

Regulation of Eukaryotic Translation Initiation by Signal Transduction

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy in the Department of  
Molecular Genetics and Microbiology in the Graduate School  
of Duke University

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ABSTRACT

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## Abstract

Eukaryotic translation initiation is a rate-limiting step of protein synthesis and is controlled by signal transduction in response to various extracellular cues and stresses. This thesis is focused on the eukaryotic initiation factor 4G (eIF4G), which participates in multiple steps of initiation: (i) eIF4G interaction with polyA-binding protein (PABP) links the 5' pre-initiation complex to the 3' poly(A) tail of mRNAs; (ii) eIF4G recruits 40S ribosomal subunit to mRNAs through interactions with the m<sup>7</sup>-G cap binding protein eIF4E; (iii) eIF4G binds to the eIF4E kinase, mitogen activated protein kinase (MAPK) interacting kinase 1 (Mnk1), modulating eIF4E phosphorylation. I studied how eIF4G function is affected by viral infection, mitogenic stimulation and during mitosis.

First, I reported that herpes simplex virus 1 (HSV-1) infection leads to re-localization of PABP to the nucleus, dissociating it from translation initiation machinery. Next, I showed that MAPK-mediated phosphorylation of Mnk1 leads to Mnk1 conformational changes, enhancing its binding to eIF4G and, hence, increasing phosphorylation of its substrate, eIF4E. Finally, I demonstrated that Cdk1/cyclin B1 directly phosphorylates eIF4G in mitosis and speculated on the role of this phosphorylation event in mitotic translation. In summary, my work demonstrated that eIF4G plays key roles in the regulation of the translational response to viral infection, growth signaling and cell cycle progression.

## **Dedication**

Dedicated to my parents.

# Contents

Abstract.....	iv
List of Tables.....	xi
List of Figures.....	xii
List of abbreviations.....	xiv
Acknowledgements .....	xviii
1. Introduction.....	1
1.1 Overview of translational regulation .....	1
1.1.1 Post-transcriptional control of gene expression.....	1
1.1.2 Regulation of protein synthesis.....	3
1.1.3 Stages of translation.....	5
1.1.4 Translation initiation.....	5
1.2 Growth signaling pathways control translation initiation.....	9
1.2.1 Overview of the receptor-induced signaling pathways.....	9
1.2.1 mTOR.....	11
1.2.1.1 Upstream activators of mTOR .....	12
1.2.1.2 mTORC1 targets translational machinery.....	15
1.2.2 Ras/MAPK pathway .....	17
1.3 eIF4G is a central protein for translation initiation .....	21
1.3.1 Structure of eIF4G .....	21
1.3.2 eIF4G isoforms.....	25

1.3.3	Initiation factors that interact with eIF4G .....	27
1.3.3.1	Cap-binding protein eIF4E .....	27
1.3.3.2	RNA helicase eIF4A .....	28
1.3.3.3	Multi-subunit complex eIF3 .....	29
1.3.3.4	Poly A-binding protein PABP .....	30
1.3.4.4	eIF4G binds translational repressors .....	32
1.4	Role of eIF4G in biology and disease.....	33
1.4.1	Depletion of eIF4G leads to growth arrest phenotypes.....	33
1.4.2	The role of eIF4G in gametogenesis and in neurons .....	37
1.4.3	Over-expression of eIF4G promotes tumorigenesis .....	38
1.4.4	Signal transduction to eIF4G.....	39
1.5	Translation in mitosis .....	42
1.5.1	Global down-regulation of translation in mitosis.....	43
1.5.2	Selective upregulation of certain mRNAs in mitosis.....	45
1.6	Regulation of translation during viral infection .....	48
1.6.1	Herpes simplex virus 1 .....	49
1.6.2	HSV-1 infection induces host protein synthesis shut-off.....	51
1.6.3	Host defense response and viral countermeasures .....	52
2.	Materials and Methods .....	57
2.1	Cell culture and stable cell lines.....	57
2.2	Expression constructs .....	58
2.2.1	Cloning for Chapter 3.1 .....	58

2.2.2 Cloning for Chapter 3.2 .....	58
2.2.3 Cloning for Chapter 3.3 .....	60
2.3 Recombinant proteins .....	63
2.4 Immunofluorescence .....	63
2.5 DNA transfections .....	64
2.6 Kinase inhibitors and activators .....	65
2.7 m7G cap pull down, glutathione pull down, immunoprecipitation.....	65
2.7.1 Chapter 3.1.....	65
2.7.2 Chapter 3.2.....	66
2.7.3 Chapter 3.3.....	67
2.8 Immunoblotting and antibodies .....	68
2.8.1 Antibodies for Chapter 3.1 .....	68
2.8.2 Antibodies for Chapter 3.2 .....	69
2.8.3 Antibodies for Chapter 3.3 .....	69
2.9 In vitro kinase assay.....	70
2.9.1. In vitro phosphorylation of eIF4E by Mnk1 kinases .....	70
2.9.2 In vitro phosphorylation of GST-eIF4G (Ct) by nocodazole-arrested cell extracts.....	70
2.10 Cell synchronization and FACS.....	71
2.11 Phosphoproteomics .....	72
2.11.1 Sample Preparation and Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) Analysis .....	72

2.11.2 Qualitative Identifications and Selected Ion Chromatograms from Raw LC-MS/MS Data .....	74
2.12 RNA transfections and Luciferase assay .....	75
3. Results .....	77
3.1 Herpes Simplex Virus 1 lytic infection affects PABPC function.....	77
3.1.1 Introduction.....	77
3.1.2 Results .....	78
3.1.2.1 Reduced association of PABPC with the translation initiation complex in HSV-1 infected cells.....	78
3.1.2.2 Identification of proteins interacting with PABPC in HSV-1 infected cells. ....	83
3.1.2.3 Effect of ectopic expression of ICP27 and UL47 on PABPC re-distribution to the nucleus.....	85
3.1.3 Discussion.....	87
3. 2 MAPK control eIF4E phosphorylation through modulation of Mnk1-eIF4G interaction.....	90
3.2.1 Introduction.....	90
3.2.2 Results .....	92
3.2.2.1 Stimulation of Erk1/2 and p38 results in increased binding of Mnk1 to eIF4G .....	92
3.2.2.2 Phosphorylation of Mnk1 active site is required for efficient Mnk1-eIF4G interaction. ....	96
3.2.2.3 Differential binding of Mnk1 isoforms and Mnk2 to eIF4G .....	100
3.2.2.4 Mnk1 C-terminal domain interferes with eIF4G binding to the Mnk1 N terminus in unstimulated cells .....	102

3.2.2.5 Mnk1 limits its own binding to eIF4G.....	104
3.2.2.6 Binding of Mnk1 to eIF4G is critical for eIF4E phosphorylation.....	107
3.2.3 Discussion.....	110
3.3 CDK1/cyclin B1 mediated phosphorylation of eIF4G modulates selective translation in mitosis .....	113
3.3.1 Introduction.....	113
3.3.2 Results.....	114
3.3.2.1 Analysis of eIF4G interactions in mitosis.....	114
3.3.2.2 Phosphoproteomics of eIF4G in mitosis and interphase.....	119
3.3.2.3 eIF4G is phosphorylated at S1232 in mitosis .....	122
3.3.2.4 Dual phosphorylation of eIF4G S1232 by Erk1/2 and CDK1/cyclin B1 in vivo .....	126
3.3.2.5 Cdk1/cyclin B1 phosphorylates eIF4G in vitro .....	128
3.3.2.6 Over-expression of the C-terminal domain of eIF4G with a S1232A mutation fails to stimulate c-myc IRES-dependent translation in mitosis.....	130
3.3.2.7 Creation of knock-down/rescue eIF4G stable inducible cell lines.....	134
3.3.3 Discussion.....	135
4. Conclusions and future directions.....	141
4.1 Insights on PABP gained from studying HSV-1 infection.....	141
4.2 Role of Mnk kinases in translation .....	144
4.3 Regulation of mitotic translation .....	147
References.....	151
Biography .....	164

## List of Tables

Table 1 List of primers used .....	62
Table 2: Proteomics analysis of eIF4G immunoprecipitates in interphase and mitosis..	117

## List of Figures

Figure 1. Model of the canonical pathway of eukaryotic translation initiation. ....	7
Figure 2 Schematic representation of mTORC1 complex .....	13
Figure 3 S6K control of translation .....	16
Figure 4 Regulation of cap-dependent translation initiation.....	18
Figure 5 eIF4GI domain structure and interacting proteins. ....	22
Figure 6 eIF4GI position in a scanning 43S complex.....	24
Figure 7 Abundance and association of translation initiation complex components in HSV-1-infected cells.....	80
Figure 8 HSV-1 infection results in PABPC redistribution to the nucleus. ....	82
Figure 9 Identification of proteins interacting with PABP in HSV-1-infected cells. ....	84
Figure 10 Effect of ectopic HSV-1 ICP27 and UL47 on PABPC subcellular distribution .	86
Figure 11 Stimulation of cells with TPA results in increased binding of Mnk1 to eIF4G.	94
Figure 12 MAPK-mediated phosphorylation of Mnk1a at T209/T214 activates Mnk1-eIF4G interaction.....	97
Figure 13 Differential regulation of Mnk1 and Mnk2 binding to eIF4G.....	101
Figure 14 The C-terminal domain of unstimulated Mnk1a blocks binding to eIF4G.....	103
Figure 15 Mnk1 kinase activity limits its binding to eIF4G. ....	105
Figure 16 MAPK regulated Mnk1-eIF4G binding is required for efficient eIF4E phosphorylation.....	108
Figure 17 Comparison of eIF4G1 phosphorylation in interphase and mitosis.....	120
Figure 18 eIF4G is phosphorylated at S1232 is during mitosis.....	123

Figure 19 CDK1-dependent phosphorylation of eIF4G phosphorylation at S1232 in vivo .....	127
Figure 20 CDK1/cyclin B1 phosphorylates eIF4G1 at S1232 in vitro. ....	129
Figure 21 C-terminal fragment of eIF4G with S1232A mutation fails to stimulate myc IRES-dependent translation .....	133
Figure 22 Creation of inducible stable knock-down/rescue cell lines for eIF4G .....	136

## List of abbreviations

AMPK - AMP-activated protein kinase

asyn – asynchronous

CAMKII - Ca<sup>2+</sup>/calmodulin-dependent kinase II

CBC- cap binding complex

CBP 80 – cap binding protein 80

CDK – cyclin dependent kinase

DDX3X - DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked

DEAD box – Asp Glu Ala Asp containing motif

Ds – double-stranded

E – early genes

eEF – eukaryotic elongation factor

eEF2K – eukaryotic elongation factor 2 kinase

EGF - epidermal growth factor

eIF – eukaryotic initiation factor

eIF4G-Ct – C-terminal fragment of eIF4G

ERK - extracellular-signal regulated kinase

Fluc – Firefly luciferase

GAP – GTPase activating protein

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GEF - guanine nucleotide-exchange factor

HCV – hepatitis C

HEAT domain - Huntington, elongation factor 3, a subunit of protein phosphatase 2A  
and target of rapamycin domains

HIF 1 – hypoxia inducible factor 1

hnRNP A1 – heterogeneous nuclear ribonucleoprotein A1

HSV-1 – herpes simplex virus 1

IBC – inflammatory breast cancer

ICP – infected cell protein

IE genes – immediate-early genes

IFN – interferon

IP – immunoprecipitation

IRES – internal ribosomal entry site

KSHV – Kaposi's sarcoma-associated herpesvirus

L – late genes

LABC – localized advanced breast cancer

MAPK – mitogen activated protein kinase

Mnk1 – MAPK interacting kinase 1

MOI – multiplicity of infection

MS – mass spectrometry

MS/MS – tandem mass spectrometry

mTOR – mammalian target of rapamycin

mTORC1 – mTOR complex 1

noc – nocodazole

ODC – ornithine decarboxylase

PABP – poly(A)-binding protein

PABPC – cytoplasmic PABP

PABPN – nuclear PABP

Paip2 – PABP interacting protein 2

PDCD4 - programmed cell death protein 4

PERK - PKR-like endoplasmic reticulum kinase

PI3K - phosphatidylinositol 3-kinase

PKC – protein kinase C

PKR - protein kinase R

Raptor - regulatory associated protein of mTOR

RHEB - Ras homologue enriched in brain

Rictor - rapamycin-independent companion of mTOR

Rluc – Renilla luciferase

RSK – 90kDa ribosomal S6 kinase

S6K – 70kDa ribosomal S6 kinase

SOX – shutoff and exonuclease protein

SRPK – splicing related protein kinase

SRSF3 - serine/arginine-rich splicing factor 3

Ss – single-stranded

TSC1 - tuberous sclerosis complex 1

uORF – upstream open reading frame

UTR – untranslated region

VEGFA - vascular endothelial growth factor A

Vhs – virion host shutoff protein

Vp – viral protein

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# 1. Introduction

## *1.1 Overview of translational regulation*

### **1.1.1 Post-transcriptional control of gene expression**

Protein synthesis consumes a substantial amount of cellular energy (four high-energy bonds per peptide bond) and resources. Thus, cells have developed a variety of mechanisms to accurately coordinate translation levels with their physiological needs. The concept of dynamic translation regulation has only recently come to the fore, as it was widely assumed that most gene expression regulation occurs via transcriptional control. Ribosomes were viewed as unalterable machinery that decoded mRNAs into proteins without much appreciation for the intricate processes involved in regulating the efficiency of this process.

In recent years, however, much evidence has accumulated in support of a prominent role of regulatory processes that control translational rates. First, in various cellular processes, changes in gene expression cannot be attributed solely to transcription. For instance, transcriptional silencing during early embryogenesis means that most of gene regulation must be executed post-transcriptionally. Another example is formation of long-term memory in neurons, which requires local translation of pre-stored mRNAs (review in (Mathews, Sonenberg et al. 2007)).

Second, translation initiation, elongation and termination factors were discovered, as well as the signaling pathways that directly modify these proteins. Specific phosphorylation events that modulate translation efficiency were identified, and aberrant phosphorylation of several translation initiation factors was shown to contribute to abnormal cellular homeostasis leading to tumorigenesis. Third, the discovery of small non-coding RNAs and RNA-binding proteins, which modulate mRNA stability and translation, revealed novel means of post-transcriptional control of gene expression.

In this thesis I will focus mostly on regulation of translation initiation. Other stages of the mRNA life cycle, such as mRNA transport, stability and localization, are also highly regulated events and contribute greatly to gene expression control. However, describing them in detail is beyond the scope of this thesis.

Also, while many exciting discoveries in the translation field were made in non-mammalian systems, such as yeast, *Xenopus* and *Drosophila*, I will focus on translation regulation in mammals, since this is the model system that I use in my day-to-day scientific endeavours.

### **1.1.2 Regulation of protein synthesis**

In prokaryotes, optimized protein expression maximizes fitness and survival chances (Dekel and Alon 2005). In eukaryotes, the translational rate is regulated primarily at the stage of initiation and is tightly controlled by multiple parameters, such as the metabolic state of the cell, extracellular cues, stress conditions and the stage of the cell cycle.

Control of translation by signal transduction to translation machinery allows cells to respond to environmental changes in an accurate fashion. Extracellular cues, such as hormones, growth factors and proliferation signals, can quickly increase/decrease levels and identity of synthesized proteins before transcriptional changes take place. On the other hand, a lack of cellular resources, such as ATP and/or amino acids, can cease translation and promote autophagy, re-directing cellular processes from anabolism to catabolism.

Similarly, various signaling pathways can down-regulate translation in response to pro-apoptotic stimuli or stress conditions, such as heat shock, oxidative stress, gamma-irradiation or viral infection. Decrease of protein synthesis in stress conditions is critical for survival of both cells and organisms. It can prevent accumulation of toxic misfolded proteins, block aberrant cell cycle progression and inhibit viral replication.

Lastly, translation also depends upon the stage of the cell cycle. For instance, during mitosis protein synthesis is drastically down-regulated, as sustaining protein synthesis is not necessary and could interfere with proper cell division.

I would like to distinguish two levels of translational control: (i) modulation of global protein synthesis rate; and (ii) template-selective translation of (a) subset(s) of mRNA(s). Usually, these occur simultaneously, because global protein synthesis suppression typically coincides with selective translation induction of certain mRNAs. Such responsive mRNAs are thought to encode regulatory proteins that are required to either restore cellular homeostasis or commit the cell to apoptosis. Translation of such mRNAs can be restricted under normal conditions and require additional factors or post-translational modification of the translation apparatus.

To summarize, translational rates are tightly linked to changes in the cellular microenvironment. Signaling pathways phosphorylate translation initiation machinery, which results in changes in global and template-selective translation. In my thesis I will describe how various conditions, such as viral infection (Chapter 3.1), growth stimulation (Chapter 3.2) and the cell cycle (Chapter 3.3) influence protein synthesis by affecting the function of eukaryotic initiation factor 4G (eIF4G). eIF4G is an important scaffolding protein that anchors the 40S small ribosomal subunit to the 5' end of

mRNAs. In Chapters 1.3 and 1.4 I will introduce this central player in translation initiation, as well as its interaction partners.

### **1.1.3 Stages of translation**

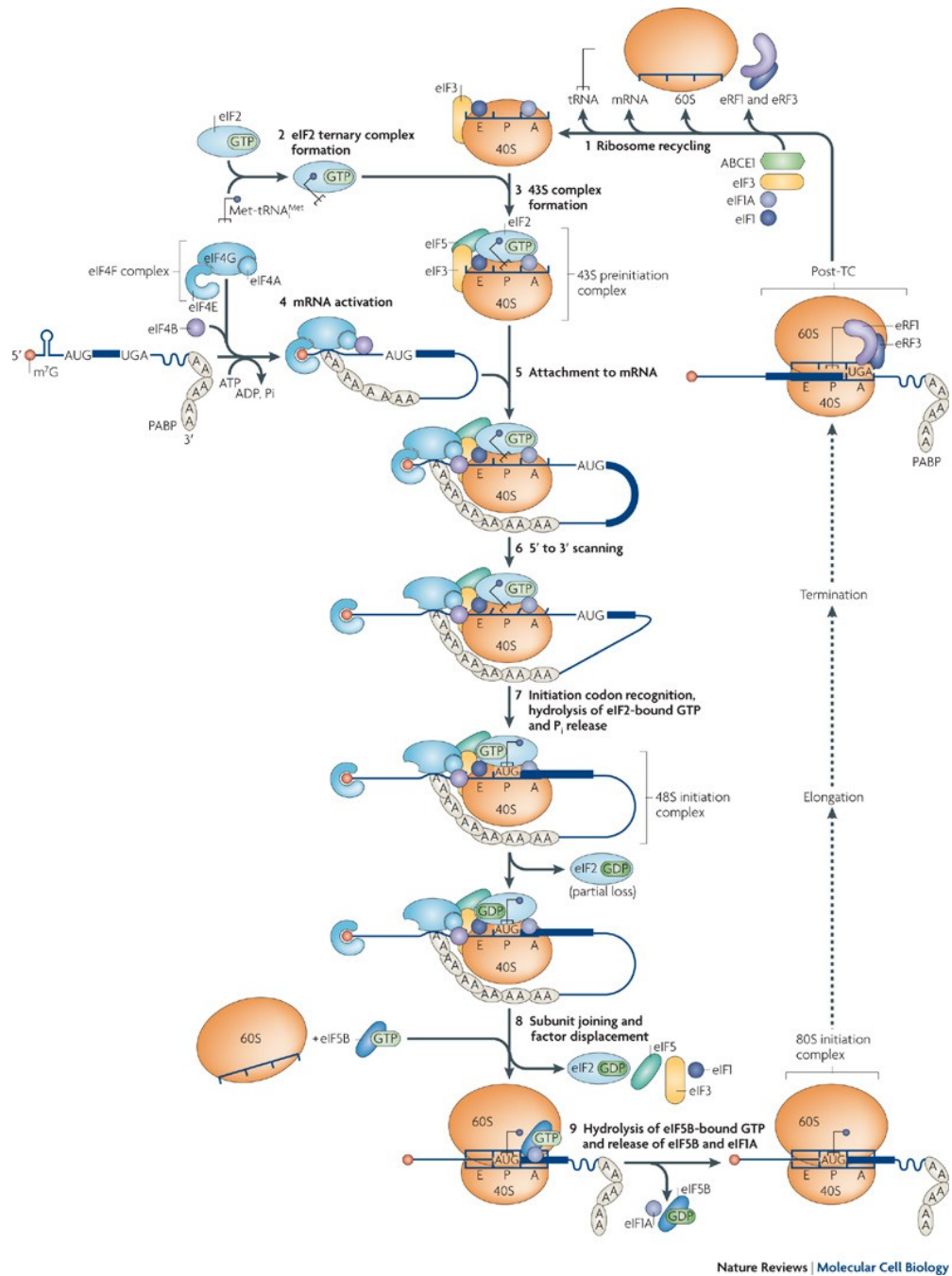
During translation several key steps must be completed. Translation initiation ensures delivery of methionyl-tRNA to the start codon, enabling the assembly of the 80S ribosome. During elongation, nucleotide triplets are decoded into peptides, peptide bonds are formed, and nascent polypeptide are co-translationally folded and targeted to the proper subcellular compartment. When ribosomes encounter a stop-codon, translation is terminated, the polypeptide is released and the ribosome either recycles to initiate a new round of translation or disassembles. Early studies of translation have established that initiation is a rate-limiting, regulated stage of translation (reviewed in (Mathews, Sonenberg et al. 2007)). Accordingly, most of the discovered control mechanisms affect translation initiation factors. Below I will illuminate mechanistic details of initiation, and, since all of my research was performed in mammalian tissue culture, I will present translation initiation as it happens in mammals.

### **1.1.4 Translation initiation**

Initiation can be sub-divided into multiple stages, which do not necessarily occur sequentially but can proceed in parallel. Several events ensure the assembly of 80S ribosomes at the correct start codon. First, the 40S ribosomal subunit associates with an

amino acid-loaded initiator tRNA. Second, this ribosomal complex is recruited to the 5' end of the mRNA and scans the 5' untranslated region (UTR) until the initiation codon is recognized. Finally, the 60S ribosomal subunit joins the 40S subunit, the initiator tRNA is positioned at the correct location in the 80S ribosome, and the initiation factors are released--allowing elongation (reviewed in (Mathews, Sonenberg et al. 2007)).

Both cis- and trans-factors affect the rate of this process. The methyl-7-guanosine cap structure, complexity of the 5'UTR, the poly(A)-tail, etc. are mRNA elements that influence the recruitment of eukaryotic initiation factors (eIFs); in some cases, this process is assisted by RNA-binding proteins. Initially, post-termination ribosomes are released from mRNA as 60S and factor-associated 40S subunits (Fig. 1, step 1). The small subunit is stably associated with eIF3, eIF1 and eIF1A. Mammalian eIF3 is a large complex (~650 kDa) that contains up to 13 subunits, and participates both in initiation and termination. Methionyl-tRNA (Met-tRNA<sub>i</sub><sup>Met</sup>) binds to eIF2-GTP to form the 'ternary complex' that is critical for recognition of the start codon in the mRNA (Fig. 1, step 2). eIF2 is a GTPase that links the initiator tRNA with the 40S small ribosomal subunit, forming the 43S pre-initiation complex. Next, the m7G-cap structure is recognized by eIF4F, which consists of the cap-binding protein, eIF4E; a DEAD box RNA helicase, eIF4A; and a scaffolding protein, eIF4G (Fig.1, step 3). Interaction of eIF4G with the polyA-binding protein (PABP) bridges the 5' and 3' ends of mRNAs and may



**Figure 1. Model of the canonical pathway of eukaryotic translation initiation.**

Please, see text for details. Adapted from (Jackson, Hellen et al. 2010)

promote ribosome recycling. eIF4G also binds eIF3, thus engaging the 43S pre-initiation complex to the 5' end of the mRNA (Fig.1, step 4). The initiation complex, which consists of 43S and eIF4F, scans the 5'UTR in the search of the correct initiation codon. Scanning is mediated by the eIF4A RNA helicase, whose activity is stimulated by eIF4B and eIF4H, both RNA-binding proteins (Fig.1, step 5). Complementarity between the initiation codon with the anti-codon of Met-tRNA<sub>i</sub><sup>Met</sup> drives recognition of the start codon, which is followed by hydrolysis of eIF2-bound GTP (Fig. 1, step 6), subunit joining and initiation factor displacement (Fig. 1, step 7). In Chapter 1.3 and 1.4 I will talk specifically about the eIF4F complex and the role of eIF4G as a central scaffolding protein in recognition of mRNAs by the 43S subunit and subsequent 5'UTR scanning.

