the RPEL repeat in complex with Ipoα, thus preventing Ipoα/Ipoβ binding. Therefore the pentamer complex of MKL1 is incompetent for import and is proposed to dominate in high G-actin levels (Hirano & Matsuura, 2011; Mouilleron et al, 2011). However, the pentamer complex can dissociate relatively easily to a trimeric complex with three actin monomers. In the trimeric complex MKL1 NLSs are uncovered and likely to recruit Ipoα/Ipoβ heterodimer for efficient nuclear import. The trimeric complex would be a conformation that dominates at low G-actin levels (Mouilleron et al, 2008; Mouilleron et al, 2011). Therefore, these structural studies confirm our model. A similar model has been proposed in studies on nuclear entry of Junction-mediating and regulatory protein (JMY): the actin-binding WH2 domain of JMY overlaps with its NLS and therefore association of actin monomers prevents its nuclear import (Zuchero et al, 2012).

Interestingly, the interaction between GST-RPEL and Ddx19 was not sensitive to the increasing amounts of LatB-actin, as opposed to the interaction between GST-RPEL and Ipoα/Ipoβ (I, Fig. 6; II, Fig.4b). Consistent with this, depletion

of Ipoβ from cytoplasmic lysates that we used in the pulldown assay, did not affect the GST-RPEL-Ddx19 interaction (II, Fig.5a). Likewise, the absence of Ddx19 did not affect binding of Ipoβ to RPEL repeat of MKL1 (II, Supplementary Fig.6), as expected based on the interaction studies with purified proteins (I, Fig. 5d).

Our observation that RPEL-Ddx19 interaction is not sensitive to actin led us to speculate on the existence of an actin-independent mechanisms of MKL1 nuclear import regulation. Although the results above clearly indicated that MKL1 nuclear import is dependent on the classical nuclear import pathway mediated by the Ipoα/Ipoβ heterodimer and regulated by actin (I), it does not exclude the possibility that atypical variants of this pathway or even different pathways may be involved. Therefore we decided to further investigate the involvement of Ddx19 in MKL1 nuclear import regulation.

4.1.7 Ddx19 regulates the localization of only longer MKL1 constructs (II)

Our previous results showed that Ddx19 interacts with the N-terminal RPEL repeat of MKL1, which includes the NLSs (see above 4.1.3). Next we asked whether Ddx19 also regulates the localization of the RPEL repeat, because it was previously shown to be sufficient for MKL1 localization (Guettler et al, 2008).

Interestingly, despite of the interaction, Ddx19 did not regulate the localization of MKL1 RPEL repeat. The RPEL fused with GFP localized similarly in Ddx19 depleted and in control cells (II, Fig. 6a,b,c). However, the localization of MKL1-C471 construct that, in addition to RPEL repeat, includes B1-box, Q- and SAP-motifs, was regulated by Ddx19 (II, Fig.6a,d,e). These results thus demonstrated that Ddx19 controls the nuclear localization of full-length MKL1 and MKL1-C471 construct (II, Fig.1;6a,d,e), but not the RPEL repeat alone (II, Fig.6b,c). Previous studies have suggested that also B1-box outside the RPEL repeat contributes to MKL1 nuclear localization (Miralles et al, 2003). However, further studies are needed to find out whether Ddx19 is required to regulate nuclear localization of MKL1 construct, where the B1-box is impaired. According to these results we hypothesize that Ddx19 would bind also other parts of the MKL1 molecule apart from the RPEL repeat.

Indeed, our co-immunoprecipitation experiments demonstrated that in addition to full-length MKL1 (II, Fig.7a,b), Ddx19 efficiently interacts with MKL1-ΔN construct that lacks the whole N-terminal part, including the RPEL repeat (II, Fig.7b). Overall, the results so far have revealed that Ddx19 interacts directly with the RPEL repeat of MKL1, but has also second binding site in the C-terminal part of the protein. Moreover, the fact that RPEL-Ddx19 interaction is not sensitive to actin led us to hypothesize an actin-independent mechanism of MKL1 nuclear import regulation. Therefore we speculate that Ddx19 could add a second level to the classical actin-based regulation of MKL1 localization.