## ***1.2 Growth signaling pathways control translation initiation***

### **1.2.1 Overview of the receptor-induced signaling pathways**

Translation in eukaryotic cells is regulated primarily at the stage of initiation. Below, I describe two major growth-signaling pathways, centered on the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and Ras/mitogen activated protein kinase (MAPK) axes, the activation of which leads to phosphorylation of translation initiation factors and modified translation levels. I will review the mechanisms of translation inhibition upon viral infection in Chapter 1.6.3, where I talk about host defense responses to viral infection.

In general, signaling pathways are induced upon binding of hormones (e.g. insulin) or growth factors (e.g. epidermal growth factor, EGF) to the corresponding plasma membrane receptors, such as receptor tyrosine kinases or G protein-coupled receptors. These receptors, in turn, mobilize downstream signal transmitters, either kinases or GTPases, which transduce the signal through the chain of phosphorylation events, eventually leading to the activation of certain transcription and translation factors, or other cellular effectors. Thus, signal transduction pathway consists of

multiple sequential players, which allows amplification of the initial signal, as well as modulation of the signal duration and noise reduction (Kholodenko 2006).

Moreover, signaling pathways are characterized by the presence of integrator molecules, which receive and process signals from multiple inputs. One of such molecules is mTOR kinase, which receives various messages and incorporates them to promote appropriate cellular responses.

GTPases (e.g. Ras) are other important components of the signaling pathways. They are active in the GTP-bound form and are inactivated upon GTP hydrolysis. The inherent catalytic activity of GTPases is low and has to be stimulated by the GTPase-activating protein (GAP), which functions when the pathways needs to be turned off. Reversely, when conditions dictate activation of the pathway, guanine nucleotide-exchange factors (GEFs) stimulate exchange of GDP to GTP on the GTPase, thus turning it back on. In this manner, cellular GTPases act as molecular switches, which may receive input from several pathways and turn a certain program 'on' or 'off'.

I would like to note that although I present signaling cascades in a relatively linear fashion, in reality these pathways are far from being linear. They contain multiple positive and negative feedback mechanisms, which can either reinforce or self-regulate

the signaling pathway. Moreover, there is extensive cross-talk between different pathways, for instance, between the Ras/MAPK and the PI3K/AKT/mTORC1 pathways (Ma and Blenis 2009). However, for the purpose of this thesis and in the interest of clarity, I present a simplified picture and focus only on the key regulators of translation and their downstream targets.

### **1.2.1 mTOR**

Protein synthesis requires adequate supply of energy (ATP, GTP) and resources (amino acids, some of which are essential, thus cannot be synthesized by the cell and need to be acquired from the environment). Thus, if extracellular cues dictate the cell to proliferate, but certain essential amino acids are lacking, the cell needs to make an important decision of whether to sustain protein synthesis, or instead suppress biosynthetic programs and induce autophagy, a controlled self-degradation process, which supplies starved cell with energy and catabolic precursors.

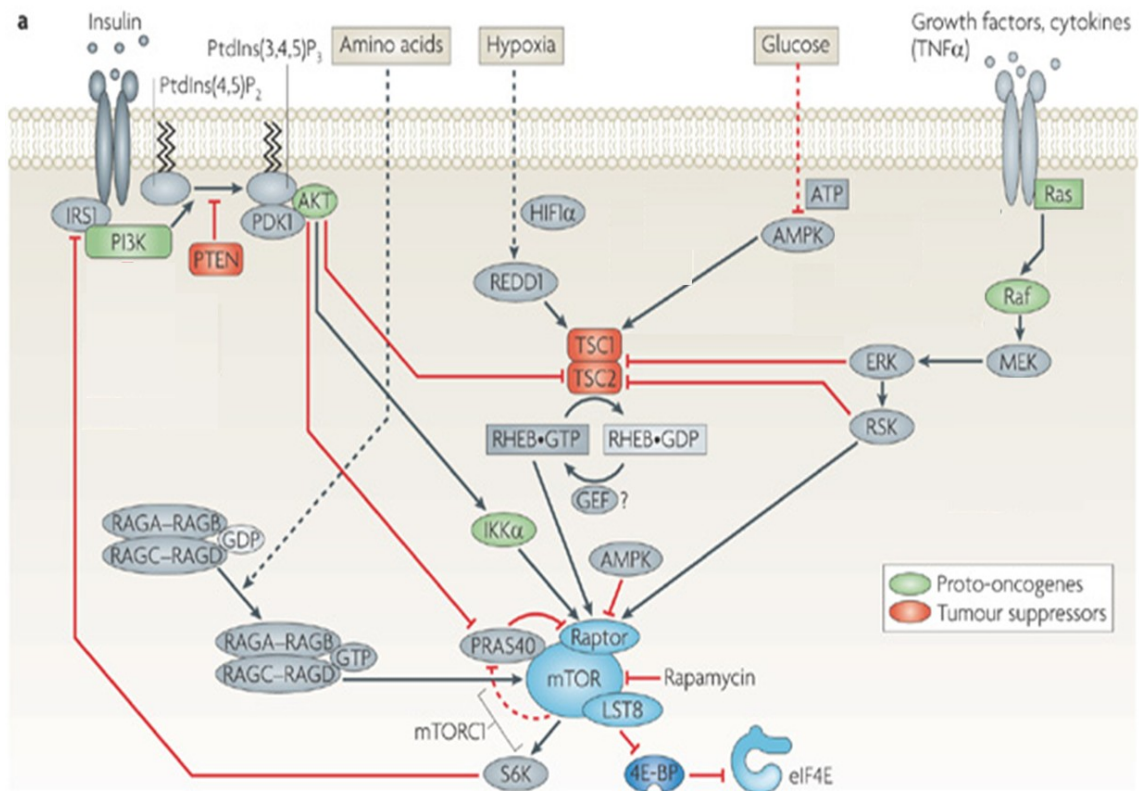
The mTOR kinase plays a key role in this decision-making process, since it processes signals from growth factors, intracellular sensors of nutrients and energy, as well as stress signaling pathways. The cumulative effects of these signals determine the cellular metabolic program. Thus, mTOR is a major controller of cellular growth and homeostasis, and deregulation of its function is implicated in metabolic disease, cancer and aging (reviewed in (Zoncu, Efeyan et al. 2011)).

The mTOR kinase exists in two separate complexes, mTOR complex 1 (mTORC1) and mTORC2. mTOR associates with accessory proteins Raptor (regulatory associated protein of mTOR) and Rictor (rapamycin-independent companion of mTOR) to form mTORC1 and mTORC2, respectively. Accessory proteins receive signals from the upstream regulators, activating mTOR, and also define its substrate specificity. In general, regulation of mTORC1 is much better understood than that of mTORC2. Also, only mTORC1 controls protein synthesis, so I will focus mostly on this complex.

#### **1.2.1.1 Upstream activators of mTOR**

Several proteins are required for the activation of the mTORC1 complex (Fig. 2). Briefly, mTORC1 catalytic activity is stimulated by the small GTPase RHEB (Ras homologue enriched in brain). In turn, RHEB is regulated by the tuberous sclerosis 1 (TSC1) - TSC2 complex, which serves as GTPase-activating protein for RHEB, converting it to the inactive GDP-bound form. Multiple signaling cascades converge on the TSC1-TSC2 complex, either promoting or inhibiting its activity (reviewed in (Ma and Blenis 2009).

Mitogenic stimulation of cells leads to increased protein production, since the cell must double in size and duplicate its cellular organelles before cell division. Key players



**Figure 2 Schematic representation of mTORC1 complex**

mTORC1 is stimulated by the active, GTP-bound form of RHEB; immediately upstream of RHEB is the tuberous sclerosis 1 (TSC1)–TSC2. TSC2 contains a GTPase-activating protein (GAP) domain that converts RHEB to its inactive, GDP-bound form. Multiple upstream signaling inputs from PI3K–AKT, Ras–ERK–RSK, AMPK and LKB1–AMPK pathways either positively or negatively regulate mTORC1 signaling. TSC2 is phosphorylated by several different kinases, including AKT, ERK and RSK, which results in the inhibition of the GAP function of TSC2 towards RHEB. Conversely, AMPK-mediated phosphorylation events positively regulate the GAP activity of TSC2 towards RHEB. In addition, Rag proteins mediate the nutrient-sensing function of mTORC1. The mTORC1 kinase is a master modulator of protein synthesis by integrating signals from growth factors and cellular conditions. In addition to its direct phosphorylation of 4E-binding proteins (4E-BPs), activated mTORC1 promotes the activation of S6Ks, which in turn phosphorylate many translation initiation factors, including eukaryotic translation initiation factor 4B (eIF4B) (adapted from (Ma and Blenis 2009)).

in this process are PI3K, which interacts with the insulin receptor, and its downstream target, AKT, which in turn inhibits the TSC1–TSC2 complex, negative regulator of mTORC1. The TSC1-TSC2 complex also receives similar input from both MAP kinase extracellular-signal-regulated kinases 1/2 (ERK1/2) and its downstream target ribosomal S6 kinase (RSK).

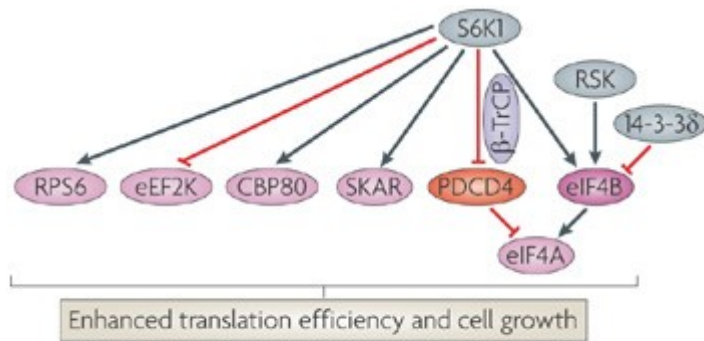
As mentioned above, mTORC1 receives signaling from environmental cues to determine if the cell has enough energy and resources to proceed with growth factor-dictated increases in protein synthesis. Lack of amino acids or ATP can block mTORC1 function. It is well established that AMP-activated protein kinase (AMPK) senses the AMP:ATP ratio and activates ATP-generating pathways, while turning off energy consuming processes via inhibition of mTORC1 (Inoki, Zhu et al. 2003). Sufficient oxygen concentration is essential for ATP production; thus in hypoxic conditions, energy-demanding processes (e.g. translation) are quickly repressed and cellular resources are re-directed to promote angiogenesis. Hypoxia-induced mTORC1 inhibition leads to the block of cap-dependent translation and increased translation of vascular endothelial growth factor A (VEGF-A) mRNA, which utilizes an alternative mode of translation initiation and is selectively active during repression of global protein synthesis (DeYoung, Horak et al. 2008). It is less clear how the cell senses amino acids. Recent reports indicate that the Rag family of GTPases plays an important role in

this process, stimulating mTORC1 in an amino acid-dependent manner (Sancak, Peterson et al. 2008).

#### **1.2.1.2 mTORC1 targets translational machinery**

Activated mTORC1 increases protein production via two mechanisms: by stimulating ribosomal biogenesis and through direct phosphorylation of translation initiation factors. Activation of mTORC1 enhances transcription of ribosomal RNAs and ribosomal proteins, leading to increased ribosomal assembly (Mayer, Zhao et al. 2004).

Two direct targets of mTORC1 are implicated in regulation of translation initiation. First on is the family of S6 kinases (S6K), which were initially identified as kinases for the ribosomal S6 protein (Fig. 3). However, despite significant effort, it is still unclear how phosphorylation of the indispensable ribosomal protein S6 influences translation (reviewed in (Meyuhas 2008)). S6 kinases also phosphorylate eIF4B, a co-stimulatory protein for the eIF4A RNA helicase (Raught, Peiretti et al. 2004), and the programmed cell death protein 4 (PDCD4), which inhibits the function of eIF4A (Dorrello, Peschiaroli et al. 2006). Thus, S6 kinases enhance eIF4A helicase activity by both stimulating its co-factor eIF4B and dissociating its inhibitor, PDCD4 (Fig. 4a).



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### Figure 3 S6K control of translation

In addition to several translation factors and RNA-binding proteins — including ribosomal protein S6 (RPS6), eukaryotic translation elongation factor 2 kinase (eEF2K), cap-binding protein 80 (CBP80), SKAR and eukaryotic translation initiation factor 4B (eIF4B) — activated 40S ribosomal protein S6 kinase 1 (S6K1) also phosphorylates PDCD4 (programmed cell death 4), a tumour suppressor that binds to eIF4A. This binding is thought to prevent translation by inhibiting the helicase activity of eIF4A, by preventing eIF4A from interacting with eIF4G, or both. S6K1-mediated phosphorylation of PDCD4 results in its ubiquitylation and subsequent degradation through the E3 ubiquitin ligase  $\beta$ -TrCP. 14-3-3 $\delta$  is required for inhibiting eIF4B activity during mitosis. In addition, ribosomal S6 kinase (RSK) and S6K1 converge on eIF4B to regulate its function in translation initiation (adapted from (Ma and Blenis 2009))

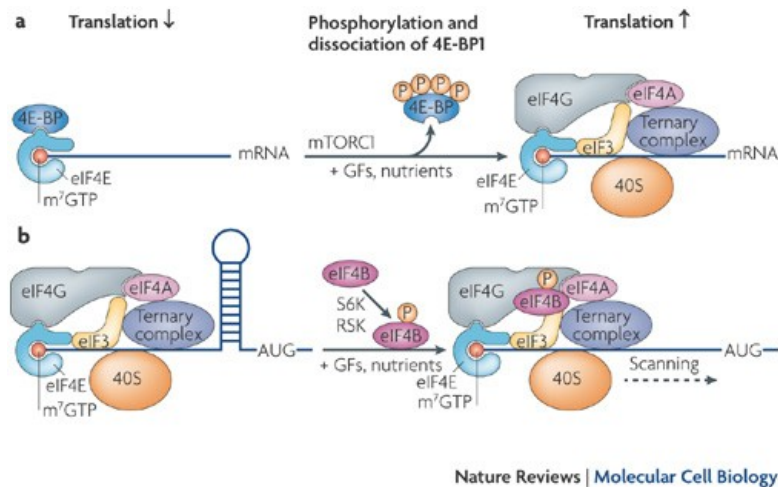
Interestingly, the S6 kinases also stimulate elongation by phosphorylating and inactivating the elongation factor 2 kinase (eEF2K). eEF2 mediates the translocation step of elongation and its inhibitory phosphorylation at Thr56 by eEF2K leads to the dissociation of eEF2 from the ribosome (Fig. 4a) (Wang, Li et al. 2001).

The second direct target of mTORC1 is a class of eIF4E-binding proteins (eIF4E-BP), which in their hypo-phosphorylated state inhibit translation by blocking eIF4E interaction with eIF4G (Fig. 4) (Gingras, Kennedy et al. 1998). Treatment of cells with insulin leads to mTOR-dependent phosphorylation of eIF3 (Harris, Chi et al. 2006) and eIF4G (Raught, 2000), however, it is unclear if mTOR is a direct kinase for these phosphosites.

In Chapter 1.3.3 I will describe in detail translation initiation factors that are downstream targets of mTOR, and how mTOR-directed phosphorylation may affect their function.

### **1.2.2 Ras/MAPK pathway**

Cellular responses to growth factor stimulation and stress conditions are mediated through the activity of MAPK signaling pathways. The MAPK pathways consists of several members, which are named according to their position in the cascade:



**Figure 4 Regulation of cap-dependent translation initiation**

**a.** The recruitment of the 40S ribosomal subunit to the 5' end of mRNA is a crucial and rate-limiting step during cap-dependent translation. A number of translation initiation factors, including the 5' cap-binding protein eukaryotic translation initiation factor 4E (eIF4E), have essential roles in this process. Signal transduction-mediated phosphorylation events regulate the function of eIF4E. For example, hypophosphorylated 4E-binding proteins (4E-BPs) bind tightly to eIF4E, thereby preventing its interaction with eIF4G and thus inhibiting translation. Mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation of 4E-BPs releases the 4E-BP from eIF4E, resulting in the recruitment of eIF4G to the 5' cap, and thereby allowing translation initiation to proceed. **b.** Another well-studied initiation factor that is targeted by signal transduction pathways is eIF4B. Following 40S ribosomal protein S6 kinase (S6K) or ribosomal S6 kinase (RSK)-mediated phosphorylation, eIF4B is recruited to the translation pre-initiation complex and enhances the RNA helicase activity of eIF4A. This is particularly important for translating mRNAs that contain long and structured 5' untranslated region sequences, because the unwinding of these RNA structures is required for efficient 40S ribosomal subunit scanning towards the initiation codon. GF, growth factor. (adapted with permission from (Ma and Blenis 2009))

MAPK kinase kinase (MAPKKK), which phosphorylates MAPK kinase (MAPKK), which in turn activates MAPK. MAPK also have downstream kinases as substrate - MAPK-activated protein kinases (MKs).

Two classes of MAP kinases are known to be important for translation: extracellular signal regulated kinases (ERK1/2), which is activated upon growth factors stimulation, and p38, which responds to stress and pro-inflammatory cytokines. Both of them activate MAPK interacting kinases 1 (Mnk1) and Mnk2, which are uniquely capable of phosphorylating eIF4E (Fukunaga and Hunter 1997; Waskiewicz, Flynn et al. 1997). Mnk1 is implicated in the pro-inflammatory response, since in T cells, it phosphorylates the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) RNA-binding protein, which is required for the translation of tumor necrosis alpha (TNF $\alpha$ ) mRNA (Buxade, Parra et al. 2005). In Chapter 3.2 I report my findings on how interaction of Mnk1 with its substrate eIF4E is controlled by MAPK signal transduction. I describe Mnk kinases function and significance of eIF4E phosphorylation in the introduction to Chapter 3.2.

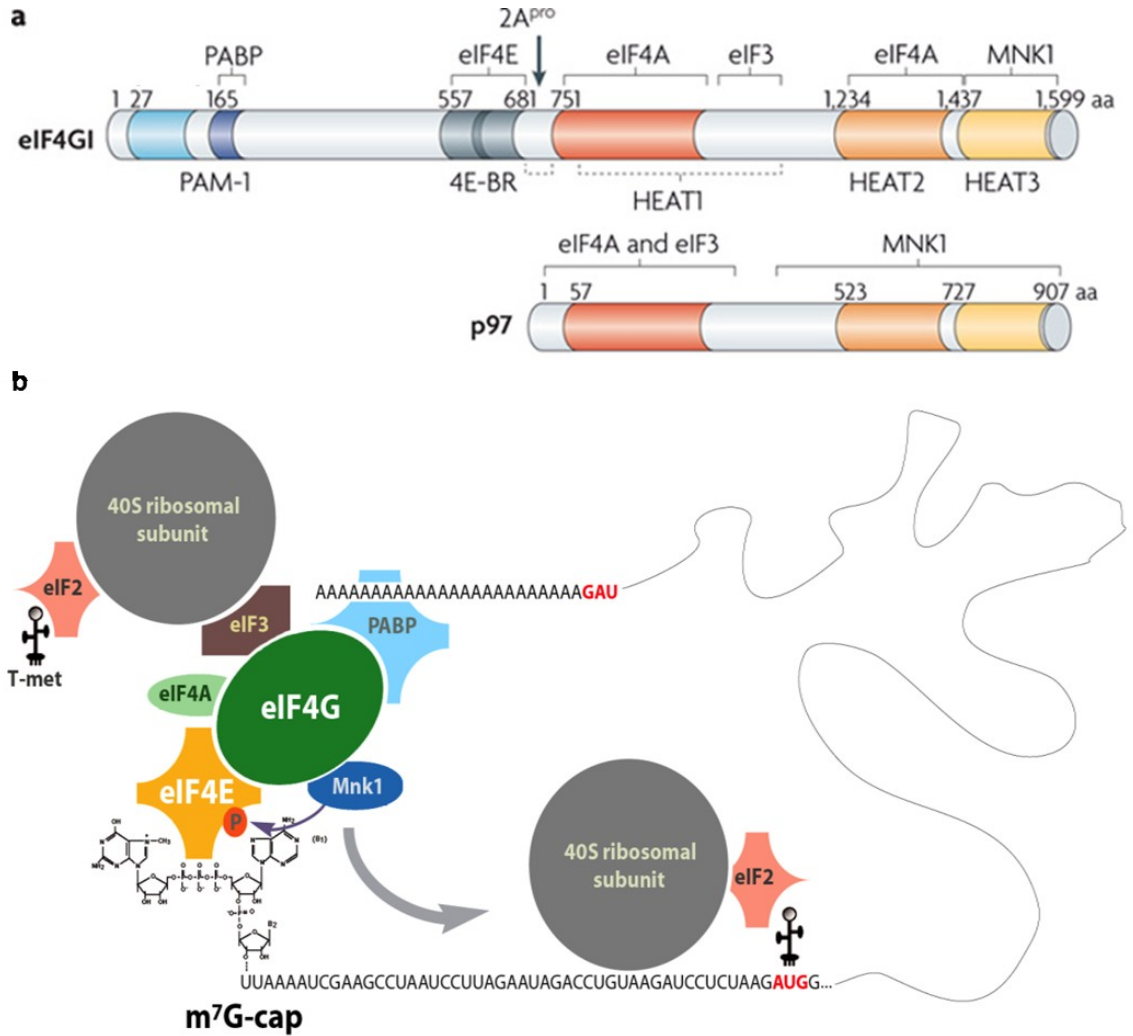
ERK1/2 phosphorylates a class of 90kDa ribosomal S6 kinases (RSK1-4), which have a set of overlapping targets with S6K, such as ribosomal protein S6, eIF4B and

eEF2K. Moreover, RSK can participate in stimulation of the mTORC1 complex through direct phosphorylation of TSC2 (reviewed in (Anjum and Blenis 2008)).