4.1.8 Ddx19 modulates MKL1 conformation (II)

Although our biochemical assays showed that Ddx19 is not required to facilitate the interaction between RPEL repeat of MKL1 and Ipoβ (I, Fig. 5d; II, Supplementary Fig.6), we decided to study the interaction between full-length MKL1 and Ipoβ in living cells. For this we utilized Förster Resonance Energy Transfer (FRET) measured by Fluorescence Lifetime Imaging (FLIM). We found that in control cells, the fluorescence lifetime of MKL1-GFP (donor fluorophore) was significantly decreased in the presence of Ipoβ-Cherry (acceptor fluorophore) indicating that these two proteins and their respective fluorophores come very close to each other to produce FRET, demonstrating that the proteins interact also in intact cells (II, Fig.8a). This interaction was shown before using a proximity ligation assay (I, Fig. 3e). In contrast, we did not observe FRET between MKL1-GFP and Ipoβ-Cherry in cells depleted of Ddx19 (II, Fig. 8a). This indicates that Ddx19 is indeed required to facilitate the MKL1-Ipoβ interaction *in vivo*. As expected based on our previous *in vitro* studies (I, 5d; II, Supplementary Fig. 6), depletion of Ddx19 did not affect the interaction between MKL1-RPEL domain and Ipoβ (II, Fig.8a). Together these results suggested that Ddx19 is required to facilitate the interaction between MKL1 and Ipoβ in the context of full-length protein. Homo- and heterodimerization among the MRTFs has been previously suggested to play a role in their nuclear export (Hayashi & Morita, 2013). However, this possibility was excluded, because Ddx19 regulated the localization of MKL1-C471 construct (II, Fig. 6a,d,e) that lacks the LZ region required for homo- and heterodimerization (Miralles et al, 2003). In this perspective, we speculate that

the RNA helicase Ddx19 could function to control intramolecular conformation of MKL1, which then can be efficiently recognized by Ipoβ.

Indeed, our subsequent FRET experiments demonstrated that the N-and C-terminal parts of MKL1 are very close to each other. Here we utilized the FRET-based reporter with mCherry and GFP-tags in N- and C-terminal parts of MKL1, respectively. The fluorescence lifetimes of mCherry-MKL1-GFP reporter were significantly shorter when compared to fluorescence lifetimes of MKL1-GFP alone, indicating a "closed" conformation of MKL1 (I, Fig.8b,c). Moreover, in cells depleted of Ddx19, the fluorescence lifetimes of mCherry-MKL1-GFP reporter were even shorter, suggesting that the absence of Ddx19 promotes this "closed" conformation (II, Fig. 8b).

The data summarized above suggest a model wherein Ddx19 is specifically required to switch MKL1 to an open conformation, which can then be recognized by Ipoα/Ipoβ heterodimer for efficient nuclear import (Fig.7). Thus, this model suggests a molecular mechanism of NLS masking through adoption of a closed conformation that is inappropriate for Ipoα/Ipoβ recognition. However, to fully understand the interplay between Ddx19 and MKL1 conformations, structural information of MKL1 is needed. A long-standing problem in the field has been the poor expression of full-length MKL1 in bacteria. Therefore the only structural information we have so far is from the RPEL repeat. However, it might be possible to produce full-length recombinant MKL1 in some other organism, for example in insect cells. This protein production would facilitate protein crystallization, which would address the conformational dynamics and mechanisms of MKL1. Interestingly, conformational changes have also been suggested to play a role in the nuclear import of Wiskott-Aldrich syndrome protein (WASP). The hyperphosphorylated WASP mutants show open conformation, which seems to have high tendency to localize in the nucleus (Looi et al, 2014). Also, conformational change seems to determine the localization of nuclear export factor Cse1. In the cytoplasm, Cse1 is found in its substrate-free closed conformation, whereas in the nucleus the open conformation allows its association with Ipoα and RanGTP (Cook et al, 2005). However, it is not known, whether this conformational switch regulates the nuclear import of Cse1.

The results above suggested an important role for Ddx19 in MKL1 nuclear import regulation. However, they did not provide an answer to our question about potential activating upstream signals here, since the interaction was not sensitive to actin.