## ***1.3 eIF4G is a central protein for translation initiation***

### **1.3.1 Structure of eIF4G**

Eukaryotic initiation factor 4G is a central scaffolding protein that anchors the small ribosomal subunit to the m<sup>7</sup>-guanosine cap structure on the 5' end of the mRNA. eIF4G is a large protein (1600 aa, predicted molecular weight ~ 176 kDa) and can be divided into three parts: the N-terminal part, which interacts with the polyA-binding protein PABP, the middle part that binds the cap-binding protein eIF4E and RNA helicase eIF4A, and the C-terminal part, which contains regions for binding of the ribosomal interacting complex eIF3, and the Mnk kinases (Fig. 5). The N-terminus of eIF4G is largely unstructured, whereas the structure of the remainder of eIF4G was solved and consists of HEAT-1 domain (Huntington, elongation factor 3, a subunit of protein phosphatase 2A and target of rapamycin domains) in the middle part and HEAT 2 and 3 domains in the C-terminal part (reviewed in (Marintchev and Wagner 2005)). The middle and C-terminal parts are connected by a flexible linker of about 200 amino acids, which contains multiple phosphorylation sites and may be important for the intermolecular interactions in eIF4G (Dobrikov, Dobrikova et al. 2011). eIF4G also plays an important role in the control of initiation in response to signal transduction, since at least three



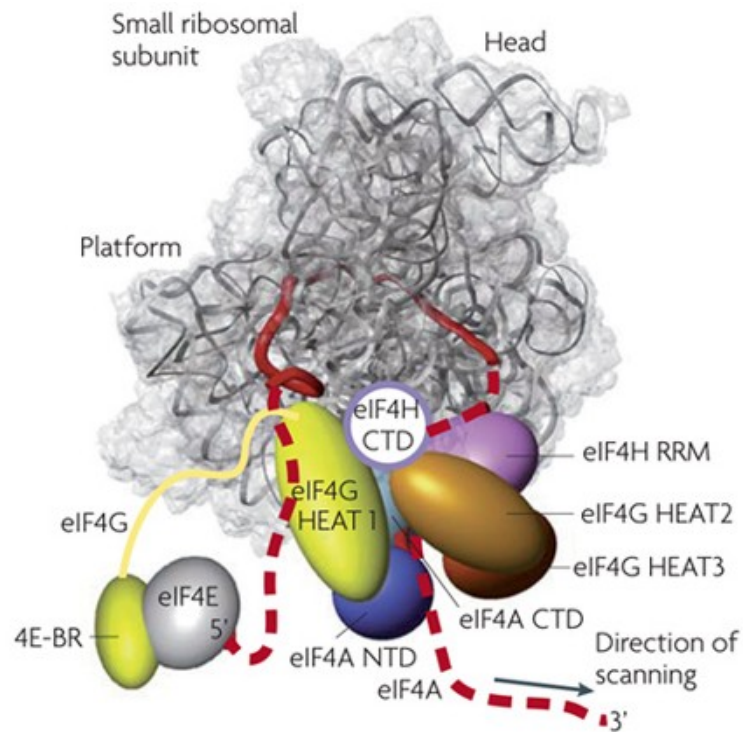
**Figure 5 eIF4GI domain structure and interacting proteins.**

**a.** Schematic representation of the longest isoform of eIF4GI and eIF4G3 (p97), showing binding sites for PABP, eIF4E, eIF4A, eIF3 and MNK1 or MNK2 and for RNA (dotted lines below eIF4GI). The amino acid residues at the amino-termini of the PABP-binding domain (PAM1), eIF4E-binding domain (4E-BR) and HEAT1, HEAT2 and HEAT3 domains are indicated, as is the cleavage site in eIF4GI for the picornavirus proteinase 2A<sup>Pro</sup>. (adapted from (Jackson, Hellen et al. 2010)) **b.** eIF4G recruits 40S ribosomal subunit associated with the ternary complex to the m<sup>7</sup>G cap structure of the mRNA via interaction with eIF4E and eIF3. Interaction of eIF4G with PABP allows mRNA circularization. Mnk1 phosphorylation of eIF4E is indicated.

kinases were shown to interact with eIF4G: Mnk1, Mnk2 and protein kinase C alpha (PKC $\alpha$ ) (Pyronnet, Imataka et al. 1999) (Dobrikov, Dobrikova et al. 2011).

Moreover, eIF4G is capable of interacting with mRNAs, although it does not contain typical RNA binding motives (the position of mRNA relative to eIF4G is indicated in Fig. 6). Two regions in eIF4G are important for RNA binding – one is located upstream of the HEAT-1 domain and another within the HEAT-1 domain (Marcotrigiano, Lomakin et al. 2001). eIF4G binding to mRNA increases the affinity of the eIF4E-eIF4G complex to the cap, thus enhancing translation of cellular mRNAs (Yanagiya, Svitkin et al. 2009). Furthermore, the RNA-binding ability of eIF4G is utilized by picornaviruses, which are uncapped and cannot recruit ribosomes via traditional eIF4E-eIF4G binding. Instead, eIF4G binds directly to the 5' UTR of picornaviral mRNAs, surpassing the requirement for eIF4E. (Lomakin, Hellen et al. 2000). Our group has shown that the C-terminal domain of eIF4G can also interact with the 5'UTR of c-myc mRNA, however, it is unclear how many eukaryotic mRNAs utilize this mode of regulation of translation (Kaiser, Dobrikova et al. 2008).

eIF4G structure is partially conserved within eukaryotes, suggesting that some of its interactions are absolutely required for translation and some have more modulatory



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**Figure 6 eIF4GI position in a scanning 43S complex.**

Hypothetical model of the scanning 43S preinitiation complex, viewed from the solvent face, showing associated factors and domains of factors, including the C-terminal and RRM domains of eIF4H and the N-terminal domain (NTD) and C-terminal domain (CTD) of eIF4A. The direction of scanning (5' to 3') is shown by an arrow. (Adapted from (Jackson, Hellen et al. 2010))

function (Morino, Imataka et al. 2000). For instance, *S. cerevisiae* eIF4G is similar to its mammalian counterpart; however, it lacks the HEAT-2 and HEAT-3 C-terminal fragment, suggesting that these domains may be required for translation during development and differentiation, which are processes specific to metazoans (Marintchev and Wagner 2005).

### **1.3.2 eIF4G isoforms**

The mammalian genome encodes three related eIF4G proteins: eIF4GI, eIF4GII and p97. eIF4GI and II have overlapping functions, since they bind the same set of translation initiation factors (Gradi, Imataka et al. 1998), whereas p97 only shares homology with the C-terminal part of eIF4G, lacking binding regions for PABP and eIF4E (Levy-Strumpf, Deiss et al. 1997). Although eIF4GI and -II share 46% overall identity, they have only 17% conservation in the so-called 'hinge' regions that link structural parts of the protein. Such flexible linkers are usually targeted by post-translational modifications, and indeed both eIF4GI (Raught, Gingras et al. 2000) and -II (Qin, Raught et al. 2003) were found to be extensively phosphorylated. Intriguingly, many of the phosphosites are not conserved between the two proteins, suggesting that eIF4GI and -II are targeted by different signal transduction pathways, and thus may have specialized functions. In Chapter 1.3.1 I will illustrate differential functions of the eIF4G proteins. eIF4GI is the most abundant and the best characterized protein of the eIF4G family (Clarkson, Gilbert

et al. 2010). Therefore, I will mostly discuss this protein (referred to as eIF4G from here on, unless otherwise specified).

Moreover, multiple isoforms of the eIF4G protein have been detected (Coldwell and Morley 2006). Transcription of the eIF4G-encoding transcripts from three promoters, as well as alternative splicing, results in transcripts of various length. Translation of these mRNAs may be initiated from several alternative start codons, giving rise to 5 isoforms of eIF4G. These isoforms vary only in the first 200 aa and bind to the same translation initiation factors, except for the shortest isoform, 197-1600, which does not bind PABP. Hence, it remains unclear whether eIF4G isoforms are functionally redundant or may have specialized functions.

Considering that eIF4G is a scaffolding protein, I will next discuss the main binding partners of eIF4G and their role in translation. Since this thesis is focused on the control of initiation by signal transduction, I will also describe known signaling to initiation factors (eIF4E, eIF4A, eIF3) and speculate on the role of these phosphorylation events for translation. I will discuss the eIF4G-interacting kinases (Mnk1 and PKC $\alpha$ ) in Chapter 3.2, where I present my findings on the control of Mnk1-eIF4G binding.

### **1.3.3 Initiation factors that interact with eIF4G**

#### **1.3.3.1 Cap-binding protein eIF4E**

eIF4E is a 26kDa, evolutionary conserved protein that binds to the 7-methylated guanosine cap structure, which is present at the 5' end of all eukaryotic mRNAs. eIF4E also binds to the middle part of eIF4G and this interaction was shown to enhance eIF4E binding to the cap (Hershey, McWhirter et al. 1999). It follows that eIF4E should facilitate translation of most cellular mRNAs. Indeed, blocking eIF4E function via eIF4E binding proteins (described below) is one of the two major mechanisms to inhibit translation (the second one is phosphorylation of eIF2, which inhibits the delivery of initiator tRNA to the start codon and is utilized to suppress translation during viral infection and the stress response; described in Chapter 1.2.3). Importantly, eIF4E is over-expressed in many cancers and high levels of eIF4E expression in transgenic mice lead to the development of B-cell lymphomas (Ruggero, 2004).

Multiple signaling pathways affect eIF4E binding both to the m<sup>7</sup>G-cap and to eIF4G. For example, the Ras/MAPK pathway stimulates the MAPK-interacting serine/threonine kinase (Mnk1), which in turn binds to eIF4G and phosphorylates eIF4E at Ser209 (Fig. 5b) (Shveygert, Kaiser et al. 2010). It is still unclear how this phosphorylation event may mechanistically affect eIF4E function; however, recent findings have shown that it decreases eIF4E affinity to the cap structure (Slepenkov,

Darzynkiewicz et al. 2006). Although eIF4E phosphorylation is dispensable for normal development and growth (Ueda, Watanabe-Fukunaga et al. 2004), a lack of eIF4E phosphorylation prevents formation of prostate cancer in PTEN<sup>-/-</sup> (phosphatase and tensin homolog) deficient mice, probably because certain anti-apoptotic and pro-growth mRNAs, such as Mcl-2 (induced myeloid leukemia cell differentiation protein 2) and c-myc, depend on eIF4E phosphorylation for their translation (Furic, Rong et al. 2010) (Wendel, Silva et al. 2007).

eIF4E availability for translation is controlled by the eIF4E-BPs, which in their hypophosphorylated form bind eIF4E and preclude its interaction with eIF4G (Fig. 3). Upon serum stimulation, the mTORC1 kinase directly phosphorylates eIF4E-BPs, leading to their dissociation from eIF4E and an increase in overall protein synthesis (Gingras, Kennedy et al. 1998). Thus, eIF4E function is controlled by two major growth signaling pathways, via Ras/MAPK and p13K/Akt/mTORC1, suggesting that eIF4E is a key player in the mitogen-induced stimulation of translation.

### **1.3.3.2 RNA helicase eIF4A**

eIF4A is an ATP-dependent RNA helicase, which unwinds secondary structure of the 5'-untranslated region (UTR) of mRNA, allowing the 'landing' of small ribosomal subunit on mRNA and scanning towards the start codon. Activity of eIF4A is greatly enhanced

by binding to eIF4G, as well as by interaction with two RNA-binding proteins, eIF4B and eIF4H (Marintchev, Edmonds et al. 2009).

The activity of eIF4A can be increased by mitogenic signaling pathways. pI3K/mTORC1 and Ras/MAPK signaling, which trigger S6K1 and RSK-mediated phosphorylation of eIF4B, stabilize its interactions with the translation initiation complex (Shahbazian, Roux et al. 2006). Moreover, S6K1 phosphorylates PDCD4, an inhibitor of eIF4A binding to eIF4G, leading to its proteosomal degradation (Dorrello, Peschiaroli et al. 2006).

### **1.3.3.3 Multi-subunit complex eIF3**

The eIF3 complex is the largest initiation factor and consists of 13 subunits in mammals and participates in almost every stage of translation initiation (reviewed in (Hinnebusch 2006)). In combination with eIF1 and eIF1A (fig 1, step 1), it prevents association of 40S and 60S ribosomal subunits, instead re-priming 40S subunits to associate with the ternary complex, forming the 43S pre-initiation complex (Majumdar, Bandyopadhyay et al. 2003). Next, it serves as an adaptor between the 43S ribosome and the eIF4F complex, assisting in ribosomal recruitment (LeFebvre, Korneeva et al. 2006). Importantly, the interaction between eIF4G and eIF3 is increased upon treatment of cells with insulin, possibly through mTOR-mediated phosphorylation of eIF3 (Harris, Chi et al. 2006).

Finally, eIF3 and eIF4G may be required for the re-initiation of translation on mRNAs that contain multiple upstream open reading frames (uORF), which are short translated regions located 5' upstream of the 'true' start codon (Poyry, Kaminski et al. 2004). Polysomal profiling demonstrated that in many mRNAs, translation initiation at uORFs (Brar, Yassour et al. 2012) can affect the translation of the main coding sequence (Hinnebusch 2005).

#### **1.3.3.4 Poly A-binding protein PABP**

Maturation of most eukaryotic mRNAs results in the addition of a ~250nt polyA-tail on their 3' end (with the rare exception of histone mRNAs, which are unpolyadenylated). The poly-A-tail plays a major role in all stages of mRNA life, including mRNA export, transport, translation and decay. In the nucleus, the poly-A-tail is bound by multiple molecules of nuclear poly-A-binding protein (PAPBN), which is replaced by cytoplasmic PABP (PABPC) upon export of the mRNA to the cytoplasm (reviewed in (Mangus, Evans et al. 2003)).

Relevant to this thesis, PABP was shown to be a canonical translation initiation factor, since its interaction with eIF4G stimulates assembly of the cap-binding complex and formation of a 'closed loop' mRNA template, which may facilitate ribosomal recycling (Kahvejian, Svitkin et al. 2005) (Wells, Hillner et al. 1998). PABP also interacts

with the GW182 protein, which is a component of the miRISC complex (miRNA loaded RNA-induced silencing complex) (Fabian, Mathonnet et al. 2009), though it remains to be determined how PABP contributes to miRNA-mediated repression (Fukaya and Tomari 2011).

The function of PABP is inhibited by polyadenylate-binding protein-interacting protein 2 (Paip2), which binds to PABP and blocks its interaction with both the polyA-tail and eIF4G (Khaleghpour, Kahvejian et al. 2001) (Karim, Svitkin et al. 2006). To date there is no described mechanisms of how PABP-Paip2 interaction are controlled, thus the physiological function of Paip2 remains unclear. Interestingly, Paip2 may be required for spermatogenesis, since mice deficient in Paip2 are male-sterile (Yanagiya, Delbes et al. 2010).

The importance of PABP for gene expression control is highlighted by the fact that many viruses either cleave PABP (picornaviruses) or re-localizing it to the nucleus (herpesviruses) (reviewed in (Smith and Gray 2010)). In Chapter 3.1 I will report how herpes simplex virus 1 infection leads to dissociation of cytoplasmic PABP from the translation initiation machinery and its nuclear re-localization.

#### **1.3.4.4 eIF4G binds translational repressors**

Not long ago, it was discovered that yeast eIF4G binds translational repressors with RGG motives (arginine-glycine-rich), such as Scd6, Np13 and Sbp1 (Rajyaguru, She et al. 2012). These proteins inhibit translation by binding eIF4G and disrupting recruitment of the 43S pre-initiation complex, thus, forming a repressed mRNP. Formation of this translationally repressed complex may be important for the storage and/or transport of mRNAs.

## **1.4 Role of eIF4G in biology and disease**

The canonical model of translation initiation proposes an essential role for eIF4G in translation (Chapter 1.1.4, Fig. 1). Previously, I described the main functions of eIF4G by focusing on its interactions with the mRNA and with other translation initiation factors. In this Chapter I will address the significance of eIF4G by answering several key questions:

- 1 Is eIF4G equally important for the translation of the entire transcriptome, or is it specifically required for the translation of a subset of mRNAs? What happens to translation if eIF4G function is inhibited?
- 2 Can different isoforms of eIF4G modulate translation of different subclasses of mRNAs?
- 3 What role does eIF4G play in tumorigenesis?
- 4 How can the function of eIF4G be modulated by signal transduction?

### **1.4.1 Depletion of eIF4G leads to growth arrest phenotypes**

Several studies tested whether eIF4G function is essential for translation of all cellular mRNAs. For instance, in yeast, depletion of eIF4G led to a 3-fold reduced protein synthesis rate and cell growth arrest (Park, Zhang et al. 2011). In contrast, depletion of

eIF3 led to a more severe impairment of translation, suggesting translation to depend to a higher degree on eIF3 than on eIF4G. Comparison of polysome-associated mRNA pools from control and depleted cells demonstrated that only a small subset of mRNAs is affected by decreased levels of eIF4G. Interestingly, these mRNAs had high translational efficiencies in control cells, and their translational efficiency was reduced to average in depleted cells, indicating that eIF4G is important for high translational levels of a subset of mRNAs.

Similar studies were performed in mammalian cells, where shRNA-mediated knock-down of eIF4GI results in a modest decrease in translation (Ramirez-Valle, Braunstein et al. 2008). However, two problems arise when assessing the results of these studies. First of all, these experiments were performed in immortalized cancer cell lines, which may already have elevated levels of eIF4GI (chapter 1.3.3). In fact, proteomic analyses in HEK293 cells identified eIF4GI among the 300 most abundant proteins (data not shown). Thus, residual levels of eIF4GI may be sufficient to sustain the bulk of translation. Moreover, it is possible that other members of the eIF4G family (eIF4GII and p97) take over eIF4GI function in the knock-down cells. Indeed, triple knock-down of all eIF4G proteins inhibits protein synthesis 60% (Ramirez-Valle, Braunstein et al. 2008). In line with previous findings in yeast, knock-down of a subunit of the eIF3 complex caused significant reductions in protein synthesis levels.

Notably, although overall translation in eIF4GI knock-down cells was not affected, cells displayed a serum-starved phenotype, characterized by cell cycle alterations and increased autophagy. Ramirez-Valle and colleagues have shown that a subset of mRNAs may be especially sensitive to eIF4G levels, many of which encode proteins that are important for cell proliferation (Ramirez-Valle, Braunstein et al. 2008). In other words, eIF4G may act as an important nutrient sensor, supporting the synthesis of certain regulatory proteins, which promote reprogramming of cellular metabolism. However, it is still unclear if these mRNAs require eIF4GI specifically, or whether they are sensitive to the total level of eIF4G proteins. For instance, recent studies in yeast have demonstrated that total eIF4G levels, but not isoform-specific activities contribute to the mRNA specific translation efficiencies (Clarkson, Gilbert et al. 2010).

Finally, knock-down of the third isoform of eIF4G, p97, which lacks the N-terminus, was shown to cause mitotic arrest and apoptosis, arguing that this isoform has a unique role in the regulation of cell division (Marash, Liberman et al. 2008).

In conclusion, several studies demonstrated that eIF4G is not essential for global translation, but instead may be important for translation of a subset of mRNAs. Nonetheless, knock-down of eIF4G has a clear phenotype, since some of the eIF4G-dependent mRNAs may encode proteins that are highly dosage sensitive.

The most interesting question is, of course, what are the cis-factors that determine whether an mRNA is dependent on eIF4G activity. It has been widely assumed that eIF4G is important for unwinding of long structured 5'UTRs of mRNAs, since it recruits the eIF4A helicase (Svitkin, Pause et al. 2001). However, in both yeast and mammalian cells eIF4G-dependent mRNAs possess 5'UTRs of average length, which suggests that eIF4A activity may be more important for 'landing' of the ribosome on mRNAs, rather than scanning of long 5'UTRs. However, in mammalian cells eIF4G-dependent mRNAs had twice as many uORF in their 5'UTR, arguing that eIF4G may be important for re-initiation after the translation of uORFs. This corresponds well with data that shows that eIF4G remains associated with ribosomes after initiation, and, given that the uORF is relatively short, may support re-association of post-terminating 40S ribosomes at a downstream start codon. In fact, polysomal profiling demonstrated that uORFs are present in many mRNAs and may regulate the translation of the main coding sequence (Brar, Yassour et al. 2012).

Of course, one major unanswered question is how mRNAs initiate translation in the absence of eIF4G. In vitro experiments showed that 43S recruitment and recognition of the start codon can happen in the absence of eIF4F, eIF4A, eIF4B, and ATP, requiring only the eIF2·GTP·Met-tRNA<sub>i</sub><sup>Met</sup> ternary complex, eIF3, eIF1, and eIF1A. However, it remains to be determined what factors may substitute for the function of eIF4G in vivo.

### 1.4.2 The role of eIF4G in gametogenesis and in neurons

Studies in model organisms may illuminate isoform-specific roles of eIF4G. For instance, in *Drosophila* eIF4GII was shown to be required for male germ cells to undergo meiotic division and proper spermatid differentiation (Baker and Fuller 2007). Furthermore, mutations in the HEAT-3 domain of eIF4GII results in failure of spermatocytes to exit meiotic prophase and male-limited infertility in mice (Sun, Palmer et al. 2010). Thus, it is plausible that eIF4GII may have specific roles in spermatogenesis.

Moreover, both eIF4GI and -II were recently implicated in neuronal translation. Genetics studies of autosomal-dominant parkinsonism revealed missense mutations in the eIF4GI gene as a possible cause for the disease. These mutations disrupt eIF4E and eIF3 binding, respectively, and render mutant cells more susceptible to oxidative stress (Chartier-Harlin, Dachsel et al. 2011). On the other hand, Ca<sup>2+</sup>/calmodulin-dependent kinase (CAMKII)-mediated phosphorylation of eIF4GII was shown to upregulate cap-dependent translation in neurons (Srivastava, Fortin et al. 2012). Translation regulation is known to be important for neuronal plasticity for almost 40 years, and these new data supports the notion that neurons are especially sensitive to alterations in translation initiation factor function.

### **1.4.3 Over-expression of eIF4G promotes tumorigenesis**

Many cancers are characterized by high levels of protein synthesis, which is uncoupled from inherent control mechanisms. Different cancers may employ both common and unique ways of increasing translation, which often vary even within a cancer type. Increased ribosome biosynthesis, over-expressed translation initiation factors and constitutively active mitogenic signaling pathways that control initiation - all of these factors contribute to tumorigenesis (reviewed in (Silvera, Formenti et al. 2010)).

Cancer cells have to proliferate under conditions that normally down-regulate translation, such as hypoxia, DNA damage, apoptosis, etc. In order to keep proliferating, tumor cells need to modify the translation apparatus to achieve two goals: (i) constitutively activate translation and overcome imposed translational blocks; and (ii) increase the translation of antiapoptotic, pro-growth and pro-angiogenic mRNAs. For instance, in local advanced breast cancer (LABC) cells eIF4G and eIF4E-BP are greatly over-expressed. Moreover, tumor cells depend on high levels of eIF4G and eIF4E-BP to cope with hypoxic stress, mainly because over-expression of these factors benefits the selective translation of pro-angiogenic factors VEGF-A and hypoxia inducible factor 1 $\alpha$  (HIF1- $\alpha$ ) (Braunstein, Karpisheva et al. 2007).

Furthermore, eIF4G function is critical for the pathogenesis of inflammatory breast cancer (IBC) (Silvera, Arju et al. 2009). IBC is the most lethal form of primary breast cancer, characterized by the formation of metastatic tumor emboli. Formation of the tumor emboli requires elevated levels of E-cadherin, which is usually considered a tumor suppressor gene. High levels of eIF4G stimulate translation of p120 beta-catenin mRNA, which stabilizes E-cadherin and promotes tumor cells interactions. Knock-down of eIF4G in IBC cells prevented the assembly of tumor emboli in the Matrigel assay without affecting cell viability or proliferation rates.

Taken together, these data indicate importance of eIF4G for the tumorigenesis of certain cancers, probably because eIF4G supports the translation of certain mRNAs that are required for tumor survival. Below, I describe how eIF4G activity can be regulated by signal transduction. It is of great interest to investigate if aberrant activation of mitogenic signaling pathways can promote tumorigenesis via phosphorylation of eIF4G. Indeed, LABCs are capable of both over-expressing and dephosphorylating eIF4E-BP, suggesting that a similar regulation mechanism may exist for eIF4G.

#### **1.4.4 Signal transduction to eIF4G**

The main role of eIF4G is to bring multiple translation initiation factors into the vicinity of the 5' end of mRNAs. Thus, it is tempting to assume that signaling pathways may

control the association of translation initiation factors with the mRNA through phosphorylation of eIF4G. Indeed, more than a decade ago, eIF4G was shown to contain phosphorylation sites that are serum-responsive and may be sensitive to rapamycin, an inhibitor of mTORC1 (Raught, Gingras et al. 2000). However, a truncation mutant of eIF4G was constitutively phosphorylated and insensitive to rapamycin, suggesting mTOR is not a direct kinase for these phosphosites. Three serum-responsive phosphosites were mapped (S1148, S1188 and S1232); however the functional outcome of these phosphorylation events remained elusive. Global phosphoproteomic studies have identified eIF4G as a target for signal transduction (Hsu, Kang et al. 2011), however, these results should be treated with caution, since no small-scale targeted validation of the phosphosites was provided.

Most of the phosphosites are located in the two unstructured regions on eIF4G, one in the N-terminus and another in the C-terminal flexible linker, which is located between HEAT-1 and HEAT-2 domains. The structure of the linker has not been solved, but by analogy with the structurally related cap binding protein 80 (CBP80) it was suggested that the linker may play a role in eIF4G intermolecular interactions, ultimately affecting eIF4G interaction with RNA or other proteins involved in translation (Marintchev and Wagner 2005).

Interestingly, the phosphosites in eIF4GI are not conserved in the two other eIF4G isoforms, indicating that post-translational modifications of eIF4G may be required for isoform specific functions. For example, eIF4GII was shown to be phosphorylated by CAMKII on sites that are not conserved in eIF4GI (Qin, Raught et al. 2003) and this phosphorylation was shown to be required for cap-dependent translation in neurons (Srivastava, Fortin et al. 2012).

Only two kinases were identified so far for eIF4GI. Our group has recently shown that PKC $\alpha$  can phosphorylate eIF4G1 at Ser1186, leading to increased interaction between eIF4G1 and Mnk1, the eIF4E kinase (Dobrikov, Dobrikova et al. 2011). Furthermore, the splicing related protein kinase (SRPK) family of kinases was recently proposed to phosphorylate eIF4G1 at Ser1148, and Mnk2 was shown to negatively control this phosphorylation through a yet to be described mechanism (Hu, Katz et al. 2012). Although eIF4G1 is integrated in various signaling pathways, the role for the majority of eIF4G1 phosphosites remains to be elucidated.

## ***1.5 Translation in mitosis***

Development and growth of an organism depends on correct cell division. Mitosis is a short stage in the somatic cell cycle, which consists of a series of well-orchestrated events that result in the correct segregation of chromosomal DNA, cytosolic content and organelles into the two daughter cells. The cellular structure is rearranged in mitosis: the nuclear envelope disassembles, chromosomes condense, microtubules assemble mitotic spindle, etc. These processes require a lot of energy, thus, other energy demanding anabolic processes, such as transcription and translation, are ceased. All cellular efforts are directed to correctly segregate the genetic material. Any mistakes that are made at this stage can potentially lead to aneuploidy, resulting in cell elimination, or in rare cases, leading to tumor formation. Thus, it is important to understand what molecular mechanisms coordinate progression of events in mitosis. Interestingly, translation is emerging as an important stage of mitotic regulation. Although global protein synthesis is suppressed, some mRNAs are still being translated, possibly ensuring continuous supply of critical mitotic regulators.

### **1.5.1 Global down-regulation of translation in mitosis**

Suppression of translation in mitosis has been studied for almost 40 years. Initial measurements of translation used metabolic labeling, which results in the incorporation of <sup>35</sup>S-radioactive molecules in newly synthesized proteins. This method depends cumulatively on both translation efficiencies and amount of mRNAs, which can be affected by changes in transcription and mRNA stability. However, cells treated with actinomycin D were able to resume protein synthesis upon mitotic exit, indicating that mRNA is not a limiting factor for translation in mitosis (Steward, Shaeffer et al. 1968). Furthermore, ribosomes per se are not inhibited in mitosis and several mRNAs were shown to be actively translated specifically during this stage (reviewed in (Le Breton, Cormier et al. 2005)). Thus, it has been assumed that in mitosis, translation is blocked at the stage of ribosomal recruitment to mRNAs, e.g. in the phase of translation initiation. (Fan and Penman 1970).

Eukaryotic translation initiation can be globally regulated via two separate mechanisms: (i) interaction of the cap-binding protein eIF4E with eIF4G is blocked by the eIF4E-BPs, which in turn are inactivated by direct phosphorylation by mTORC1 (chapter 1.2.1 and 1.3.3.1); (ii) stress-induced phosphorylation of eIF2 $\alpha$  prevents the formation of initiator tRNA-loaded ribosomes (chapter 1.5.3).

Surprisingly, the mTORC1 complex was recently shown to be active during mitosis (Ramirez-Valle, Badura et al. 2010), (Gwinn, Asara et al. 2010). Two groups have reported that the cyclin-dependent kinase 1 (CDK1)/cyclin B1 complex mediates phosphorylation of Raptor, up-regulating mTORC1 activity and leading to hyperphosphorylation of eIF4E-BPs. Another study indicated that CDK1/cyclin B1 may directly phosphorylate eIF4E-BP in mitosis, also rendering them inactive (Heesom, Gampel et al. 2001). Moreover, CDK1 also phosphorylates and inactivates the eEF2 kinase, which is a known inhibitor of elongation (see chapter 1.2.1) (Smith and Proud 2008). Yet, another group recorded the opposite effect of CDK1 on elongation. Sivan et al. argue that an elongation block in mitosis may be achieved through CDK1-mediated phosphorylation of the catalytic delta subunit of eEF1, which may potentially hinder tRNA delivery to elongating ribosomes (Sivan, Kedersha et al. 2007). (Sivan, Aviner et al. 2011).

Despite its apparent importance for regulation of translation initiation, phosphorylation of eIF2 $\alpha$  in mitosis was not properly investigated. Two groups proposed increased phosphorylation of eIF2 $\alpha$  in mitosis, but the evidence is scarce and unconvincing (Tinton, Schepens et al. 2005) (Sivan, Kedersha et al. 2007). Moreover, although signaling to eIF2 $\alpha$  is well defined, the status of these pathways was not investigated in mitosis.

Finally, it was suggested that 14-3-3 $\sigma$  binding to eIF4B, eIF2 $\alpha$  and eEF1 in mitosis may be responsible for suppression of translation, since cells lacking 14-3-3 $\sigma$  fail to down regulate cap-dependent translation (Wilker, van Vugt et al. 2007). However, no mechanistic evidence was provided in this relatively short study.

In conclusion, although multiple attempts were made to address the molecular mechanisms behind suppression of translation in mitosis, the results are still far from satisfactory. It is plausible that different cell lines, cell cycle synchronization techniques, etc may lead to discrepancies in the literature. Likewise, many studies were performed with HeLa cells, which are characterized by aneuploidy and multinucleation, and thus cannot faithfully recapitulate proper progression of events in mitosis. Nevertheless, it is becoming increasingly clear that both translation initiation and elongation factors are extensively modified in mitosis and that the CDK1 kinase is a key player in this process.

### **1.5.2 Selective upregulation of certain mRNAs in mitosis**

Findings that mTORC1 is active in mitosis seem counter-intuitive; however, it is possible that mTORC1 activation is required for selectively increased translation of mRNAs that encode critical mitotic regulators. Indeed, several mRNAs were shown to be actively translated in mitosis, such as ornithine decarboxylase (ODC), c-myc and PITSLRE CDK11/p58, etc (Pyronnet, Pradayrol et al. 2000). Serum stimulation of cells led to

mTORC1-dependent selective upregulation of translation of several mRNAs (for instance, c-myc), which are poorly translated in unstimulated conditions. mTORC1 increases the availability of the eIF4F complex, as well as the activity of the eIF4A RNA helicase, which may be important for the recruitment of the ribosome to mRNA (reviewed in (De Benedetti and Graff 2004)). However, it remains to be determined if mTORC1 can similarly stimulate translation of mitotic mRNAs.

The mechanism for translation upregulation of the mRNA encoding the CDK11 kinase has been well documented. This mRNA contains two in-frame AUG start codons: the first initiates protein synthesis of a larger, 110 kDa isoform and the second is used for the synthesis of a shorter, 58 kDa isoform. Interestingly, the second start codon is used only in mitosis and relies on an unconventional, cap-independent mechanism of translation initiation, sometimes called an internal ribosomal entry site (IRES) (Cornelis, Bruynooghe et al. 2000). It was shown that the RNA-binding protein UNR, which itself is synthesized at higher rates during mitosis, binds to the CDK11 mRNA and via an undetermined mechanism, directs small ribosomal subunits to the second start codon (Schepens, Tinton et al. 2007). Moreover, mitotic expression of the p58 isoform is required for centrosome maturation and bipolar spindle morphogenesis (Petretti, Savoian et al. 2006).

Whereas translational regulation of p58/CDK11 has been extensively studied, it remains to be determined what fraction of mRNAs are actively translated in mitosis and what are the mechanisms governing this selective upregulation of translation. In Chapter 3.3 I present our findings on CDK1/cyclin B1-mediated phosphorylation of eIF4G in mitosis and speculate how this phosphorylation may contribute to mitotic translation.

## ***1.6 Regulation of translation during viral infection***

Viruses are obligate parasites of cells and depend on host biochemical machinery to proliferate. They vary in size and complexity from small RNA viruses, which may encode only a dozen proteins, to large DNA viruses with ~200 kb genomes. Viruses also differ in their survival strategies: some go through rounds of rapid lytic infection, often killing the host, while others can establish lifelong latent infection, accompanied by occasional re-activation and shedding of the viral particles. Depending on the size and type of their genome, viruses either encode their own enzymes or hi-jack cellular enzymes to replicate their genome. For instance, large DNA viruses, such as poxviruses, supply their own RNA and DNA polymerases and most of the RNA viruses must encode their own RNA-dependent RNA polymerases, since this enzymatic activity is not present in cells. Importantly, viruses are especially dependent on the translational apparatus of the host cell, since even the largest viruses cannot afford to encode and assemble their own ribosomes/translation apparatus.

All viruses must accomplish a balance between two major goals: maximal viral production and evasion of host immune responses. Below I will discuss how HSV-1 manipulates host translation to achieve these goals. I focus on HSV-1, the subject of my

research, since it has developed multiple strategies to adjust protein synthesis machinery to suit its needs.

Viruses have historically been beneficial for the discovery of many critical translation elements, such as the poly-A-tail, the 7-methyl guanosine cap and initiator tRNAs (reviewed in (Mathews, Sonenberg et al. 2007)). Since viruses have evolved under strong selection, they have adapted highly efficient mechanisms to control protein synthesis machinery. Thus, studying viral interactions with translation machinery can provide insight on the normal cellular regulation of translation.

### **1.6.1 Herpes simplex virus 1**

HSV-1 is an enveloped double-stranded DNA virus that has both lytic and latent stage of infection. HSV-1 initially infects epithelial cells, which results in cell lysis, and can also establish lifelong latent infection in sensory neurons.

HSV-1 is very efficient in blocking both innate and adaptive immune responses, which allows it to successfully reactivate from latency and initiate secondary infection of epithelial cells. Multiple lines of evidence suggest that the ability of HSV-1 to evade immune responses depends on the suppression of host gene expression very early in infection (reviewed in (Smiley 2004)).

During HSV-1 infection, viral nucleic acids and lipoproteins are recognized by pattern recognition receptors (PRRs), leading to expression of type I interferon (IFN-I). Early in infection, HSV-1 shuts off host protein synthesis, thus blocking production of IFN-I and escaping innate immune responses. Host protein synthesis shut-off also down-regulates expression of major histocompatibility complex I (MHCI) components, thus preventing recognition of infected cells by cytotoxic T lymphocytes (Smiley 2004).

HSV-1 has a large genome (~150 kb), and encodes about 80 proteins, as well as miRNAs that promote the establishment and maintenance of viral latency (Umbach, Kramer et al. 2008). Although the role for many viral proteins has not been clearly defined, it is assumed that these factors contribute to the fitness of the virus, since they were kept under strong selective pressure.

The viral gene expression program can be divided into several sequential stages, which result in the expression of viral immediate-early (IE), early (E) and late (L) genes. IE gene products are required for the induction of viral transcription and suppression of host protein synthesis, E proteins facilitate the replication of the viral genome and L gene products mediate capsid assembly, maturation and viral egress (Paladino and Mossman 2009). Interestingly, several viral proteins are pre-packaged into the virion (so called tegument proteins) and can act immediately after virus entry into host cells. One

such protein, viral protein 16 (Vp16), stimulates viral transcription and another, virion host shut-off protein (vhs) is an RNase that degrades cellular mRNAs, leading to rapid decrease of the mRNA content in the infected cell (Smiley 2004).

HSV-1 affects every level of host gene expression. Initially, viral tegument (vhs) and immediate-early (infected cell protein 27, ICP27) proteins lead to the shut-off of host protein synthesis to prevent development of immune responses. Later, infected cells attempt to block mass production of viral proteins via global down-regulation of translation. In return, HSV-1 aims to foster translation of viral mRNAs. Below I will briefly describe this complex interplay between the virus and the host cell that centers on translation.

### **1.6.2 HSV-1 infection induces host protein synthesis shut-off**

HSV-1 inhibits host protein synthesis at multiple levels, including mRNA degradation, suppression of transcription and splicing. After viral entry into cells, virions releases the vhs protein, which has endonuclease activity and preferentially cleaves mRNAs, but cannot distinguish between viral and cellular mRNAs (Taddeo and Roizman 2006) (Kwong and Frenkel 1987). Vhs is critical for suppressing the interferon-mediated antiviral response and a Vhs-deletion mutant is severely impaired in replication in mouse models (Leib, Harrison et al. 1999). It has been suggested that interaction of vhs

with translation initiation factors stimulates endonuclease activity and targets vhs to mRNAs, but not other RNA species (Page and Read 2010). Why vhs does not distinguish between cellular and viral mRNAs remains enigmatic. However, it has been proposed that vhs-mediated degradation of viral mRNAs may help sharpen the transitions between different expression programs, allowing tight correlation between transcription levels and protein synthesis output (Kwong and Frenkel 1987).

HSV-1 also inhibits cellular transcription and splicing. Products of immediate-early genes, such as ICP27, re-direct RNA II polymerase to the viral transcription compartment, sequestering it away from cellular promoters (Jenkins et al., 2001 JV). ICP27 also affects spliceosome assembly, thus suppressing host mRNA splicing (Sciabica, Dai et al. 2003). Most of the viral genes do not contain introns (with the exception of immediate-early genes), thus, expression of viral genes is not affected by inhibition of splicing. ICP27-deficient virus is replication incompetent; hence inhibition of cellular transcription and splicing is essential to the virus ((McCarthy, McMahan et al. 1989).

### **1.6.3 Host defense response and viral countermeasures**

Virus infection results in major rearrangements of cellular transcription and translation, which, in turn, triggers apoptosis to prevent virus replication. Cellular recognition of

extraneous viral proteins and nucleic acid rapidly initiates IFN production, stimulating IFN-stimulated genes (ISGs) and signaling to other cells the presence of the virus. Inhibition of translation is one of the key features of both apoptosis and the IFN response (review in Walsh D Mohr 2011).

Down-regulation of translation is achieved through phosphorylation of eIF2 $\alpha$ , which severely impairs translation initiation. eIF2 is a GTPase which, in its GTP-bound conformation, interacts with initiator methionyl-tRNA (Met-tRNA<sub>i</sub><sup>Met</sup>) to deliver this essential component of translation initiation to 40S ribosomal subunit. After the recognition of the start codon by the initiation complex, eIF2 hydrolyzes GTP and dissociates from initiator tRNA, followed by formation of the 80S ribosome. Phosphorylation of the eIF2 $\alpha$  subunit blocks the activity of eIF2B, which is a GEF for eIF2, thus leading to accumulation of GDP-bound eIF2, which is incapable of binding Met-tRNA<sub>i</sub><sup>Met</sup> (Dever et al., 2007 CSHL).

Phosphorylation of eIF2 $\alpha$  is an evolutionary conserved mechanism of translation regulation, since eIF2 $\alpha$  kinases are present in all eukaryotes. Two eIF2 $\alpha$  kinases are pertinent to viral infection. The first one, protein kinase R (PKR), is conserved among vertebrates and is activated upon binding to double-stranded (ds) RNA regions. Under normal conditions somatic cells of vertebrates lack dsRNA, however, these elements are

abundant upon viral infection. All RNA virus genomes contain dsRNA regions and most DNA viruses have packed genomes, thus encode over-lapping ORFs on opposite strands, which may result in the temporary formation of double-stranded RNA. Thus, recognition of dsRNA regions, plays a major role in the anti-viral response (Gale and Katze 1998). The second eIF2 $\alpha$  kinase, PKR-like endoplasmic reticulum kinase (PERK), is activated upon endoplasmic reticulum (ER) stress, which is a host defense response to infection. Recognition of HSV-1 by toll-like receptors leads to rapid production of reactive oxygen species, which are targeted to clear the pathogen (Schachtele, Hu et al. 2010). Oxidative stress interferes with the formation of disulfide bonds in the ER compartment and leads to accumulation of unfolded protein, causing ER stress. Stress-induced activation of PERK down-regulates translation and causes the induction of chaperone-encoding genes (Lu, Jousse et al. 2004)). Enveloped viruses use the ER for synthesis of their membrane associated proteins, thus they have developed mechanisms to resolve ER stress.

The importance of eIF2 $\alpha$  phosphorylation as a host defense mechanism is emphasized by a variety of mechanisms that unrelated viruses have evolved to block this phosphorylation. For instance, the HSV-1 protein Us1 binds PKR and prevents PKR activation by dsRNA. Moreover, the viral protein  $\gamma_1$ 34.5 binds the PP1 $\alpha$  cellular phosphatase and redirects it to dephosphorylate eIF2 $\alpha$ . Hepatitis C virus (HCV) (ssRNA



virus) and vaccinia virus (dsDNA virus) both encode a pseudo substrate for PKR, whereas Epstein Barr virus (dsDNA virus) encodes Epstein Barr encoded RNAs (EBER) that bind PKR and prevent its activation. Influenza virus (ssRNA virus) is uniquely capable of activating a cellular PKR inhibitor (reviewed in (Walsh and Mohr 2011))

These examples demonstrate that maintaining translation initiation at its normal levels (or even stimulating it) is essential for viral replication. In the introduction to Chapter 3 I will describe specific tactics that HSV-1 employs to stimulate translation and report our findings on how HSV-1 infection affects the function of the cytoplasmic polyA-binding protein 1 (PABPC), an important translation initiation factor.

## **2. Materials and Methods**

### ***2.1 Cell culture and stable cell lines***

HSV-1 (Kos) was grown and titrated in Vero cells.

HeLa, HEK293, Vero cells and immortalized fibroblasts from Mnk1/Mnk2 double-knockout (DKO) or wild-type (WT) mice were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids (all from Invitrogen Carlsbad, CA). HEK 293 stable, tetracycline (Tet)-inducible cell lines were established using the Flp-In T-Rex system (Invitrogen) according to the manufacturer's instructions and grown in DMEM supplemented with 10% FBS, nonessential amino acids, hygromycin B (100 $\mu$ g/ml; Mediatech), and Blasticidin S HCl (15 $\mu$ g/ml; Invitrogen). HEK 293 stable, (Tet)-inducible cell lines expressing myc-eIF4G1-flag with stable, tet-inducible knock-down of endogenous eIF4G1 were established as followed. First, HEK293 Flpn T-Rex cells (Invitrogen) were transfected with pcDNA3.1 TO/miR-4G and selected with G418 (Gibco). Stable clones were selected and analyzed for the efficiency of eIF4G1 knock-down. To generate eIF4G1 rescue cell line, stable clone that has demonstrated maximum knock-down was transfected with wt and S1232A pcDNA/FRT/TO myc-eIF4G1-flag and pcDNA5/FRT/TO plasmids according to the manufacturer's instructions (Flp-In T-Rex system, Invitrogen).

## **2.2 Expression constructs**

### **2.2.1 Cloning for Chapter 3.1**

Annealed complementary oligonucleotides were used to insert a Flag tag into NheI-HindIII sites of pcDNA3.1 (Invitrogen) (pair 21/22, Table I). The ICP27 open reading frame (ORF) was PCR amplified from pM27, kindly provided by S. Rice (University of Minnesota, Minneapolis, MN) with the primer pair 23/24 or the pair 24/25 for cloning Flag-tagged or untagged forms into BamHI/NotI sites of Flag-pcDNA3.1 or HindIII-NotI sites of original pcDNA3.1 vector, respectively.

The UL47 ORF generated by PCR from HSV-1 genomic DNA with the primer pair 26/27 was inserted downstream of the Flag epitope using BamHI/XhoI sites. To generate the GST-PABP bacterial expression plasmid, the PABP ORF was PCR amplified from pTYB2-PABP (Bradrick, Dobrikova et al. 2007) using the primer pair 28/29 and inserted into BamHI-NotI sites of pGEX-4T-1 (GE Healthcare).

### **2.2.2 Cloning for Chapter 3.2**

pcDNA5/FRT/TO myc-eIF4GI-flag was generated by modifying pcDNA5/FRT/TO myc-eIF4GI-b (Kaiser, Dobrikova et al. 2008) with a C-terminal Flag tag. Briefly, a C-terminal eIF4G fragment fused to Flag protein was generated by PCR using primers 1 and 2 (Table 1), digested with NheI-NotI, and inserted into the pcDNA5/FRT/TO myc-eIF4GI-

b backbone. pcDNA5/FRT/TO-HA was created by inserting a hemagglutinin (HA) epitope, generated with complementary oligonucleotides 3 and 4, into Acc65I-BamHI-digested pcDNA5 FRT/TO. pcDNA5/FRT/TO-HA-Mnk1a, -Mnk1b, and -Mnk2a expression constructs were generated as follows. Fragments encoding Mnk1a, Mnk1b, and Mnk2a were reverse transcription-PCR (RT-PCR) amplified from HEK-293 total RNA by using primer pairs 5/6 (Mnk1a and Mnk1b) and 7/8 for Mnk2a. The PCR fragments were digested with BamHI-NotI and inserted into the pcDNA5/FRT/TO-HA backbone. Mnk1a mutants with T2A2, D191A, and A362P mutations and a deletion of the C-terminal 24 amino acids containing the MAPK binding site ( $\Delta 24$ ) were generated by overlapping PCR using primer pair sets 5/10 and 6/9, 5/14 and 6/13, 5/18 and 6/17, and 5/12 and 6/11, respectively. The overlapping fragments were fused in the second PCR with primer pair 5/6 and inserted into pcDNA5/FRT/TO-HA. To generate the HA-Mnk1a $\Delta 12$  variant, which lacks the first 12 codons of Mnk1a, a Mnk1a N-terminal fragment was PCR amplified from full-length HA-Mnk1a by using primer pair 15/16, digested with BamHI-Bsu36I, and inserted into pcDNA5/FRT/TO-HA-Mnk1a. To create HAMnk1a with eIF4G deleted ( $\Delta 4G$ ), the mutant pcDNA5/FRT/TO-HA-Mnk1a backbone was digested with BamHI-BsrGI, gel purified, filled in with Klenow fragment, and religated. pcDNA3.1 HA-Mnk1a, HA-Mnk1b, HA-Mnk2a, and HA-Mnk1a mutants were generated by digesting the corresponding pcDNA5/FRT/TO expression vectors with Acc65I-NotI and ligating with the pcDNA3.1 backbone. pcDNA3.1 myc-eIF4E has

been described previously (Bradrick and Gromeier 2009). To create pGEX4T-1 glutathione S-transferase (GST)-eIF4E, the eIF4E coding region was PCR amplified from pcDNA3.1 myc-eIF4E by using primers 17 and 18, digested with BamHI-NotI and inserted into the pGEX4T-1 backbone.