4.1.9 Functional RNA-binding domain of Ddx19 is required for MKL1 nuclear import (II)

We were interested in the biochemical properties of Ddx19 in MKL1 nuclear import, since in mRNA export and translation termination Ddx19 utilizes its characteristic helicase activity (Alcazar-Roman et al, 2010; Bolger et al, 2008; Schmitt et al, 1999).To study the biochemical requirements of Ddx19 in MKL1 nuclear import, we generated Ddx19 mutants, where the helicase activity, Nup214- or RNA-binding ability was abolished (II, Fig.3a). All these properties of Ddx19 have been shown to be critical in Ddx19-mediated mRNA export (Hodge et al, 2011; Noble et al, 2011; Schmitt et al, 1999).

First we confirmed the functionality of the mutants by RNA FISH. As expected from the previous studies (Hodge et al, 2011; Noble et al, 2011; Schmitt et al, 1999), none of the mutants could rescue the mRNA export defect caused by Ddx19 depletion (II, Supplementary Fig. 4a,b). In contrast, the helicase defective mutant (Ddx19-E242Q,V385N) and the two mutants that are defective in Nup214-binding (Ddx19-R261A and Ddx19-R261D) could rescue MKL1 nuclear localization (II, Fig. 3c). Interestingly, the only mutants that could not rescue MKL1 nuclear localization were the two mutants (Ddx19-R371G and Ddx19-R428Q), where the RNA-binding ability was eliminated (II, Fig. 3). Moreover, these mutants acted in a dominant negative manner, because their expression inhibited MKL1 nuclear localization in serum stimulated conditions (II, Supplementary Fig. 5a,b). Therefore, these results indicated that neither helicase activity nor Nup214-binding is necessary for Ddx19-mediated regulation of MKL1 nuclear import, while RNA-binding capacity appears to be critical.

Previous studies have indicated that the ability of Ddx19 to remodel mRNPs requires its helicase/ATPase activity (Schmitt et al, 1999). In contrast, our results demonstrated that the helicase activity of Ddx19 was not required in MKL1 nuclear import and therefore indicated that Ddx19 modulates MKL1

conformation independently of its helicase activity. Consistent with this, Gle1, which is required to induce the helicase activity of Ddx19 (Alcazar-Roman et al, 2006; Weirich et al, 2006), did not play a role in MKL1 nuclear localization (II, Fig. 2a). Also, other studies have proposed helicase-independent remodeling activities for RNA helicases (Gebhard et al, 2012; Uhlmann-Schiffler et al, 2006; Yang & Jankowsky, 2005). For example, both human Ddx42p and yeast DEAD1 RNA helicases can anneal RNA independently of ATP hydrolysis (Uhlmann-Schiffler et al, 2006; Yang & Jankowsky, 2005).

Since the interaction of Ddx19 with Nup214 was not required for MKL1 nuclear import, these results indicated that Ddx19 functions in the cytoplasm to promote MKL1 nuclear import. This conclusion is further supported by our *in vitro* study, which showed that RanGTP can block the interaction between MKL1 and Ddx19 (II, Fig. 4a). Therefore, in addition to translation termination, this is the second cytoplasmic function proposed for Ddx19. Nup214 binding to Ddx19 has been shown to enhance its ADP release (Noble et al, 2011). In this respect, the cytoplasmic ADP release factor awaits its discovery.

The dependency of a functional RNA-binding domain in MKL1 nuclear import was surprising. However, it was confirmed by the use of two distinct mutants that are both defective for RNA-binding (Ddx19-R371G and Ddx19-R428Q) (II, Fig. 3a). Structural studies have shown that RNA binding of Ddx19 leads to a conformational change, which is required to enhance its ATPase activity (Montpetit et al, 2011). Our data, however, showed that MKL1 import did not require Ddx19 ATPase/helicase activity. These findings therefore suggested that the conformational change, which is caused by RNA binding, must be the key triggering event for the formation of MKL1 nuclear import complex.