### **2.2.3 Cloning for Chapter 3.3**

pcDNA5/FRT/TO myc-eIF4GI-flag constructs expressing either wt or S1232A phosphomutant were described previously (Dobrikov, Dobrikova et al. 2011). General principles of creating an expression plasmid encoding artificial miRNA are described in detail in Zeng et al., 2005, *Methods in Enzymology*. pcDNA 3.1/TO miR-4G plasmid, expressing artificial miRNA targeting the 3'UTR region (5183-5205) of eIF4G1 transcript (GenBank/EMBL/DDBJ accession no. AY082886), was generated as follows. pCMV-miR-30 (Zeng, Cai et al. 2005) plasmid was generously provided by B. Cullen (Duke University, Durham, NC USA). miR-30 cassette fragment was PCR-amplified from pCMV-miR-30 plasmid using the following primers (31/32). PCR fragment was then digested with NotI-ApaI and inserted in the NotI-ApaI-digested pcDNA5/FRT/TO backbone, creating pcDNA5/FRT/TO miR-30 expression plasmid. This plasmid was digested with MluI-ApaI and the fragment containing pCMV promoter, tetracycline operator (TO) and miR-30 cassette was inserted into the MluI-ApaI digested pcDNA3.1 backbone, generating pcDNA 3.1/TO miR-30. To create pcDNA 3.1/TO miR-4G plasmid

two pairs of oligonucleotides (33/34) were annealed to each other, PCR extended, digested with XhoI and inserted into the XhoI-digested pcDNA 3.1/TO miR30.

Reporter plasmids encoding Renilla luciferase ORF under control of VEGF and c-myc IRES and Firefly luciferase under control of b-globin 5'UTR were previously described (Kaiser, Dobrikova et al. 2008) (Goetz, Everson et al. 2010).

**Table 1 List of primers used**

No.	Name	Sequence
1	5'flag-tag	5'-cgg agc ttc agc aag gaa gtg g-3'
2	3'flag-tag	5'-gtg cgg ccg ctc act tat cgt cgt cat cct tgt aat cgt tgt ggt cag act cct cct ctg c-3'
3	5'HA-tag	5' -gt acc atg tac cca tac gac gtc cca gac tac gct g-3'
4	3'HA-tag	5' -ga tcc agc gta gtc tgg gac gtc gta tgg gta cat g-3'
5	5'BamHI-Mnk1a	5' -gc ggatcc gta tct tct caa aag ttg gaa aaa cct ata gag atg ggc-3'
6	3'NotI-Mnk1a	5' -gag cgg ccg ctc aga gtc ctg tgg gcg ggc tcc t-3'
7	5'BamHI-Mnk2a	5' -gc ggatcc gtg cag aag aaa cca gcc gaa ctt cag-3'
8	3'NotI-Mnk2a	5' -ga gcgccgc tca ggc gtg gtc tcc cac cag gac-3'
9	5'hT2A2	5' -ata acc gca cca gag ctg acc gcc cca tgt-3'
10	3'hT2A2	5' -aca tgg ggc ggt cag ctc tgg tgc ggt tat-3'
11	5'Mnk1Δ 24	5' -ctc cct gca agt gac gcc tgg ccc-3'
12	3'Mnk1Δ 24	5' -ggg cca ggc gtc act tgc agg gag-3'
13	5'D191A	5' -cag tga aaa tct gtg cct ttg act tgg gca-3'
14	3'D191A	5' -tgc cca agt caa agg cac aga ttt tca ctg-3'
15	5'BamHI-Mnk1-Δ12	5' -gc ggatcc ggc agt agc gaa ccc ctt-3'
16	3'Bsu36I-Mnk1-Δ12	5' -gc cctcagg ggc cat gta ttc tgc aga-3'
17	5'A362P	5'-acg ctc ttc gca cct gag gcc atc-3'
18	3'A362P	5'-gat ggc ctc agg tgc gaa gag cgt-3'
19	5'BamHI-eIF4E	5' -gc ggatcc cgc act gtc gaa ccg g-3'
20	3'NotI-eIF4E	5'-ga gcgccgc tta aac aac aaa cct att ttt agt ggt gg-3'
21	5'NheI-flag	5'-ctagcaccatggattacaaggatgacgacgataaga-3'
22	3'HindIII-flag	5'-agctccttatcgtcgtcctccttgaatccatgggtg-3'
23	5'BamHI-ICP27	5'-ttggatccgcgactgacattgatatgct-3'
24	3'NotI-ICP27	5'-aagcggccgctaaaacagggagttgca-3'
25	5'HindIII-IPC27	5'-ggaagcttaccatggcgactgacattgatatgctaattg-3'
26	5'BamHI-UL47	5'-aaggatcctcggtcgcgaacccgc-3'
27	3'XhoI-UL47	5'-ttctcagcttatggcggtggcg-3'
28	5'BamHI-PABP	5'-gcggatccatgaacccagtgccccag-3'
29	3'NotI-PABP	5'-gcgcccgccttaaacagttggaacaccggtggc-3'
30	5'AflIII-miR30	5'-cttaagtaccagcctacagtcggaaaccatc-3'
31	3'ApaI-miR30	5'-gggcccacaactgtagtctcttcaag-3'
32	5'4G-5183	5'-gctcagatctgcgatgtgatgtgc ctgaactaataagtgaagccacagatg-3'
33	3'4G-5183	5'-gctcagaggatccgcagtgatgtgtctgaactaataacatctg tggcttac-3'

### **2.3 Recombinant proteins**

Recombinant proteins GST-PABP, GST-eIF4E and GST-eIF4G (Ct) were generated as previously described (Kaiser, Dobrikova et al. 2008). Briefly, *Escherichia coli* BL21 cells transformed with expression plasmids were induced with isopropyl- $\beta$ -D-thiogalactopyranoside ([IPTG] 0.5 mM) and cultured for 6 h at 30°C before harvesting and lysis by sonication. Recombinant proteins were purified using a GSTrap FF column (GE Healthcare) and dialyzed against hypotonic buffer (10 mM HEPES at pH 7.5, 0.5 mM MgOAc<sub>2</sub>, 10 mM KOAc, and 2 mM dithiothreitol, where Ac is acetate). For GST-eIF4E The GST tag was removed with PreScission protease (GE Healthcare) according to the manufacturer's instructions.

### **2.4 Immunofluorescence**

Immunostaining was performed essentially as described previously (Kedersha, Anderson 2007). Briefly, 105 HeLa cells were plated on 12-mm glass coverslips and incubated for 24 h, infected with HSV-1 at an MOI of 10 for 7 h, or transfected with the ICP27 and/or UL47 expression construct for 18 h. Cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 20°C methanol for 10 min. The cells were treated for 1 h with blocking buffer (10% goat serum

in PBS) and incubated with primary antibodies diluted in blocking buffer at 4° overnight. Cells were washed and incubated for 45 min at room temperature with a mixture of FITC-conjugated anti-mouse IgG (Sigma) and TRITC-conjugated anti-rabbit IgG (Sigma) at dilutions of 1:150 and 1:500, respectively. Coverslips were washed three times and mounted on glass slides using ProLong Gold antifade reagent with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen). Images were collected using an XI50 Olympus microscope, DP70 digital camera, and DPController/DPManager software. Images were processed and analyzed using Adobe Photoshop software.

## ***2.5 DNA transfections***

Transfection of pcDNA3.1 expression plasmids for IP experiments was performed with HEK-293 tet-inducible cells grown in 10 cm dishes. Cells were transfected with 2.5 µg DNA and 6.5 µl lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. We used relatively low quantities of plasmid cDNA for transfections, since high levels of transfection reagent is toxic to cells and leads to undesirable activation of stress pathways and eIF4E phosphorylation (data not shown). Six hrs post-transfection, the media were changed to the starvation media (-FBS) containing tet. After 18 hrs of incubation, the cells were stimulated with the indicated compounds, harvested and lysed as described below.

## **2.6 Kinase inhibitors and activators**

Inhibitors of MEK (UO126) (Cell Signaling, Danvers, MA), CDK1 (aminopurvalanol A; roscovitine) (EMD, San Diego, CA), Mnk (CGP57380) (Sigma, St. Louis, MO) and p38 (SB203580) (Sigma) were dissolved in DMSO and used at concentrations as indicated. TPA, anisomycin, sodium arsenite (Sigma), nocodazole (Tocris, Minneapolis, MN), thymidine (EMD) were dissolved in DMSO, dH<sub>2</sub>O (sodium arsenite) or PBS (thymidine) and used at concentrations as indicated.

## **2.7 m7G cap pull down, glutathione pull down, immunoprecipitation**

### **2.7.1 Chapter 3.1**

To prepare cell lysates, cells were scraped, washed with cold phosphate-buffered saline (PBS), and pelleted by centrifugation at 4°C. The pellet was resuspended in an equal volume of lysis buffer (150 mM NaCl, 50 mM HEPES-KOH, pH 7.3, 10% glycerol, 1 mM EDTA, 5 mM EGTA, 0.5% NP-40, 2.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor cocktail (Sigma) and Halt phosphatase inhibitor (Pierce). Lysates were incubated on ice for 5 to 10 min and stored in aliquots at -80°C. Thereafter, cellular debris was separated by centrifugation of the total cell lysate at 14,000 g for 10 min, and total protein concentration was determined by Bradford assay

Cell lysates (5 mg of mock- or HSV-1-infected cells) was used for GST-PABP pull-downs. Glutathione-Sepharose beads (GE Healthcare) were preswollen in NT2 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 0.05% NP-40) supplemented with 1% bovine serum albumin and incubated with 8 to 20µg of recombinant GST-PABP or GST overnight at 4°C. Unbound proteins were washed off three times with 1 ml of cold NT2 buffer, and beads were incubated with precleared mock- or HSV-1-infected lysates for 4 h at 4°C. Thereafter, beads were rinsed five times with NT2 buffer, and bound proteins were resolved by electrophoresis in 4 to 12% Bis-Tris NuPAGE gels (Invitrogen) for subsequent Coomassie staining (Denville Scientific) or Western blot analysis. The same protocol was used for 7-methyl-GTP-Sepharose (GE Healthcare) pull-downs. Immunoprecipitation (IP) reactions were carried out with protein G-Sepharose beads (GE Healthcare) coated with 10 µg of primary antibodies and 1 to 1.5 mg of mock- or HSV-1-infected cell lysates.

### **2.7.2 Chapter 3.2**

Cell lysates were prepared as described for Chapter 3.1, except that low-salt lysis buffer was used (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% NP-40, 2 mM dithiothreitol [DTT]). Prior to IP or cap pulldown, Flag- or HA-Sepharose (Sigma) or 7-methyl-GTP Sepharose 4B (GE Healthcare, Piscataway, NJ) was blocked with 1% bovine serum albumin (BSA) for 30 min. Reaction mixtures were incubated for 2 to 4 h for IP or for 1 h for cap pulldown and contained 25 µl of Sepharose slurry and 1.5 mg of protein

lysate. Precipitates were washed with a buffer identical to lysis buffer but containing 0.05% NP-40 and 1 mM MgCl<sub>2</sub>. Beads were resuspended in either sample buffer for SDS-PAGE or wash buffer with 0.1 mg/ml Flag peptide or 0.1 mg/ml HA peptide (Sigma) for elution of Flag- or HA-tagged proteins, respectively.

### **2.7.3 Chapter 3.3**

Cell lysates were created as described for Chapter 3.1. Prior to immunoprecipitation (IP) or pull-down beads were blocked with 1% bovine serum albumin (BSA) for 30 min at +4. Following sepharoses were used: flag-sepharose (Sigma), protein G sepharose, glutathione sepharose (GE Healthcare, Piscataway, NJ) or p13suc1 sepharose (Thermo Scientific). All of the steps were performed at +4C.

For anti-cyclin B1 immunoprecipitation 25 ul of protein G sepharose was incubated with anti-cyclin B1 antibody for 2 hours, followed by the 2 hour incubation with 200 ug of lysate. For p13suc1 pull-down, 200 ug of lysate was incubated with 25 ul of slurry for 2 hours. For glutathione pull-down of GST-eIF4G Ct, lysates containing GST-eIF4G Ct were incubated with glutathione sepharose for 30 min. After the completion of IP/pull-down supernatants were collected and stored, and beads were washed 4 times with the wash buffer (10 mM HEPES, pH 7.4 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% NP-40) and resuspended in the sample buffer for SDS-PAGE. For large scale precipitation of flag-sepharose x amount of lysate was incubated with amount of

flag sepharose for 2 hours, washed 4 times with the wash buffer. Last wash was performed with 50 mM ammonium bicarbonate and flag-tagged eIF4G was eluted with elution buffer (50 mM ammonium bicarbonate, 0.1 mg/ml Flag peptide) for 30 minutes, concentrated on the column with 10 mW cut-off (Millipore) and submitted for phosphoproteomics.

## ***2.8 Immunoblotting and antibodies***

Immunoblotting was performed as described previously (Kaiser et al., 2008). Briefly, cell lysates were resolved by SDS gel electrophoresis in precast 4%–12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to PROTRAN nitrocellulose membrane (Whatman).

### **2.8.1 Antibodies for Chapter 3.1**

Polyclonal anti-Paip2 and anti-PABPC antibodies were kindly provided by Y. Svitkin and N. Sonenberg (McGill University, Montreal, Canada) and by J. Keene (Duke University, Durham, NC), respectively. Antibodies used included those against eIF4G1, eIF4A, eIF4E, and GAPDH (Cell Signaling Technology); antibodies against Flag (catalog no. F7425/F3165), c-myc (catalog no. M4439), PABP (catalog no. P6246), Paip2 catalog no. P0087), tubulin (catalog no. T6074), rabbit immunoglobulin G/tetramethyl rhodamine isothiocyanate [IgG/TRITC] catalog no. T6778), and mouse IgG/FITC (catalog no. F0257) (Sigma); antibody against ICP27 (catalog no. P1119) (Virusys); and antibodies against VP5 (catalog no. ab6508) (Abcam) and against HSV-1 glycoprotein (DakoCytomation).

## 2.8.2 Antibodies for Chapter 3.2

Antibodies used were against eIF4G1 (#2649), Mnk1, eIF4E, HA, Erk1/2, eIF4A, eIF4E-P, Mnk1-P, Erk1/2-P (#4370) (Cell Signaling), c-myc, and poly(A) binding protein (PABP) (Sigma). Phospho-eIF4E immunoblot signals were quantified by densitometry using the FluorChem FC2 imaging system (Cell Biosciences, Santa Clara, CA) and analyzed using the AlphaEase FC program. The phospho-eIF4E signal in the vector control lane was set as the background and subtracted from the values for the WT Mnk1a or  $\Delta$ 4G Mnk1a lane. The resulting values were normalized to the values of the respective immunoblot signals for unphosphorylated eIF4E in the same samples.

## 2.8.3 Antibodies for Chapter 3.3

Antibodies used were against eIF4G1 (#2649), eIF4E, Erk1/2, eIF4A, GST (#2625), cdk1 (#9112), RSK1/RSK2/RSK3 (#9355), phospho-Erk1/2 (T202, Y204) (#4370), phospho-eIF4G1 (S1148), phospho-histone H3 (S10) (#9701), phospho histone H3 (S10) Alexa Fluor® 647 Conjugate (#9716), phospho-p90RSK (S380) (#9341) (Cell Signaling), c-myc, and poly(A) binding protein (PABP) (Sigma), phospho-eIF4G1 (S1232)(Novus Biologicals), cyclin B1 (Millipore).

## **2.9 In vitro kinase assay**

### **2.9.1. In vitro phosphorylation of eIF4E by Mnk1 kinases**

HEK-293 cells were transfected with various HA-Mnk1a constructs and lysed as described above. HA-Mnk1 was immunoprecipitated as described above with the following modifications: the last wash and elution with HA peptide were performed in kinase buffer (20 mM HEPES-KOH, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 25 μM ATP). Eluates were either assayed by immunoblotting to analyze equal loadings of HA-Mnk1 kinase or incubated with 3 μg of recombinant purified eIF4E and 10 μCi of [ $\gamma$ -<sup>33</sup>P]ATP at 30°C for 1 h. Reactions were stopped by the addition of sample buffer, the volumes were subjected to SDS-PAGE, and proteins were visualized by autoradiography.

### **2.9.2 In vitro phosphorylation of GST-eIF4G (Ct) by nocodazole-arrested cell extracts**

HEK-293 cells were arrested in mitosis by sequential double thymidine-nocodazole block and scraped in ice-cold PBS and lysed with Dounce homogenizer in the kinase buffer (20 mM HEPES-KOH, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM DTT) containing protease and phosphatase inhibitors. Cell lysates were aliquoted, snap frozen and stored at -80°C. 10 μg of lysates were incubated with 2 μg of recombinant purified GST-eIF4G Ct and with or without inhibitors at 30°C for the indicated amount of time. Reactions were stopped

by the addition of ice-cold wash buffer and GST-eIF4G Ct was isolate using glutathione sepharose as described above.

## **2.10 Cell synchronization and FACS**

Cells synchronization was achieved by double thymidine block and/or nocodazole treatment. In brief, cells were synchronized at the G1/S boundary by incubation with 5 mM thymidine for 12hrs, followed by an 12 hr incubation in growth media prior to the addition of 5 mM thymidine for another 12 hr. Then cells were released from thymidine block in growth media containing 100 nM nocodazole for 12-14 hrs. To release from nocodazole-induced mitotic arrest, cells were washed and growth medium was added. To isolate mitotic cells by mechanic shake-off, HeLa cells were grown to about 60-80% confluency, and mitotic cells were separated from adherent cells by tapping on the flask 30-40 times. Adherent cells were then removed from the flask by trypsinization.

For synchronization experiments, cells were trypsinized and washed twice in ice-cold PBS, and fixed with 90% methanol overnight. Then cells were washed once with PBS, incubated for 10 minutes with 0.25% Tween in PBS, washed with PBTB (PBS, 0.1% Tween, 0.5% BSA) and stained with anti-H3-phospho APC-conjugated antibody (1:200) in PBTG (PBTB, 1% normal goat serum) for one hour. After incubation cells were washed with PBTB prior to staining with 10 ul of 7-AAD (BD, Franklin Lanes, NJ) in 100 ul PBS containing 200 ug/ml RNase A (Qiagen, Valencia, CA). DNA histograms and

analysis of histone H3-phosphorylation was acquired using FACS CANTO flow cytometer (BD) and analyzed using Flow Jo software (Tree Star).

## **2.11 Phosphoproteomics**

### **2.11.1 Sample Preparation and Nano-Flow Liquid Chromatography**

#### **Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS)**

##### **Analysis**

Samples delivered in 50 mM ammonium bicarbonate, pH 8.0 were supplemented with 0.1% Rapigest SF surfactant (Waters Corp). Samples were reduced with 5 mM dithiothreitol for 30 min at 70°C and free sulfhydryls were alkylated with 10 mM iodoacetamide for 45 min at room temperature. Proteolytic digestion was accomplished by the addition of 500 ng sequencing grade trypsin (Promega) directly to the resin with incubation at 37°C for 18 hours. Supernatants were collected following a 2 min centrifugation at 1,000 rpm, acidified to pH 2.5 with TFA and incubated at 60°C for 1 h to hydrolyze remaining Rapigest surfactant. Insoluble hydrolyzed surfactant was cleared by centrifugation at 15,000 rpm for 5 min. Samples were then dried using vacuum centrifugation and resuspended in 100 uL 80% acetonitrile, 1% TFA, 50 mg/mL MassPrep Enhancer, pH 2.5 (Waters Corp). Peptides were subjected to phosphopeptide enrichment using a 200 uL TiO<sub>2</sub> Protea Tip (Protea Bio) and subsequently washed with 200 uL 80% acetonitrile, 1% TFA, 50 mg/mL MassPrep Enhancer followed by 200 uL 80%

acetonitrile, 1% TFA. Peptides were eluted in 50  $\mu$ L 20% acetonitrile, 5% aqueous ammonia, pH 10.5 and then acidified to pH 2.5 with formic acid prior to drying using vacuum centrifugation.

Samples were resuspended in 10  $\mu$ L 2% acetonitrile, 0.1% formic acid, 10 mM citric acid and subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7  $\mu$ m BEH130 C<sub>18</sub> 75  $\mu$ m I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 5  $\mu$ L injection, peptides were trapped for 5 min on a 5  $\mu$ m Symmetry C<sub>18</sub> 180  $\mu$ m I.D. X 20 mm column at 20  $\mu$ L/min in 99.9% A. The analytical column was held at 5% B for 5 min then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 300 nL/min. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10  $\mu$ m tip orifice and coupled to an LTQ-Orbitrap XL mass spectrometer through an electrospray interface. The instrument was set to acquire a precursor MS scan in the Orbitrap from  $m/z$  400-2000 with  $r = 60,000$  at  $m/z$  400 and a target AGC setting of  $1e6$  ions. In a data-dependent mode of acquisition, MS/MS spectra of the three most abundant precursor ions were acquired in the Orbitrap with  $r = 7500$  at  $m/z$  with a target AGC setting of  $2e5$  ions. Max fill times were set to 1000 ms for full MS scans and 500 ms for MS/MS scans with minimum MS/MS triggering thresholds of 5000 counts. For all

experiments, fragmentation occurred in the LTQ linear ion trap with a CID energy setting of 35% and a dynamic exclusion of 60 s was employed for previously fragmented precursor ions.

### **2.11.2 Qualitative Identifications and Selected Ion Chromatograms from Raw LC-MS/MS Data**

Raw LC-MS/MS data files were processed in Mascot distiller (Matrix Science) and then submitted to independent Mascot database searches (Matrix Science) against SwissProt (*human* taxonomy) containing both forward and reverse entries of each protein. Search tolerances for LTQ-Orbitrap XL data were 10 ppm for precursor ions and 0.8 Da for product ions using trypsin specificity with up to two missed cleavages. Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M) and phosphorylation (+79.9663 Da on S, T, and Y) were considered a variable modifications. All searched spectra were imported into Scaffold (Proteome Software) and protein confidence thresholds were set using a Bayesian statistical algorithm based on the PeptideProphet and ProteinProphet algorithms which yielded a peptide and protein false discovery rate of 1.0%. (Keller, Nesvizhskii et al. 2002; Nesvizhskii, Keller et al. 2003) Phosphorylation site localization was assessed by exporting peak lists directly from Scaffold into the online AScore algorithm ([ascore.med.harvard.edu](http://ascore.med.harvard.edu)). Phosphopeptide abundances across metaphase and interphase samples were obtained by generating selected ion chromatograms (20 ppm window

around most abundant charge state of precursor ion with seven point Boxcar smoothing) from raw LC-MS data. MS Response at peak apex was used for quantitating abundance.

## **2.12 RNA transfections and Luciferase assay**

Reporter plasmids were linearized with Sall (c-myc and VEGF) and BamHI (b-globin) and used for in vitro transcription with T7 RNA polymerase using the MegaScript Kit T7 (Ambion) for uncapped c-myc and VEGF IRES Rluc reporter RNAs, and mMessage mMachine T7 Kit (Ambion) to produce m7G-capped b-globin 5'UTR Fluc reporter RNA. Reactions were treated with DNase and RNAs were purified by RNeasy (Quiagen), inspected for quality by agarose gel electrophoresis, and quantified by spectrometric analysis. In vivo luciferase expression assays were performed as described before (Bradrick, Dobrikova et al. 2007).

Briefly, HEK293 were transfected with equimolar amounts of reporter RNA using DMRIE-C reagent (Invitrogen). Seven to eight hours post-transfection, the cells were lysed with passive lysis buffer (Promega) and Rluc and Fluc activity was assayed in a Glowmax 20/20 luminometer (Turner Biosystems). Rluc values were first normalized to FLuc (capped m7G b-globin), and then analyzed as fold stimulation by eIF4G Ct (dox-induced/mock-induced) for each respective cell lines.

At least two independent transfection experiments were carried out, each containing at least five replicates, and the data is shown for one representative experiment with standard error indicated.

## **3. Results**

### ***3.1 Herpes Simplex Virus 1 lytic infection affects PABPC function***

#### **3.1.1 Introduction**

In chapter 1.6, I introduced the HSV-1 virus and discussed strategies that this virus employs to down-regulate host gene expression as a means to avoid induction of innate immune responses. I also presented the mechanisms of the host defense responses to viral infection at the translational level, exemplified by phosphorylation of eIF2 $\alpha$ , which is a potent measure to down-regulate translation and block production of viral particles. Thus, herpesviruses have developed multiple mechanisms to boost translation of viral mRNAs.

First of all, HSV-1 increases translation efficiency by activating signal transduction to translation initiation machinery. The viral protein Us3 is a Ser/Thr kinase that acts as an AKT surrogate, phosphorylating the TSC2 complex, which is an inhibitor of the mTOR kinase (Chuluunbaatar, Roller et al. 2010). mTORC1 phosphorylates the eIF4E-BPs, resulting in their dissociation from the eIF4E cap-binding protein and activation of cap-dependent translation. Moreover, viral protein ICP6 interacts with the

scaffolding protein eIF4G and stimulates eIF4F-cap binding complex formation (Walsh and Mohr 2006; Walsh, Casadei et al. 2006).

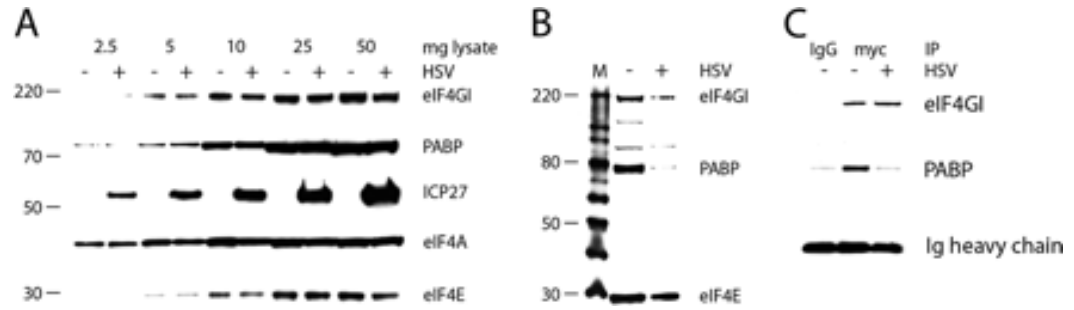
In this chapter, I describe how HSV-1 infection affects the function of the cytoplasmic polyA-binding protein (PABPC). PABPC binds the poly-A tail of mRNAs and has a critical function in mRNA translation and stability. It directly binds eIF4G, enabling mRNA circularization and ribosomal recycling. PABPC both protects mRNAs from degradation and recruits the deadenylation complex. PABPC is targeted by both RNA and DNA viruses, highlighting its central role in gene expression control. We report that in HSV-1 infected cells, PABPC is dissociated from the eIF4F cap-binding complex and relocalizes to the nucleus (Shveygert, Dobrikova et al. 2010). We identify novel viral proteins that interact with eIF4G and demonstrate that ectopic expression of viral protein ICP27 leads to the accumulation of PABPC in the nucleus.

### **3.1.2 Results**

#### **3.1.2.1 Reduced association of PABPC with the translation initiation complex in HSV-1 infected cells.**

To evaluate the effects of HSV-1 infection on translation initiation factors in HeLa cells, we examined the relative abundance of all eIF4F components and PABP in mock- and HSV-1-infected cells. Immunoblot analysis demonstrated that the abundance and

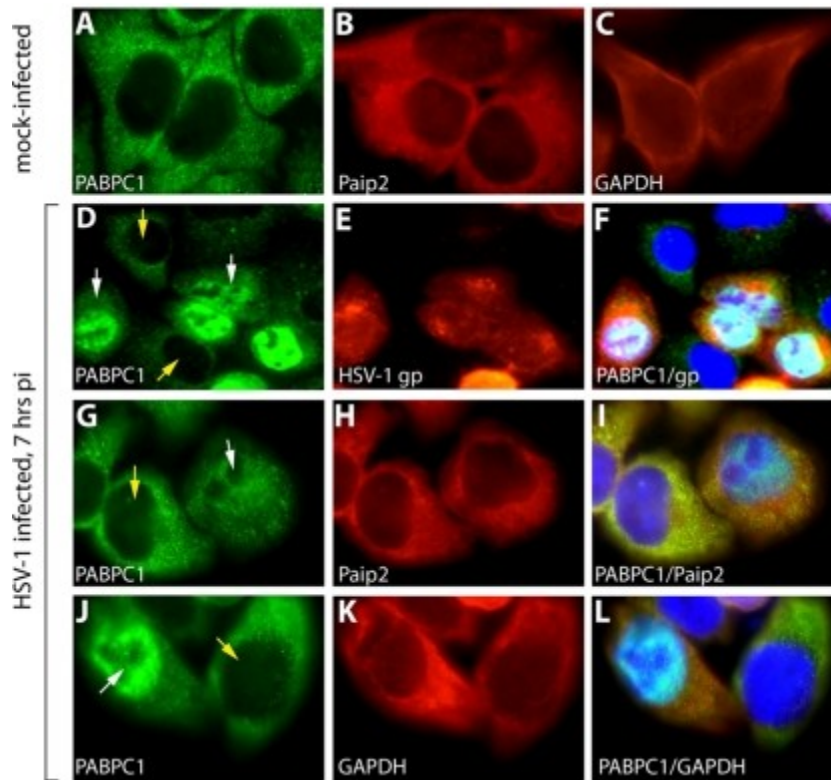
integrity of these proteins were unchanged upon HSV-1 infection (Fig. 7A). Next, to test functional interaction of translation factors, we analyzed eIF4F assembly at the m7G cap. Lysates prepared from mock- or HSV-1-infected HeLa cells were subjected to m7G cap-Sepharose pull-down, and precipitated proteins were analyzed by immunoblotting (Fig. 7B). Although equal amounts of translation initiation factors were present in input lysates (Fig. 7A), we consistently recovered slightly reduced amounts of eIF4E and eIF4G from HSV-1-infected samples. A more pronounced difference was observed for PABPC's association with eIF4F, with significantly less PABPC recovered by m7G cap-Sepharose pull-downs in HSV-1-infected cells (Fig. 7B). Since PABPC associates with eIF4F through eIF4G, we performed co-immunoprecipitation (co-IP) of these two proteins. Because IP of endogenous eIF4G is inefficient with available antibodies (data not shown), lysates were prepared from mock- or HSV-1-infected HeLa cells expressing tetracycline (Tet)-inducible myc-tagged eIF4GI-b (Kaiser, Dobrikova et al. 2008). IP with anti-c-myc antibody recovered equal amounts of eIF4GI from mock- and HSV-1-infected cells, but significantly less PABPC was coprecipitated from infected lysates (Fig. 7C). This suggests reduced PABP association with the m7G cap-binding complex in HSV-1-infected cells (Fig. 7B). All assays were performed with antibodies specifically recognizing PABPC.



**Figure 7 Abundance and association of translation initiation complex components in HSV-1-infected cells.**

(A) Increasing amounts of mock- and HSV-1(Kos)-infected HeLa lysates were loaded to compare steady-state levels and integrity of m<sup>7</sup>G cap-binding complex components (eIF4G, eIF4A, and eIF4E) and PABP. ICP27 was included to mark HSV-1 infection. (B) Equal amounts (1 mg of total protein) of lysates prepared from mock- or HSV-1-infected HeLa cells were incubated with m<sup>7</sup>G-Sepharose, and precipitated proteins were analyzed by immunoblot. (C) Lysates prepared from mock- or HSV-1-infected HeLa cells expressing myc-tagged eIF4G after Tet induction were subjected to IP with anti-myc antibody or isotype-matched mouse IgG1 control (Dobrikova, Shveygert et al. 2010).

prompted us to test whether PABPC may re-localize to the nucleus upon HSV-1 infection. HeLa cells were infected at a multiplicity of infection (MOI) of 10 for 7 h and subjected to immunofluorescent labeling of PABPC and HSV-1 glycoproteins (Fig. 8D to F). Our immunofluorescence experiments detect only PABPC, since the antibody was developed to the C-terminal domain of PABP, which is missing the nuclear form of PABP. In mock-infected cells, PABPC was present exclusively in cytoplasm (Fig. 8A), but HSV-1 infection produced PABPC redistribution to the nuclear compartment (Fig. 8D, G, and J). This effect was observed only in infected cells since nuclear PABPC signal overlapped with HSV-1 glycoproteins (Fig. 8F). The distribution of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as our control cytoplasmic protein (Fig. 8C), did not change upon HSV-1 infection (Fig. 8H and I). Similarly, Paip2, an established PABP interaction partner with cytoplasmic distribution (Khaleghpour, Svitkin et al. 2001), also remained in the cytoplasm in HSV-1-infected cells (Fig. 8B,K, and L). These findings suggest that HSV-1 infection promotes relocalization of cytoplasmic PABP, but not its native binding partner Paip2, to the nucleus.

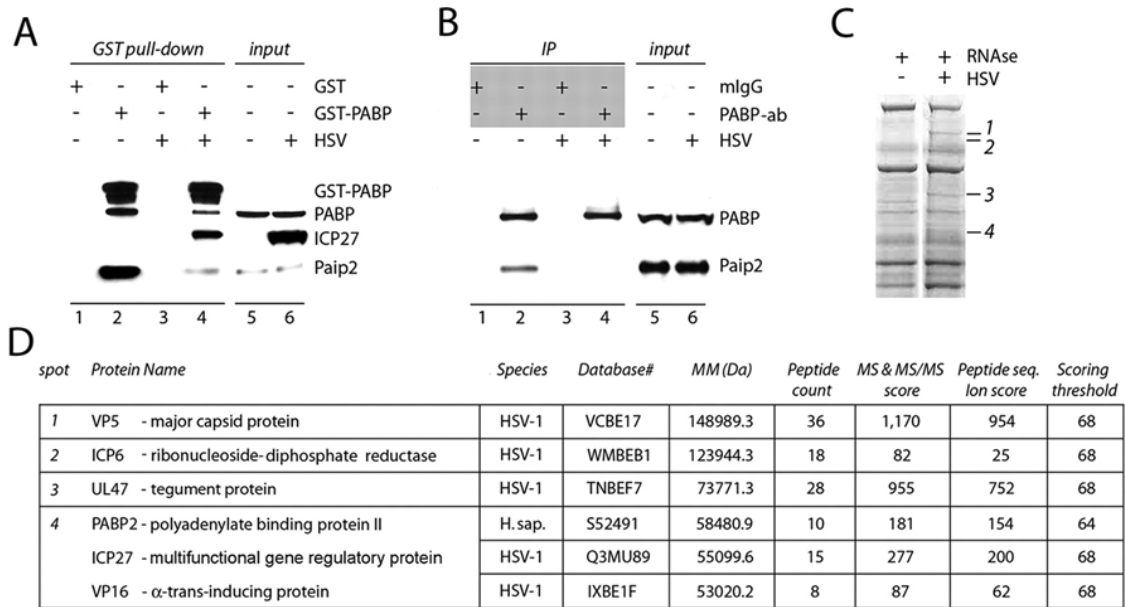


**Figure 8 HSV-1 infection results in PABPC redistribution to the nucleus.**

HeLa cells were mock infected (A to C) or infected at an MOI of 10 with HSV-1 for 7 h (D to L). PABPC (A), Paip2 (B) and GAPDH control (C) are shown in mock-infected HeLa cells. HSV-1 infection results in PABPC relocalization to the nucleus in infected (D; white arrow) but not uninfected (D; yellow arrows) cells in the same culture. (E) Infection was confirmed by staining with anti-HSV-1 glycoprotein antibody (gp). (F) Overlapping nuclear PABPC/HSV-1 glycoprotein is visible in the merged image. PABPC (G and J), Paip2 (H), and GAPDH (K) are shown in presumably uninfected cells (yellow arrow) and infected cells (white arrow). Merged images show overlapping nuclear PABPC/DAPI signal and markedly reduced cytoplasmic PABPC signal only in infected cells (I and L). pi, postinfection (Dobrikova, Shveygert et al. 2010)

### **3.1.2.2 Identification of proteins interacting with PABPC in HSV-1 infected cells.**

Immunofluorescent experiments suggest that in HSV-1 infected cells PABP no longer interacts with its inhibitor Paip2. To investigate this further we biochemically probed the interactions of Paip2 with PABP. Equal amounts of Paip2 were detected by immunoblotting from total lysates prepared from mock- and HSV-1-infected cells (Fig. 9B and C, lanes 5 and 6), however the amount of protein present in GST-PABPC pull-downs of infected lysates was much less than the amount in mock lysates (Fig. 9B, compare lanes 2 and 4). We were concerned by the fact that we reproducibly observed less PABP bound to recombinant GST-PABPC in HSV-1-infected lysates (Fig. 9B, compare lanes 2 and 4), which might be the reason for reduced Paip2 co-IP. To exclude this possibility, we performed IP of mock- and HSV-1-infected lysates with anti-PABPC (Fig. 9C) antibody. In this experiment, similar amounts of PABPC were precipitated from both extracts, but no detectable Paip2 was coprecipitated in infected cells (Fig. 9C, compare lanes 2 and 4), suggesting that PABP-Paip2 interaction is indeed disrupted by HSV-1 infection. One prominent band in GST-PABPC pull-downs of infected lysates corresponded by size with ICP27 (Fig. 9A, band 2), which has been shown to interact with PABP in HSV-1-infected cells (Fontaine-Rodriguez, Taylor et al. 2004). Immunoblot analysis confirmed that ICP27, indeed, is precipitated with GST-PABPC in infected extracts (Fig. 9B, lane 4).



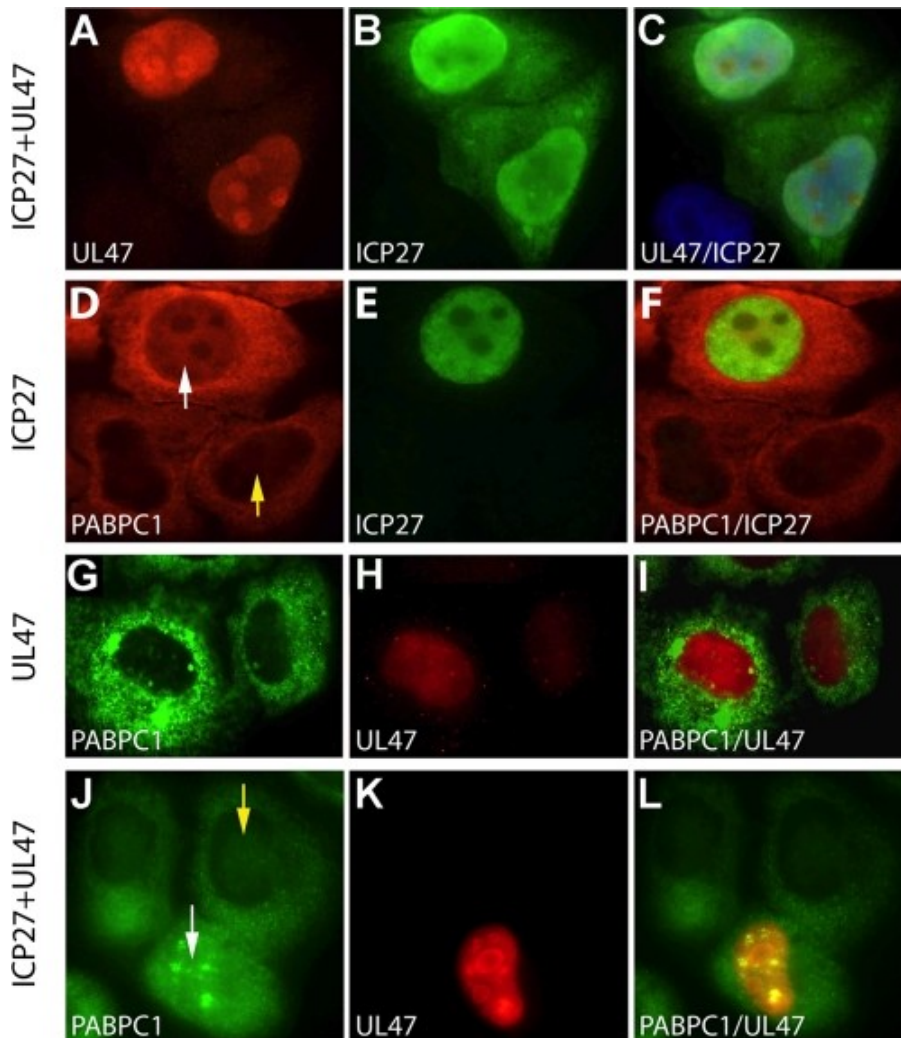
**Figure 9 Identification of proteins interacting with PABP in HSV-1-infected cells.**

(A) Mock- or HSV-1-infected HeLa cell lysates were subjected to pull-down with GST alone (negative control; lanes 1 and 3) or GST-PABP (lanes 2 and 4). (B) Co-IP of Paip2 with PABP in mock- and HSV-1-infected cells. Antibodies used for IP (isotype-matched mouse IgG1 [mIgG] or anti-PABP) are indicated in a gray-shaded box. (C) Proteins bound to GST-PABP were resolved by SDS-PAGE and stained with Coomassie blue, and bands 1 to 4 present in HSV-1-infected but not in mock-infected lysates were submitted for MS. (D) List of PABP-associated proteins identified in HSV-1-infected cell lysates. MM, molecular mass. Modified from (Dobrikova, Shveygert et al. 2010)

To test whether additional viral proteins may associate with PABPC we performed GST-PABPC pull-down with mock- and HSV-1-infected HeLa cells, and bound proteins were resolved by SDS-PAGE and visualized by Coomassie blue (Fig. 9D). Bands present in infected but absent in mock lysates were analyzed by tandem mass spectrometry (MS/MS). We identified five viral proteins associating with PABP in HSV-1-infected cells (Fig. 9E). These were the HSV-1 major capsid protein VP5, ICP6, the 73.8 tegument protein (UL47), ICP27, and VP16 (Fig. 9E). The only Homo sapiens protein identified was PABP2, a known binding partner of PABPC (Fig. 9E) (6). We again confirmed ICP27 binding to PABP in our screen, but VP5 and UL47 were novel PABP interacting proteins. ICP6 and VP16 did not have very high MS MS/MS scores; they may have precipitated in our assay via known interactions with eIF4G (Walsh (Walsh and Mohr 2006) and UL47 (Vittone, Diefenbach et al. 2005), respectively. Since ICP27 and UL47 associated with PABPC in RNase-treated extracts, we presume that binding occurs due to direct protein-protein interactions.

### **3.1.2.3 Effect of ectopic expression of ICP27 and UL47 on PABPC re-distribution to the nucleus.**

ICP27 and UL47 are nucleo-cytoplasmic shuttling proteins that, through their interaction with PABPC, might be involved in its redistribution (Donnelly 2001 JV, Mears 1996 JV). To test this hypothesis, we performed immunostaining of HeLa cells after transfection of ICP27 and Flag-UL47 expression constructs (Fig. 10). In accordance



**Figure 10 Effect of ectopic HSV-1 ICP27 and UL47 on PABPC subcellular distribution**

HeLa cells were transfected with ICP27 and/or UL47 expression constructs (as indicated on the left of each panel) 18 h prior to immunostaining. UL47 (A) and ICP27 (B) colocalize in the nucleus of transfected cells (C). ICP27 expression leads to nuclear accumulation of PABPC in transfected (D; white arrow) cells but not their nontransfected neighbors (D; yellow arrow). Nuclear PABPC and ICP27 (E) colocalize in nuclei, as shown in merged images (F). In UL47-transfected cells, PABPC (G) and UL47 (H) do not colocalize (I). In cells cotransfected with ICP27 and UL47, PABPC redistribution (J; white arrow) occurs in cells expressing UL47 (K) but not in untransfected neighbors (J; yellow arrow). The merged image shows nuclear colocalization of PABPC and UL47 (L). (Dobrikova, Shveygert et al. 2010)

with published data, both UL47 (Donnelly and Elliott 2001) and ICP27 (Mears and Rice 1996) assumed a predominantly nuclear distribution in transfected cells (Fig. 10A to C). ICP27 expression mediated PABPC redistribution to the nucleus, similar to the effect observed in HSV-1-infected cells compare Fig. 10D to F with Fig. 8G and J). UL47 expression alone did not alter PABPC distribution (Fig. 10G to I), reflecting its inability to associate with the protein on its own. Combined expression of ICP27 and UL47 did not significantly change PABPC subcellular distribution compared to ICP27 alone (Fig. 10J to L).

### **3.1.3 Discussion**

In this chapter we first demonstrate that in HSV-1 infected HeLa cells, PABPC dissociates from translation initiation complex, as determined by m7G-cap binding assays and eIF4G co-immunoprecipitation. Next we performed immunofluorescence experiments to demonstrate re-distribution of PABPC to the nucleus in HSV-1 infected cells. To elucidate viral activities that lead to PABPC relocalization, we performed mass-spectrometry of proteins that interact with PABPC specifically in infected cells. We analyzed GST-PABP pull-down from mock and HSV-1-infected cells and identified VP5, ICP6, ICP27, UL47 and VP16 among viral proteins that interact with GST-PABP. We confirmed interactions of UL47 with ICP27 with PABP and determined that ICP27 bridges UL47 and PABP (data not shown). Finally, we demonstrated that ectopic expression of viral protein ICP27 leads to PABP relocalization to the nucleus.

Our data were partially confirmed by Salaun and colleagues, who also reported the loss of PABP interaction with the m<sup>7</sup>G-cap binding complex and relocalization of PABPC to the nucleus (Salaun, MacDonald et al. 2010). However, they showed that in cells infected with ICP27-deletion virus, PABPC still relocalizes to the nucleus, suggesting that PABP accumulation in the nucleus does not depend solely on ICP27. They confirmed that over-expression of ICP27 leads to PABP re-distribution, probably because ectopic expression of ICP27 blocks splicing and export of mRNAs, leading to accumulation of unspliced polyadenylated mRNAs in the nuclear compartment (Burgess, Richardson et al. 2011).

Interestingly, Salaun et al. argued that relocalization of PABPC to the nucleus is due to the stressed conditions of infected cells, since oxidative stress can result in a similar accumulation of PABPC in the nucleus (Salaun 2010). These data suggests that the redistribution of PABP is a result of viral-induced stress, rather than an active viral measure. However, a similar effect on PABPC was observed during infection with the  $\gamma$ -herpes virus, Kaposi Sarcoma Herpes Virus (KSHV) (Kanno, Sato et al. 2006). KSHV1-1 expresses the shutoff and exonuclease (SOX) protein that actively imports PABP into the nucleus (Lee and Glaunsinger 2009). The fact that two different herpesviruses have

evolved with different mechanisms to re-distribute PABPC, supports the hypothesis that this event is advantageous to the virus.

How does depletion of PABPC from the cytoplasm benefit the virus if viral mRNAs are capped and polyadenylated and also require PABP for translation? First, nuclear PABPC contributed to hyperadenylation and nuclear retention of mRNAs, suppressing host gene expression (Kumar and Glaunsinger 2010). However, it is unclear how viral mRNAs escape this down-regulation mechanism, since presumably they are transcribed and exported in a manner similar to cellular mRNAs. Secondly, at any given time, about 20% of total PABP is still present in the cytoplasm and translation of at least some viral mRNAs is not affected (Salaun, MacDonald et al. 2010), suggesting that viral mRNAs are to an extent insensitive to the depletion of PABP, possibly because of the lack of competition with cellular mRNAs. Defining how PABPC relocalization to the nucleus is advantageous to herpesviruses remains an important future direction.

## **3. 2 MAPK control eIF4E phosphorylation through modulation of Mnk1-eIF4G interaction**

### 3.2.1 Introduction

In eukaryotes, initiation of protein synthesis is facilitated by eIF4F, a complex consisting of the scaffolding factor eIF4G and its interaction partners, the m<sup>7</sup>G-cap-binding protein eIF4E and the helicase eIF4A. In the pre-initiation complex, eIF4G serves as a central ribosome adaptor module, attracting 40S ribosomal subunits to the 5' end of mRNAs via direct association with eIF3 (Gingras, Raught et al. 1999). Interaction of eIF4E with the m<sup>7</sup>G-cap and eIF4G are recognized as rate-limiting steps in translation. They are tightly controlled by key mitogenic signals such as the PI3K/mTOR and Ras/MAPK signal transduction pathways. A myriad of effects of PI3K/mTOR and MAPK mitogenic signals on translation of discrete mRNA subsets and the global transcriptome have been reported (Rajasekhar, Viale et al. 2003).