Overall, the dependency of a functional RNA-binding domain was an interesting finding. It might indicate that MKL1 localization in cells could react to general mRNA levels. However, it is not known whether Ddx19 could also bind other RNA species apart from mRNA, because no specificity for a particular RNA has been described (Tieg & Krebber, 2013). Therefore the interplay between mRNA levels and MKL1 import remains to be discovered. Also, it is not known whether Ddx19 binds all mRNAs or only a subset of them, because no sequence specificity for Ddx19 mRNA binding has been described. This specificity aspect would be an interesting issue for future

studies, because it could give clues to whether MKL1 localization responds to a specific set of mRNAs through Ddx19-mRNA interaction. Sequence specificity between RNA and proteins can be studied for example by RNA immunoprecipitation followed by sequencing (RIP-seq) (Lu et al, 2014).

Collectively, the results above suggest that Ddx19 regulates MKL1 nuclear import by modulating MKL1 conformation in the cytoplasm and thus facilitating the interaction between MKL1 and its nuclear import complex Ipoα/Ipoβ heterodimer. This modulation does not require the ATPase activity of Ddx19, which is crucial for its role in mRNA export. However the question remains, if this conformational change mediated by Ddx19 facilitates the actin-mediated regulation of the protein, or whether it provides a completely new MKL1-SRF regulatory pathway.

4.2 The RPEL repeat of Phactr4 is required to maintain the cellular actin balance (III)

Phactr family proteins share a similar (37% identical) actin-binding RPEL repeat with MRTFs family members, which underscore our interest to study whether Phactr4, which is the main family member expressed in the NIH 3T3 fibroblasts (Puusaari, unpublished) is also regulated by this repeat. The RPEL repeat of MKL1 is both necessary and sufficient for controlling the nucleo-cytoplasmic shuttling in response to actin dynamics (Guettler et al, 2008). However, our results showed that the RPEL repeat of Phactr4 does not determine its localization in cells (III, Fig.2). Although the RPEL repeat of Phactr4 bound efficiently to actin (III, Fig. 4), its localization was independent of actin dynamics (III, Fig. 2). This observation is supported by a concurrent study, which showed that Phactr1 appears to be the only family member, whose localization is sensitive to actin signaling through RPEL motifs (Wiezlak et al, 2012). Instead, our results demonstrated that the RPEL repeat of Phactr4 was required to facilitate the competitive interaction between G-actin and PPI for the RPEL repeat. An increase in actin amounts correspondingly decreased the interaction between Phactr4 and PPI (III, Fig. 5c). Moreover, increasing actin input lowered the PPI phosphatase activity (III, Fig.5d). Together these results suggested that the RPEL repeat of Phactr4 senses the actin concentrations in

cells, which controls the formation of Phactr4-PPI complex and thus phosphatase activity of PPI.

PPI is an enzyme that can dephosphorylate numerous cellular substrates. One of them is cofilin, whose dephosphorylation is required for its activity (Agnew et al, 1995). Indeed, we observed that Phactr4 regulates cofilin activity through PPI, and this was dependent on actin monomer levels (III, Fig. 6). Activated form of cofilin can depolymerize and sever actin filaments, which leads to elevated G-actin levels (Lappalainen & Drubin, 1997). Certainly, we observed an increase in G-actin levels in cells which overexpressed Phactr4 and this was dependent on cofilin activity (III, Fig.7). Together these experiments revealed that Phactr4 responds to decreased cellular G-actin levels and activates cofilin through PPI-binding and thus increases actin monomer levels. Therefore our data pointed to an important role for Phactr4 in a feedback system, where actin monomers can locally regulate their own abundance (III, Fig.8).

Despite of the fact that the RPEL repeats are similar between Phactr4 and MKL1, they respond to actin dynamics very differently. In MKL1, actin-binding inhibits the NLS recognition by Ipoα/Ipoβ and thus, nuclear import (see above 4.1.6). With Phactr4, actin binding inhibits the complex formation with PPI and therefore its activation (III, Fig. 5). However, when activated, the outcomes of both pathways are similar: regulated actin dynamics. Thus, the RPEL repeat, depending on the protein context, can maintain the cellular actin balance by means of different mechanisms. In the context of MKL1 the RPEL repeat regulates actin dynamics through transcriptional control of cytoskeletal genes and in the context of Phactr4 the RPEL repeat modulates actin dynamics locally by regulating cofilin activity.