Deregulated translational control is a significant factor in tumorigenesis and constitutes a prominent target for therapy. Thus, it is of central interest to mechanistically unravel the effects of mitogenic signals on the translation apparatus. A signature oncogenic signaling effect is eIF4E phosphorylation at Ser209 upon activation

of Erk1/2 or p38 MAPKs (Fukunaga and Hunter 1997; Waskiewicz, Flynn et al. 1997). Erk1/2 and p38 MAPK signals converge on Mnk, which is uniquely capable of catalyzing eIF4E Ser209 phosphorylation (Ueda, Watanabe-Fukunaga et al. 2004). eIF4E has been implicated in tumorigenesis (Lazaris-Karatzas, Montine et al. 1990; Mamane, Petroulakis et al. 2004), and Ser209 phosphorylation has been shown to be required for eIF4E's oncogenic potential (Wendel, Silva et al. 2007) (Furic, Rong et al. 2010). Unraveling mechanisms of protein synthesis modulation due to eIF4E phosphorylation has been pursued intensely, but the consequences of eIF4E phosphorylation for the regulation of translation initiation remain a matter of debate (Scheper and Proud 2002).

The Mnk proteins are serine/threonine kinases encoded by two distinct genes, *Mnk1* and 2 (Scheper, Morrice et al. 2001). Both *Mnk1* and 2 transcripts are subject to alternative splicing, giving rise to full length (*Mnk1a/2a*) as well as truncated versions (*Mnk1b/2b*) lacking the MAPK binding domain (O'Loughlen, Gonzalez et al. 2004). *Mnk1a* and 2a are activated by p38 and/or Erk1/2 MAPKs. However, *Mnk2* is active even in unstimulated cells with high basal levels of catalytic activity towards eIF4E phosphorylation (Scheper, Morrice et al. 2001). Interestingly, Mnks do not form a stable binary complex with eIF4E to achieve Ser209 phosphorylation. Instead, they interact with the scaffolding protein eIF4G, bringing the kinase and its substrate into physical

proximity (Pyronnet, Imataka et al. 1999). Consequently, association of Mnk with eIF4G is essential for eIF4E Ser209 phosphorylation.

We examined how MAPK signaling modulates interaction of eIF4G with Mnk1. Through co-immunoprecipitation assays, we show that MAPK-mediated phosphorylation of the Mnk1 active site controls eIF4G binding. Utilizing a naturally occurring splice variant, we demonstrate that the C-terminal domain of Mnk1 restricts its interaction with eIF4G, preventing eIF4E phosphorylation in the absence of MAPK signaling. Moreover, using a small-molecule Mnk1 inhibitor and kinase-dead mutant, we establish that Mnk1 autoregulates its interaction with eIF4G, releasing itself from the scaffold after phosphorylation of its substrate. Thus we demonstrate that phosphorylation of Mnk1 by p38 or Erk1/2 MAPKs not only activates its kinase activity, but also modulates Mnk1 interaction with eIF4G, thereby facilitating eIF4E phosphorylation. MAPK-mediated control over Mnk1-eIF4G binding constitutes an added level of regulation over eIF4E phosphorylation.

## **3.2.2 Results**

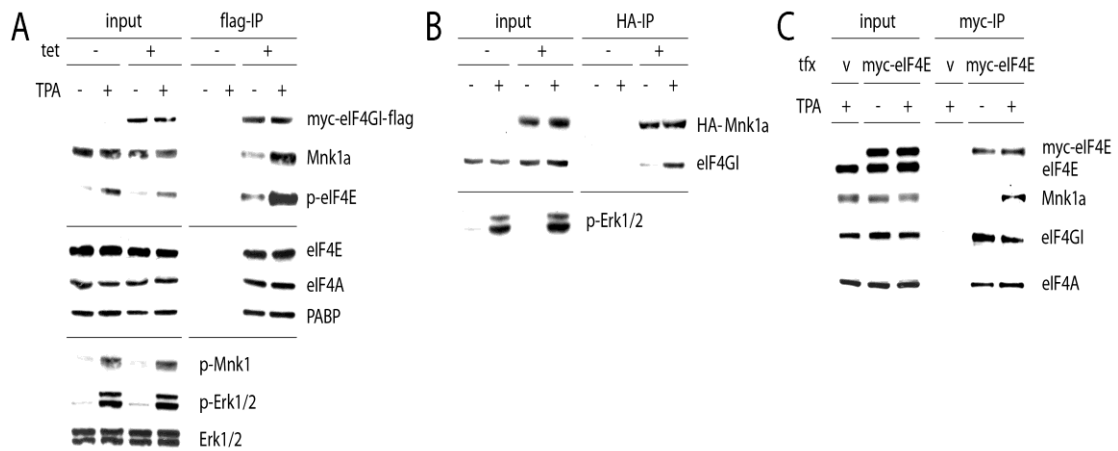
### **3.2.2.1 Stimulation of Erk1/2 and p38 results in increased binding of Mnk1 to eIF4G**

Stimulation of the Ras/MAPK pathways with phorbol esters results in increased translation. We have investigated whether activation of MAPK signaling can result in

change binding of eIF4G with its interaction partners. Since antibodies to the endogenous eIF4G do not work well in immunoprecipitation, we had to over-express exogenous full-length eIF4G that has a myc tag on the N-terminus and flag-tag on the C-terminus. We immunoprecipitated flag-tagged eIF4G with flag-sepharose and then eluted the protein complex from the agarose by incubating with an excess of a flag peptide. This elution procedure is milder than traditional boiling with Laemmli SDS-PAGE loading buffer, so that protein precipitates are not contaminated with heavy and light chains of the anti-flag antibody, thus allowing the detection of proteins that run in the 26-30 and 50-60 kDa range.

To achieve high expression of exogenous eIF4G we generated a stable tet-inducible HEK-293 cell line (HEK-293<sup>eIF4G</sup>). (Fig. 11A). Treatment of these cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) leads to activation of Erk1/2 with subsequent phosphorylation of Thr209/Thr214 in the Mnk1 active site and phosphorylation of Mnk1 target eIF4E (Fig. 11A). In all the subsequent experiments we use Erk1/2 phosphorylation as a control for successful activation of the PKC/Ras/MAPK pathway.

Lysates of TPA-treated cells were subjected to anti-flag immunoprecipitation of over-expressed eIF4G and precipitates were analyzed by immunoblot. Interestingly, binding of the major eIF4E partners, such as PABP, eIF4E and eIF4A was not affected by



**Figure 11 Stimulation of cells with TPA results in increased binding of Mnk1 to eIF4G.**

(A) HEK-293<sup>eIF4G</sup> cells were un-induced (-) or tet-induced (+), serum-starved for 18 hrs and treated for 15 min with DMSO (-) or 100 nM TPA (+). Cell lysates were either analyzed by immunoblot or subjected to anti-flag IP. Dividers separate immunoblot results obtained with different gels from the same experiment contained within the panels. (B) Cell extracts of HEK-293<sup>Mnk1a</sup> cells were prepared as described in Fig. 11A and subjected to anti-HA IP. (C) HEK-293 cells were transiently transfected with myc-eIF4E and cell lysates were used for anti-myc IP and analysis of the co-IP complex. (Shveygert, Kaiser et al. 2010)

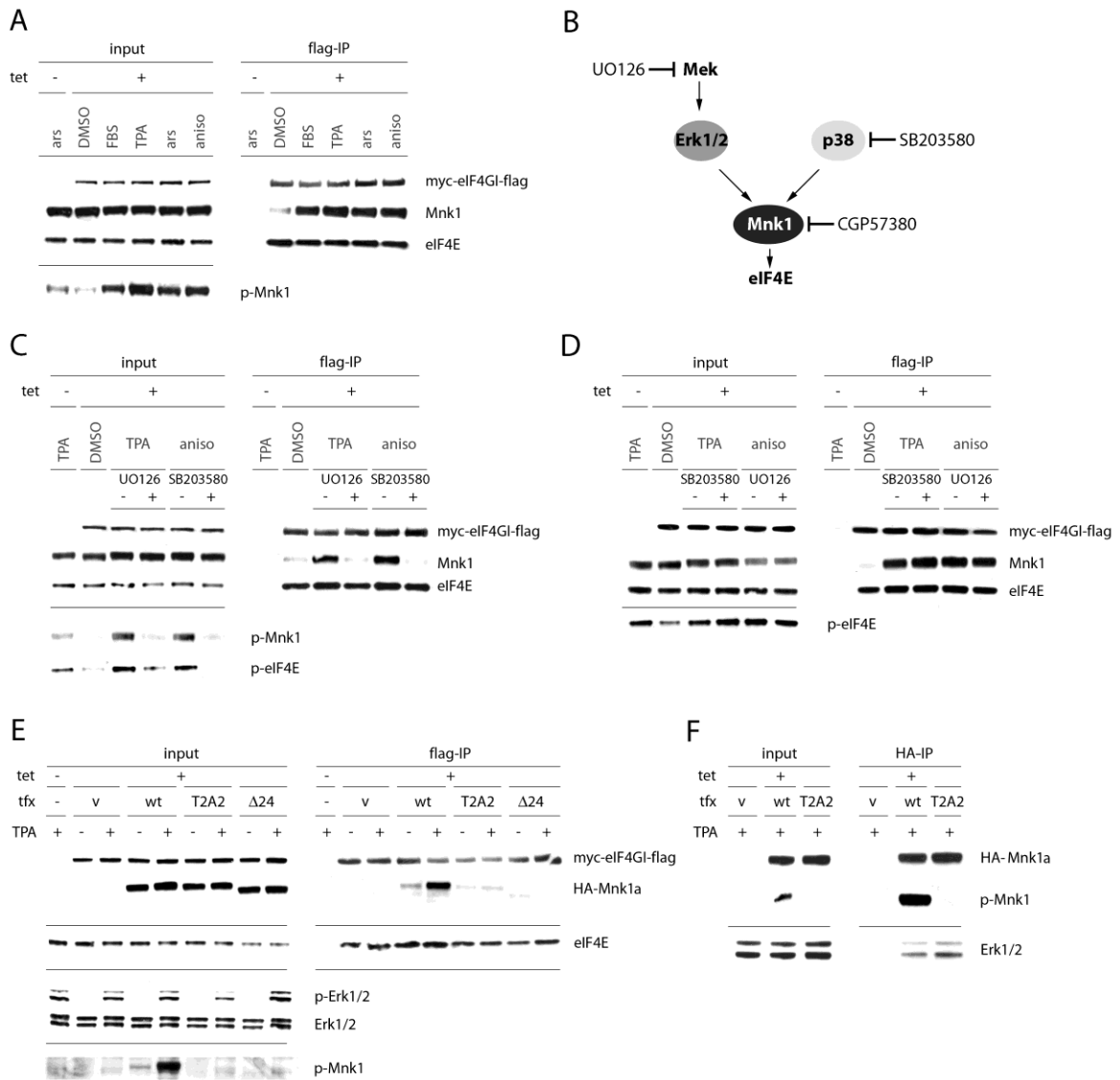
TPA treatment, however we observed a major increase of Mnk1 interaction with eIF4G in TPA-treated samples (Fig. 11A). No proteins were observed in tet- samples, confirming the specificity of co-immunoprecipitation. We also observed increased phospho-eIF4E co-precipitating with eIF4G, which stems from increased amounts of phosphorylated eIF4E in the lysate.

Since over-expression of tagged proteins can sometimes lead to erroneous results, we tested if a similar effect would be observed if we check binding of over-expressed tagged Mnk1 to endogenous eIF4G (Fig. 11B). To this end, we generated stable HEK-293 cells (HEK-293<sup>Mnk1</sup>) expressing hemagglutinin (HA)-tagged human Mnk1a in a tet-inducible fashion. In line with previous results, treatment of cells with TPA enhanced binding of endogenous eIF4G to HA-Mnk1 (Fig. 11B). To demonstrate that Erk1/2 activation enhances the physical proximity of Mnk1 and its substrate, we over-expressed myc-eIF4E and performed anti-myc IP (Fig. 11C). Again, TPA stimulation of cells led to enhanced co-IP of Mnk1 with eIF4E, while the eIF4E:eIF4G interaction remained unchanged (Fig. 11C). Thus, PKC activation stimulates Mnk1 binding to eIF4G and, subsequently enhances the interaction of Mnk1 with its substrate, eIF4E.

### **3.2.2.2 Phosphorylation of Mnk1 active site is required for efficient Mnk1-eIF4G interaction.**

Multiple scenarios can explain how eIF4G-Mnk1 binding interaction is stimulated by TPA treatment. It is possible that either eIF4G or Mnk are phosphorylated by one of the kinases in this signaling pathway, and these phosphorylation events result in changes in the protein conformations in favor of increased binding. Another possibility is that some inhibitory molecule is removed upon pathways activation.

Since it has been shown that Mnk1 is phosphorylated as a result of TPA treatment, we decided to test if this phosphorylation event is responsible for the increase in Mnk1-eIF4G interaction. Both p38 and Erk1/2 MAPKs can activate eIF4E phosphorylation, so we employed multiple treatments to induce either signaling pathway (Fig. 12A). All conditions that stimulated Mnk1 phosphorylation markedly increased Mnk1-eIF4G association (Fig. 12A). Importantly, this effect was abrogated by the inhibition of MEK (immediately upstream of Erk1/2) with UO126 or p38 inhibition with SB203580 (Fig. 12B, C). Next, we verified if down-regulation of Mnk1-eIF4G binding by inhibitors is due to decreased activity of their respective kinase targets. SB203580 and UO126 failed to inhibit Mnk1-eIF4G binding stimulated by Erk1/2 or p38 MAPK activation, respectively (Fig. 12D). This implies that both inhibitors block Mnk1-eIF4G association through specific effects on their target kinases only.



**Figure 12 MAPK-mediated phosphorylation of Mnk1a at T209/T214 activates Mnk1-eIF4G interaction.**

(A) HEK-293eIF4G cells were un-induced or tet-induced, serum-starved for 18 hrs and treated for 15 min with DMSO, 20% fetal bovine serum (FBS), 100 nM TPA (Erk1/2 stimulation); 0.1 mM sodium arsenite (ars; broad stimulation), or 10  $\mu$ g/ml anisomycin (aniso; p38 stimulation). Cell extracts were subjected to anti-flag IP. (B) Schematic representation of MAPK signal transduction to eIF4E and corresponding kinase inhibitors. (C, D) HEK-293eIF4G cells were serum-starved for 18 hrs, incubated

for 2 hrs with DMSO, 20  $\mu$ M UO126 (MEK inhibitor) or 10  $\mu$ M SB203580 (p38 inhibitor) and were treated for 15 min with DMSO (-), 100 nM TPA or 10  $\mu$ g/ml anisomycin. Cell extracts were subjected to anti-flag IP. (E) HEK-293eIF4G cells were transfected with the indicated HA-Mnk1a variants for 24 hrs and stimulated for 15 min with DMSO (-) or 100 nM TPA (+). Cell extracts were subjected to anti-flag IP. (F) HEK-293 cells were transfected with the indicated HA-Mnk1a variants for 24 hrs and treated for 15 min with 100 nM TPA (+). Cell extracts were subjected to anti-HA IP. (Shveygert, Kaiser et al. 2010)

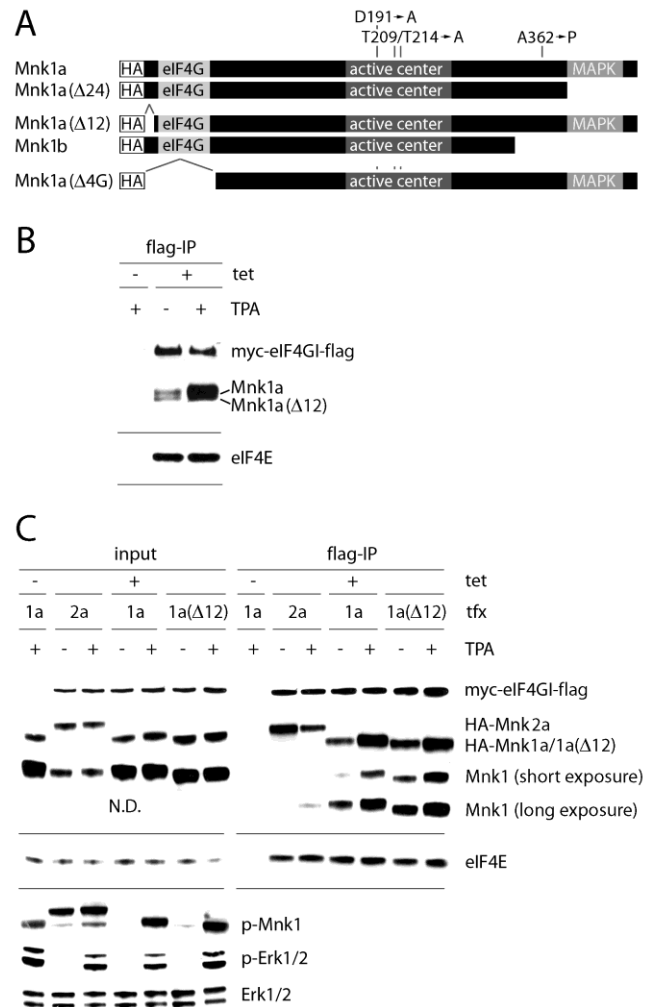
Both p38 and Erk1/2 can directly phosphorylate Mnk1 on the phosphosites Thr209/Thr214, which are located in the active site of the kinase. Phosphorylation of these sites results in structural rearrangements of the kinase domain and leads to the activation of the enzyme. eIF4G binds to the unstructured region in the N-terminal part of Mnk1, thus, crystal structure data cannot predict what happens to eIF4G binding after phosphorylation of the active site. However, since activation of both p38 and Erk1/2 increases Mnk1-eIF4G binding, it is plausible to conclude that phosphorylation of Mnk1 is responsible for the increased binding.

Alternatively, these MAPKs may post-translationally modify eIF4G, which also contains phosphorylation sites sensitive to serum stimulation (Raught, et al. 2000). To distinguish between these two possibilities, we created a mutant of Mnk1 in which Thr209/Thr214 were mutated to Ala (Mnk1a T2A2) and a deletion variant, which lacks the C-terminal 24 amino acids containing the MAPK binding site (Mnk1a $\Delta$ 24) (Ueda, Watanabe-Fukunaga et al. 2004) (Fig. 12A). Neither of these proteins can be phosphorylated by MAPKs. We used these expression constructs to transfect HEK-293<sup>eIF4G</sup> cells and test binding to flag-eIF4G (Fig. 12E). In both cases binding to eIF4G was severely impaired. It is possible that the Mnk1a T2A2 mutant did not bind eIF4G due to misfolded conformation of the mutated protein. However, T2A2 HA-Mnk1a protein was still capable of binding Erk1/2 to the same extent as wt HA-Mnk1 (Fig. 12F). Thus,

phosphorylation of the Mnk1 active site is not only required for kinase activity, but also determines binding to eIF4G.

### **3.2.2.3 Differential binding of Mnk1 isoforms and Mnk2 to eIF4G**

Two isoforms of Mnk1a have been reported that arise from alternate use of AUG's 12 codons apart (Mnk1a and -1a $\Delta$ 12; Fig. 13A) and may exhibit intrinsically distinct eIF4G binding activity (Pyronnet 2000). Using anti-Mnk1 antibody, we consistently detected two distinct bands in total cell lysates, both of which decreased in mobility upon TPA stimulation (data not shown). Using electrophoresis conditions that achieve higher resolution, we observed two bands in flag-eIF4G precipitates, likely representing both isoforms (Fig. 13B). When over-expressed, both Mnk1a isoforms exhibited similar eIF4G binding responses upon TPA treatment of cells (Fig. 13C). We also investigated if Mnk2-eIF4G interaction is regulated by MAPK signals. Mnk2 shares 71% sequence identity with Mnk1 and phosphorylates eIF4E *in vivo* (Scheper, Morrice et al. 2001). Since we lack reliable anti-Mnk2 antibodies, we over-expressed HA-tagged Mnk2. Binding of HA-Mnk2 to eIF4G did not increase upon addition of TPA but, instead, was slightly diminished (Fig. 13C). We attribute this to competition of HA-Mnk2 with endogenous Mnk1, which upon activation with TPA binds more strongly to eIF4G in the same lysate (Fig. 13C). These data suggest that Mnk2-eIF4G binding is secondary to Mnk1-eIF4G interaction. Thus, Mnk2-eIF4G binding does not appear to be directly regulated by



**Figure 13 Differential regulation of Mnk1 and Mnk2 binding to eIF4G.**

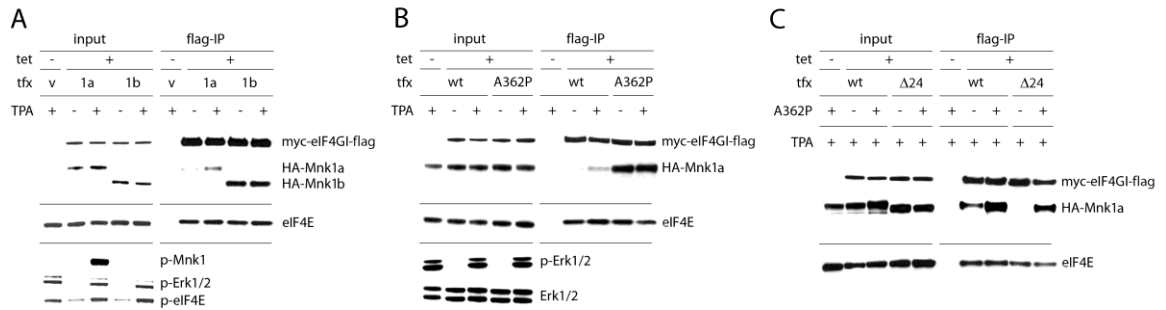
(A) Schematic view of Mnk1 constructs used in this study. The HA-tag, activation loop, truncation mutants, amino acids manipulated in this study, and the eIF4G and MAPK binding sites are indicated. (B) HEK-293<sup>eIF4G</sup> cells were serum-starved for 18 hrs and treated with DMSO or 100 nM TPA for 15 min. Flag-IPs were run on a 10% Bis-Tris gel to separate two similar-sized isoforms of Mnk1a and analyzed by immunoblot. (C) HEK-293<sup>eIF4G</sup> cells were transfected with the indicated constructs and after 24 hrs stimulated for 15 min with DMSO (-) or 100 nM TPA (+) as shown. Cell extracts were subjected to anti-flag IP. Various exposures are shown for the Mnk1 co-IP to visualize faint signal with endogenous protein. (Shveygert, Kaiser et al. 2010)

MAPK signal transduction, but responds indirectly to competition with Mnk1.

#### **3.2.2.4 Mnk1 C-terminal domain interferes with eIF4G binding to the Mnk1 N terminus in unstimulated cells**

Next, we investigated Mnk-eIF4G binding with the natural splice variant Mnk1b, which lacks 84 C-terminal amino acids compared to Mnk1a (Fig. 13A) (O'Loghlen, Gonzalez et al. 2004). Compared to HA-Mnk1a, Mnk1b had a substantially higher basal level of binding to eIF4G (Fig. 14B) and was nonresponsive to TPA stimulation (Fig. 14B). This suggests that a specific region within the Mnk1a C-terminus, upstream of the terminal 24 amino acids, interferes with binding of eIF4G, and this interference is alleviated upon stimulation by MAPK. Our results are in agreement with previous data proposing that the C-terminal region of Mnk1a occludes access to the catalytic domain (Scheper, Parra et al. 2003; Goto, Yao et al. 2009).