Interestingly, also Phactr1 can form a complex with PPI competitively with G-actin. Similarly to Phactr4, the Phactr1-PPI complex formation is inhibited by an increase in the cytoplasmic G-actin levels. This mechanism is used to regulate the phosphorylation status of myosin and as such, actomyosin assembly (Wiezlak et al, 2012). Combining these results, we propose that Phactr proteins, in response to G-actin levels, regulate PPI activity. Thus Phactrs can regulate the actin cytoskeleton dynamics by controlling the phosphorylation status of cofilin and myosin. In the future, it would be interesting to know if the other family members, Phactr2 and 3, also share the

common mode of regulation and whether they also participate in regulating actin dynamics, as they both have the highly conserved RPEL repeat and PPI binding sites.

5. CONCLUSIONS

The present work provides new insights into how MKL1 nuclear import is regulated. This information is needed to understand how SRF activity in cells is controlled. By using cell biological and biochemical approaches, this study shows that MKL1 nuclear import is a manyfold process that requires an interplay between its newly characterized nuclear import factors, Ipoβ and Ddx19, as well as actin. The results reveal that both Ipoβ and Ddx19 are essential components of the signaling pathway that contributes to SRF activity. This study demonstrates that in MKL1 nuclear import Ipoβ associates with Ipoα and therefore MKL1 utilizes the classical nuclear import pathway. The results reveal that monomeric actin competes with Ipoα/Ipoβ heterodimer for access to the MKL1 bipartite NLS, which is located in the actin binding RPEL repeat. This explains the observed inhibitory effect that actin binding has on MKL1 nuclear localization (Miralles et al, 2003; Vartiainen et al, 2007). In conclusion this work describes a molecular mechanism, by which actin can regulate the nuclear import of one of its binding partners (Fig. 7). Previous studies have shown that actin binding enhances MKL1 nuclear export (Vartiainen et al, 2007), perhaps by providing a structure that is more optimal to its nuclear export receptor, Crm1. This prospect, as well as the inhibitory effect of actin on MKL1 nuclear activity, will be exciting topic for future studies.

This work also reveals that the RNA helicase Ddx19 is required to modulate the conformation of MKL1, which affects its nuclear import status in cells. The results demonstrate that N- and C-terminus of MKL1 associate with each other, thus predicting a closed conformation, which is incompetent for nuclear import. Since Ddx19 is needed to facilitate the interaction between full-length MKL1 and its nuclear import receptor Ipoβ, we speculate that Ddx19 is specifically required to maintain MKL1 in an open conformation, which makes MKL1 accessible for the importin complex to mediate its efficient nuclear import. The results show that the ATPase cycle of Ddx19 needed for mRNA export is not required for MKL1 nuclear import. Therefore MKL1 nuclear import and mRNA export mediated by Ddx19 have different mechanisms. As the MKL1-Ddx19 interaction is not dependent on actin, we propose that MKL1 conformational control occurs upstream of the actin-mediated regulation (Fig.

7). However, in the future, structural studies on MKL1 conformation are required to confirm this hypothesis. Also, mapping the exact Ddx19 interaction sites could give us valuable information about the regions that are required for MKL1 intramolecular structure and conformational dynamics.

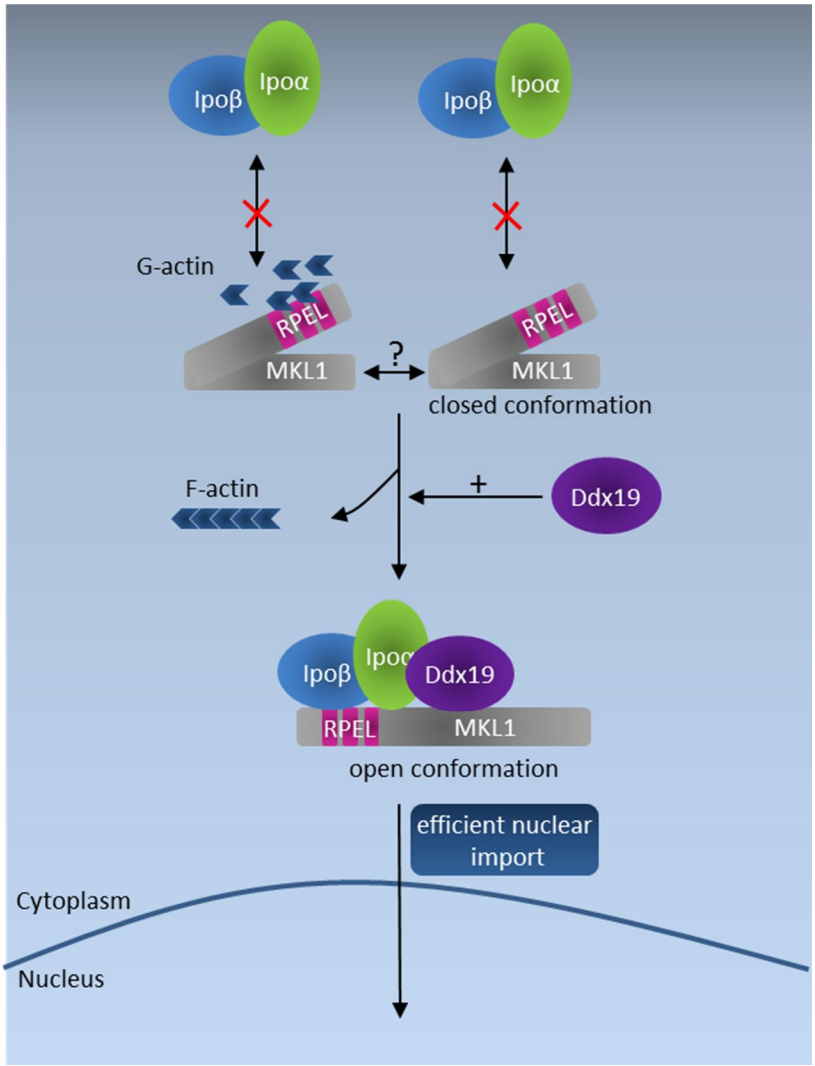

Figure 7. A schematic model on MKL1 nuclear import regulation. Binding of actin to the RPEL repeat of MKL1 inhibits its nuclear import, because it prevents Ipoα/Ipoβ binding. Upon actin polymerization (F-actin), actin monomer levels decrease and the NLSs of actin-free MKL1 are exposed to Ipoα/Ipoβ complex, which leads to efficient import of MKL1 into the nucleus. Also in its closed conformation, MKL1cannot recruit Ipoα/Ipoβ heterodimer and is thereby incompetent for nuclear import. Ddx19 facilitates the acquisition of an open conformation that allows the recruitment of Ipoα/Ipoβ for efficient nuclear import of MKL1. Whether this conformational change, mediated by Ddx19, occurs upstream of actin-mediated regulation of MKL1 nuclear import remains to be determined.

This study suggests that MKL1 nuclear import is dependent on Ddx19 ability to bind RNA. Therefore, in addition to actin dynamics, MKL1-SRF pathway could respond to general mRNA levels in cells. As Ddx19 directly regulates the

general mRNA levels in the cytoplasm by controlling their nuclear export, it is tempting to speculate that these events are linked. However, the possible interplay between these two processes remains to be studied.

This study expands the multifunctional nature of Ddx19. Previously, Ddx19 has been linked to several cellular processes including transcription, mRNA export and translation termination. This study proposes a novel role for it in nuclear import of a key transcriptional regulator. Interestingly, all of these processes are involved in gene expression. Therefore in the future it will be interesting to know whether Ddx19 is required to coordinate these events. In this light MKL1 might not be the only transcription factor regulated by this RNA helicase.

Collectively, this thesis work describes the molecular mechanisms by which nuclear import of MKL1 is controlled. This knowledge is needed to understand how SRF activity and thus cytoskeletal genes are controlled. In addition, this work describes how Phactr4, by regulating PPI and cofilin activity, can modulate local levels in the actin monomer pool. Although both Phactr4 and MKL1 respond to the same stimuli, decreased actin monomer levels, they might react to it differently. Small changes in local actin monomer pool could be altered locally by modulating cofilin activity by Phactr4, but drastic changes would need transcriptional activation through MKL1. Therefore this work emphasizes the significance of RPEL repeat as a general actin monomer sensor and regulator of actin homeostasis in cells.

6. ACKNOWLEDGEMENTS

This thesis work was carried out between years 2008 and 2015 at the Institute of Biotechnology, University of Helsinki. I wish to thank the former and current directors of the Institute, Professors Mart Saarma, Tomi Mäkelä and Howy Jacobs for providing such a high quality research facilities and friendly working environment.

Without a doubt I own my deepest gratitude to my supervisor, Docent Maria Vartiainen. I started my career as an undergraduate student in Maria's lab and she has thought me virtually everything I know about cell biological research. Thank you for giving me this opportunity to work with and learn from you. Your example as a successful and brave women scientist has also encouraged me to continue and follow my passion.

I wish to thank my follow-up group members, Professor Pekka Lappalainen and Docent Mikko Frilander for their friendly and valuable guidance over the years. Mikko also served as a pre-examiner of this thesis together with Professor Guido Posern, and I am grateful for their thoughtful review and insightful comments.

I wish to acknowledge all the co-authors for fruitful collaborations: Professor Richard Treisman, Dr. Rafael Pawlowski, M.Sc Tiina Viita, Salla Kyheröinen, M.Sc Marko Civaro and Doctors Guillaume Huet, Kari-Pekka Skarp and Joseph Dopie. The staff members of Image Unit, "the microscopy-boys", especially M.Sc Marko Civaro, M.Sc Mika Molin and Dr. Kimmo Tanhuanpää are warmly thanked for their excellent technical assistance with the imaging. In addition, many thanks to Marko for his valuable help with data analysis and statistics.

For the financial and educational support, I wish to thank Viikki Doctoral Programme in Molecular Biosciences (VGSB), now part of Integrative Life Science Doctoral Program (ILS) and Professors Dennis Bamford, Hannes Lohi and Assistant Professor Ville Hietakangas, the former and current heads of the programs. I also want to acknowledge VGSB/ILS coordinators Doctors Ulla Tuomainen and Erkki Raulo for organizing excellent courses and student activities.

Over the years in Maria's lab I have had the privilege to work with many marvelous and smart people, and many of them have become my dear friends. Guillaume, your willingness to help colleagues and especially us students has been invaluable. Thank you for sharing your knowledge and for being my personal mentor. All the post-docs in the lab, Guillaume, Alise, Henna and Ilya are warmly thanked for careful examination of this Thesis and all the valuable comments and suggestions, which really helped me to improve it. Many thanks also for the peer support during past few months. Alise, thank you for sharing the ups and downs in the lab and life in general, thank you for always listening, supporting and understanding. You are a true friend! Rita, thank you for always keeping my back and for seeing the bright side in everything and everyone. I am very lucky to have you as my friend. I wish to thank Henna for your kindness, support, friendship and willingness to help. Many thanks to Tiina for being such lively company in the lab and on our conference trips and always ready to help. Katia is thanked for taking care of MAL in the future and Paula, for making my work much easier. Former lab members, K-P, Joseph and Johanna are thanked for the good old times. Thank you for taking me in to your team and incorporating me in the lab work. I really miss you guys! New lab members, Jori, Hilal, Salla and Mariel are thanked for keeping the lab energetic and lively.

Many hugs for all the past and present people on the 5th floor. Specifically, I wish to acknowledge Sari and Hongxia for being important role models. And I'm so glad to remain in contact with Sari through our future collaborator. Thank you for Johanna and Maarit for the continuing friendship. I am also grateful for Ansku, Martina, Juha, Elena, Minna, Geri, Markku, Jaakko, Yaming, Karita, Merja, Pirta, Enni and Anna-Liisa, for their help and advices, for lunch, coffee and traveling company, and for many nice discussions over the years.

I warmly thank my family, close relatives, friends and other people outside the lab for making my life so good. Specifically, I thank my Mom and Dad and my dear brothers Mikko and Matti, for always encouraging me in the choices I have made and for staying behind me in any circumstances.

Finally, I would like to thank my beloved husband Olli and my little son Paavo: without your continuous support, love and believe in me I would have never had the courage to finish this project. Thank you for making me so incredibly happy!

Helsinki, August 2015 Kaisa

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