To test if the Mnk1a C-terminus can indeed block binding to eIF4G, we utilized an alanine to proline mutation (A362P) that has been proposed to disrupt the  $\alpha$ -helical conformation of the Mnk1 C-terminus (Goto, Yao et al. 2009). Indeed, A362P HA-Mnk1 exhibited significantly enhanced eIF4G binding (which was unresponsive to TPA; Fig. 14B), supporting the notion of an inhibitory effect of the Mnk1a C-terminal domain. The



**Figure 14 The C-terminal domain of unstimulated Mnk1a blocks binding to eIF4G.**

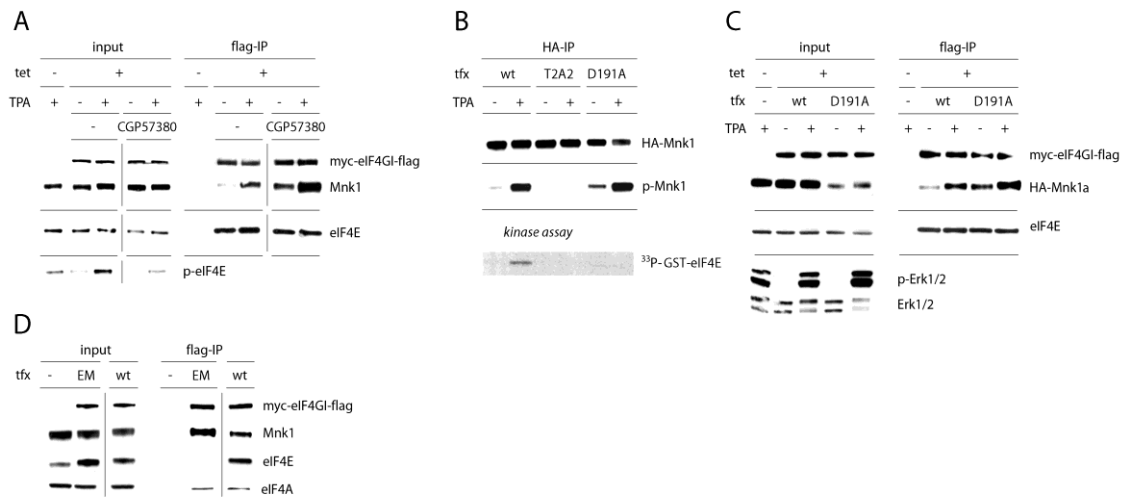
HEK-293<sup>eIF4G</sup> cells were uninduced or tet-induced and transfected with the following expression plasmids: (A) control vector, HA-Mnk1a or HA-Mnk1b; (B) wt or A362P HA-Mnk1a; (C) wt or Δ24 HA-Mnk1a with the A362P mutation either absent (-) or present (+). After 24 hrs cells were stimulated for 15 min with DMSO (-) or 100 nM TPA (+). Cell extracts were subjected to anti-flag IP. (Shveygert, Kaiser et al. 2010)

A362P mutation even rescued deficient binding of Mnk1a<sup>Δ24</sup> (lacking the MAPK binding domain) to eIF4G (Fig. 14C). This suggests that destabilizing the inhibitory C-terminal region of Mnk1a bypasses requirement for MAPK activation of Mnk1a to induce eIF4G binding.

### 3.2.2.5 Mnk1 limits its own binding to eIF4G

Next, we investigated why phosphorylation of Thr209/Thr214 is important for eIF4G binding. Since Mnk1 may be capable of autophosphorylation (Fukunaga and Hunter 1997), the active kinase may assume altered conformation favoring eIF4G binding upon phosphorylation of (a) secondary site(s). To investigate this possibility, we used a competitive inhibitor of ATP (CGP57380) to suppress Mnk1 kinase activity. Surprisingly, while this inhibitor diminished the levels of phospho-eIF4E, it strongly stimulated Mnk1 binding to eIF4G in the absence or presence of TPA (Fig. 15A). This finding indicates that catalytic activity of Mnk1 is not required for eIF4G binding and that inhibiting Mnk1 enzymatic activity increases eIF4G interaction.

CGP57380 is a compound with broad inhibitory spectrum. Thus, it could indirectly affect Mnk1-eIF4G interaction via secondary effects on kinases other than Mnk1. To exclude this possibility we confirmed our findings using a kinase-dead



**Figure 15 Mnk1 kinase activity limits its binding to eIF4G.**

(A) HEK-293<sup>eIF4G</sup> cells were un-induced or tet-induced, serum-starved for 18 hrs and incubated for 2 hrs with 10  $\mu$ M CGP57380 (Mnk1 inhibitor), and then stimulated for 15 min with DMSO (-) or 100 nM TPA (+). Cell extracts were subjected to anti-flag IP. (B) HEK-293<sup>eIF4G</sup> cells were un-induced or tet-induced and transfected with wt, T2A2 or D191A mutants of HA-Mnk1a for 24 hrs and stimulated for 15 min with DMSO or 100 nM TPA. Cell extracts were subjected to anti-HA IP. Precipitates were either analyzed by immunoblot with HA or p-Mnk1 antibodies (top panels) or used in *in vitro* kinase reactions with recombinant eIF4E as a substrate (bottom panel). (C) Cell extracts from B were subjected to anti-flag IP and precipitates were analyzed by immunoblot. (D) HEK-293 cells were transfected with wt or EM myc-eIF4G-flag and after 24 hrs stimulated for 15 min with 100 nM TPA. Cell extracts were subjected to anti-flag IP. (Shveygert, Kaiser et al. 2010)

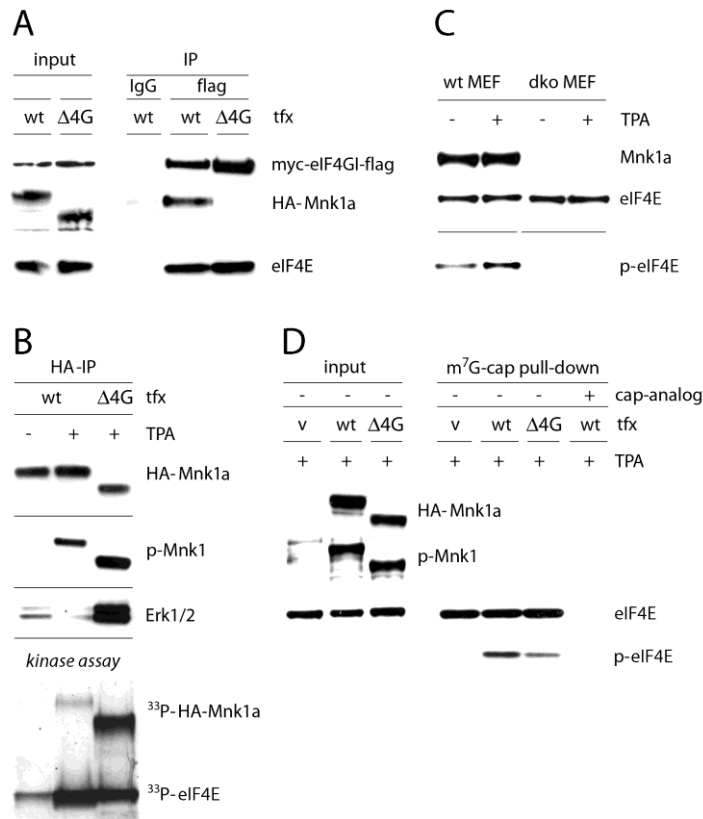
version of Mnk1. To this end, we established an Asp191→Ala (D191A) mutant which disrupts the metal-coordinating site in Mnk1 and abolishes kinase activity of the enzyme (Knauf, Tschopp et al. 2001). We confirmed that D191A was efficiently activated by TPA, because it was readily recognized by the phospho-specific anti-Mnk1 antibody (Fig. 15B). However, D191A was unable to phosphorylate recombinant GST-eIF4E in an *in vitro* kinase reaction as expected (Fig. 15B). Co-IP with eIF4G revealed that despite significantly lower expression of D191A compared to wt HA-Mnk1a, the former exhibited substantially enhanced binding to eIF4G in the absence or presence of TPA (Fig. 15C). Thus, considering these two independent lines of investigation, we conclude that inhibiting Mnk1 catalytic activity results in elevated interactions between the kinase and the eIF4G scaffold.

One plausible explanation for these findings could be that blocking phosphoryl transfer with CGP57380 or by the D191A mutation prevents Mnk1 from disassociating from eIF4G. Thus, Mnk1 may change its conformation after it completes phosphorylation of its substrate, and subsequently dissociate from eIF4G. If this were correct, then elevated binding of Mnk1 to eIF4G would be observed in situations where Mnk1 lacks potential substrates. To test this hypothesis, we performed co-IP of Mnk1 with either wt eIF4G or eIF4G mutated in the eIF4E binding domain (Mader, Lee et al. 1995). Confirming our hypothesis, Mnk1-eIF4G binding was enhanced with eIF4G that is

incapable of eIF4E binding, whereas the level of eIF4A remained unaffected (Fig. 15D). However, the differential in binding was not as pronounced as with CGP57380-treated cells or with cells expressing the D191A mutant. This could be due to additional Mnk1 substrates whose phosphorylation may also rely on Mnk1-eIF4G binding (Buxade, Parra et al. 2005).

### **3.2.2.6 Binding of Mnk1 to eIF4G is critical for eIF4E phosphorylation**

Ultimately, it was critical to demonstrate what contribution Mnk1-eIF4G binding makes to eIF4E phosphorylation independently of Mnk1 catalytic activity. To address this problem we created a deletion mutation of Mnk1 that lacked the eIF4G binding site (Mnk1a $\Delta$ 4G; Fig. 13A) and investigated whether it can phosphorylate eIF4E *in vivo*. First, using a co-IP approach, we validated that HA-Mnk1a $\Delta$ 4G did not bind eIF4G (Fig. 16A). Next, we examined if deletion of the eIF4G binding domain impairs the kinase activity of Mnk1. This is unlikely given that the eIF4G binding site lies outside of the catalytic domain (Jauch, Cho et al. 2006). We immunoprecipitated either wt or  $\Delta$ 4G Mnk1a proteins and tested their ability to phosphorylate recombinant eIF4E in an *in vitro* kinase reaction. Both wt and mutant Mnk1a phosphorylated eIF4E equally well (Fig. 16B).



**Figure 16 MAPK regulated Mnk1-eIF4G binding is required for efficient eIF4E phosphorylation.**

(A) HEK-293<sup>eIF4G</sup> cells were tet-induced, transfected for 24 hrs with either wt or Δ4G HA-Mnk1a and stimulated for 15 min with 100 nM TPA. Cell extracts were subjected to IP with either non-specific IgG (anti-HA sepharose) or anti-flag sepharose. (B) HEK-293 cells were transfected for 24 with either wt or Δ4G HA-Mnk1a and stimulated for 15 min with DMSO (-) or 100 nM TPA (+). Cell extracts were subjected to anti-HA IP. Precipitates were either analyzed by immunoblot with HA, p-Mnk1, Erk1/2 antibodies (top panels) or used in *in vitro* kinase reactions with recombinant eIF4E as a substrate (bottom panel). (C) Immortalized fibroblasts from Mnk1/Mnk2 double knock-out (dko) and wt mice were serum-starved for 18 hrs, and then stimulated for 15 min with DMSO (-) or 100 nM TPA (+). Cell extracts were analyzed by immunoblot. (D) Mnk1/Mnk2 dko cells were transfected for 24 hrs with the indicated constructs and stimulated for 15 min with 100 nM TPA. Cell extracts were subjected to cap pull-down without (-) or with (+) m<sup>7</sup>G-cap analog. (Shveygert, Kaiser et al. 2010)

Interestingly, we consistently detected significantly enhanced Mnk1a phosphorylation with the  $\Delta 4G$  mutant, either by immunoblot with phospho-Mnk1 specific antibody or with an *in vitro* kinase assay (Fig. 16B). Interestingly, HA-Mnk1a $\Delta 4G$  co-immunoprecipitated significantly higher levels of Erk1/2 than wt HA-Mnk1a, which probably explains higher levels of phosphorylation (Fig. 16B). Since the MAPK binding site lies at the very C-terminus of Mnk1a, this phenomenon further indicates that the N-terminus of Mnk1a functionally interacts with the C-terminus.

To investigate the inherent catalytic activity of Mnk1a $\Delta G$  *in vivo* we used a system with categorically absent eIF4E phosphorylation. Immortalized mouse embryonic fibroblasts (MEF) from Mnk1/Mnk2 double-knockout cells [dko MEF (Ueda, Watanabe-Fukunaga et al. 2004)] lack any activity that can phosphorylate eIF4E (Fig. 6C). We utilized these cells to transfect either wt or HA-Mnk1a $\Delta 4G$ . To enrich phospho-eIF4E, we performed m<sup>7</sup>G-cap pull-downs (Fig. 16D). TPA stimulation of transfected cells produced efficient phosphorylation of both forms of Mnk1a, but eIF4E phosphorylation levels varied significantly. While exogenous HA-Mnk1a $\Delta 4G$  retained eIF4E phosphorylation capacity, the level of phospho-eIF4E was significantly higher with wt Mnk1a. This further indicates that Mnk1a-eIF4E binding is indeed required for efficient eIF4E phosphorylation *in vivo*.

### 3.2.3 Discussion

In this study, we investigated how MAPK signaling pathways control Mnk1 binding to eIF4G and regulate phosphorylation of the principal Mnk substrate eIF4E. We demonstrated that activation of either p38 or Erk1/2 MAPKs enhances Mnk1 binding to eIF4G. Our results are at variance with previously published work. In studies with mouse Mnk1, Scheper *et al.* reported decreased Mnk1 interaction with eIF4G upon TPA stimulation (Scheper, Morrice et al. 2001). Several factors may explain these divergent findings. First, Scheper *et al.* ectopically expressed mouse Mnk1 in HEK-293 cells. There are important differences between murine and human Mnk1; the former lacks the N-terminal 12 amino acids and features distinct amino acid sequences in the eIF4G binding site and C-terminal portion of the protein. Since our data suggest that intra-molecular interactions regulate Mnk1-eIF4G binding, variant primary sequence in the mouse protein may alter regulation of Mnk1-eIF4G interaction. Second, Orton *et al.* showed that interaction of mouse Mnk1 with eIF4G is negatively regulated by the Pak2 kinase (Orton, Ling et al. 2004). However, in human Mnk1 a corresponding Pak2 phosphorylation site is substituted with glycine, thus eliminating this mode of regulation. Lastly, over-expressed murine Mnk1 in human cells, may compete with endogenous Mnk1 for interaction with eIF4G. If human Mnk1 has higher affinity for eIF4G, it may displace exogenous mouse Mnk1 subsequent to TPA stimulation similarly to apparent competition between Mnk1 and 2 (Fig. 12C).

Since activation of either p38 or Erk1/2 MAPKs stimulates Mnk1-eIF4G binding, we speculate that phosphorylation of Thr209/Thr214 triggers conformational changes in Mnk1 that coordinately promote both Mnk1-eIF4G interaction and Mnk1 kinase activity. Accordingly, neither the T2A2 mutant nor the  $\Delta$ 24 MAPK-binding site deletion mutant responded to TPA stimulation with enhanced eIF4G binding. In the absence of MAPK signaling, the C-terminal part of Mnk1 abrogates Mnk1-eIF4G interaction. Upon disruption of the structural integrity of the C-terminal domain (A362P) or in its absence (Mnk1b), eIF4G binding is constitutive and no longer requires MAPK activation.

An additional level of regulation of Mnk1-eIF4G binding appears to directly involve Mnk1 enzymatic activity since blocking Mnk1 either by the D191A mutation or with CGP57380 increased Mnk1-eIF4G interaction. It could be argued that the D191A mutation or binding of CGP57380 could simply alter the conformation of Mnk1 so that it constitutively binds eIF4G, as is the case for the A362P mutant. However, TPA stimulated eIF4G association with the D191A mutant protein or with wild-type Mnk1 in the presence of CGP57380, suggesting that at least two factors may influence eIF4G interaction: MAPK-mediated conformational changes of Mnk1 and dissociation of ADP-bound Mnk1 after phosphoryl transfer is complete. The latter may be physiologically significant, since it would enable Mnk1 to bind successive eIF4F complexes, thus allowing rapid turnaround of the substrate.

Our findings suggest that after MAPK activation, Mnk1 not only becomes catalytically active, but it also actively recruited to the vicinity of eIF4E through increased interaction with the scaffolding protein eIF4G. The latter is biologically significant, because the  $\Delta 4G$  Mnk1 mutant, which is a fully active kinase but cannot bind eIF4G, is unable to reconstitute a full eIF4E phosphorylation response to TPA stimulation in Mnk1/2 dko MEFs.

The data presented here uncover an additional level of control over Mnk1 activity and consequently eIF4E phosphorylation. This complex regulation is likely influenced by other aspects not addressed in this study. For example, we showed that phosphorylation of eIF4G by PKC $\alpha$  at S1186 increases Mnk1 association with eIF4G (Dobrikov, Dobrikova et al. 2011). Considering that phosphorylation of eIF4E by Mnk proteins appears to be critical for malignant transformation (Wendel, Silva et al. 2007), it will be of interest to fully define both the events that govern this post-translational modification and the consequences it has for control of mRNA translation.

### ***3.3 CDK1/cyclin B1 mediated phosphorylation of eIF4G modulates selective translation in mitosis***

#### **3.3.1 Introduction**

Protein synthesis is robust during interphase and decreases greatly when cells enter mitosis. Interestingly, several specific mRNAs escape global down-regulation of translation during mitosis. Multiple explanations were offered how translation is down-regulated in mitosis (see Chapter 1.5); however, this subject was never vigorously investigated. In this study we tested if eIF4G function is regulated in mitosis.

Multiple pieces of data indicate that eIF4G function may be important in mitosis. First, knock-down of one of the eIF4G isoforms, p97, causes mitosis-specific caspase-dependent apoptosis, indicating that translation mediated by p97 may be important to maintain cell survival during mitosis (Marash, Liberman et al. 2006). The same study suggested that p97 function is required for mitotic translation of anti-apoptotic protein Bcl-2 (B-cell lymphoma 2). Moreover, eIF4G knock-down in HeLa cells results in the multinucleated phenotype, indicating potential defect in cytokinesis (Coldwell and Morley, 2006).

eIF4G contains multiple phosphorylation sites that are regulated in a serum-dependent fashion (thus, may depend on the stage of the cell cycle). However, neither the role for these phosphosites nor kinases responsible for the modifications have been determined. Here we identify multiple phosphorylation sites on eIF4G that are increased in mitosis and define CDK1/cyclin B1 as a kinase for one of the sites, S1232. I also present the results of some preliminary experiments that probe the significance of this phosphorylation event for eIF4G function.

### **3.3.2 Results**

#### **3.3.2.1 Analysis of eIF4G interactions in mitosis.**

Multiple high throughput phosphoproteomics studies identified eIF4G as a target for signal transduction (Dephoure, Zhou et al. 2008) (Hsu, Kang et al. 2011). More than 30 phosphosites were identified, however, only few sites were confirmed in a low-throughput fashion. Moreover, for most phosphosites, the functional outcome of these phosphorylation events, as well as upstream activators are unknown. To elucidate the potential role of eIF4G phosphorylation in mitotic translation, we performed mass spectrometry identification of *in vivo* phosphorylation of eIF4G in interphase and metaphase. In order to successfully and reliably identify phosphorylation sites with this approach, a substantial amount of protein has to be derived. Since it was not feasible to immunoprecipitate sufficient amounts of endogenous eIF4G, we utilized an exogenous

over-expressed tagged variant of full-length eIF4G that was derived from previously described stable dox-inducible HEK-293 cell line (HEK-293eIF4G) (Shveygert, Kaiser et al. 2010). The cells were dox-induced and a double thymidine block was applied to arrest the cells in G1/S transition. This population of cells was used as an “Interphase” sample. To obtain a “Metaphase” sample, the cells were released from the double thymidine block in media containing nocodazole, a microtubule-stabilizing drug that arrests cells in the metaphase stage of mitosis. Lysates from both samples were used in anti-flag immunoprecipitation to purify over-expressed eIF4G, which was then subjected to mass spectrometry as described in Materials and Methods. Equal amount of protein was submitted for both samples (data not shown).

First, we analyzed if eIF4G interaction patterns change between interphase and mitosis. We did not observe changes in association of eIF4G with its main binding partners, such as eIF4E, eIF4A, eIF4A or PABP (data not shown). Thus, in the conditions of our assay, the formation of the eIF4F binding complex is not affected in mitotic cells. In Table 2, we present all proteins that interacted with eIF4G either in mitosis or interphase. Most of them are either components of eIF3 initiation complex or 40S small ribosomal subunit. All of them interacted with eIF4G specifically, rather than with flag sepharose, since they were absent in the anti-flag immunoprecipitates from un-induced cells. The minimum detection level was set at 2 peptides per protein and the quantitative

value (amount of assigned spectra normalized to the molecular weight of the protein) for each condition is presented. For each given protein the value from positive immunoprecipitation in mitosis (M) was divided by the respective value in interphase (I). We assumed that proteins with an M/I ratio  $> 1.5$  bound better to eIF4G in mitosis (five proteins), whereas proteins with an M/I ratio  $< 0.75$  are relatively depleted from eIF4G precipitates (nine proteins).

Interestingly, two proteins that were enriched in eIF4G mitotic precipitates, SRSF3 (serine/arginine-rich splicing factor 3) and DDX3X (DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked), were previously implicated in cap-independent viral translation (Fitzgerald and Semler 2011) (Geissler, Scholze et al. 2011). Although this data may be helpful for an initial analysis of eIF4G function, additional verification is required. In these experiments we did not access the total levels of the interacting proteins between mitosis and interphase, thus we may observe less binding due to the decreased amounts of the total protein in the nocodazole-treated cells. For instance, PDCD4 decreased in mitotic precipitates down to 20% of interphase levels; however, PDCD4 is an unstable protein that may be degraded at higher rates during mitosis. Thus, these mass spectrometry results require additional confirmation in immunoblotting assays.

**Table 2: Proteomics analysis of eIF4G immunoprecipitates in interphase and mitosis.**

MW – molecular weight, I - Interphase, M - mitosis, Neg - negative control, Pos – experiment. See text for more detail. Proteins whose quantitative values M/I ratio is higher than 1.5 or lower than 0.75 are highlighted in bold.

Gene name	MW	Quantitative value				Ratio M/I
		Neg M	Neg I	Pos M	Pos I	
<b>SRSF3</b>	19 kDa	0,0	0,0	2,2	0,0	<b>N/A</b>
<b>HNRPG</b>	42 kDa	0,0	0,0	2,2	0,0	<b>N/A</b>
<b>GRP78</b>	72 kDa	2,5	0,0	8,1	3,8	<b>2,1</b>
<b>EIF1AX</b>	16 kDa	0,0	0,0	1,5	0,8	<b>1,9</b>
<b>DDX3X</b>	73 kDa	0,0	0,0	3,7	2,3	<b>1,6</b>
EIF4A2	46 kDa	0,0	0,0	13,2	9,8	1,3
EIF3H	40 kDa	0,0	2,8	8,8	6,8	1,3
RS18	18 kDa	0,8	1,8	3,7	3,0	1,2
EIF3F	38 kDa	1,7	2,8	7,3	6,1	1,2
EIF4A1	46 kDa	1,7	1,8	19,8	16,6	1,2
PABP1	71 kDa	1,7	0,0	5,1	4,5	1,1
EIF3A	167 kDa	9,2	8,3	30,1	27,2	1,1
EIF3C	105 kDa	2,5	4,6	22,0	20,4	1,1
EIF4G1	175 kDa	3,4	7,4	48,4	45,4	1,1
EIF3G	36 kDa	1,7	1,8	9,5	9,1	1,1
EIF3L	67 kDa	0,8	1,8	12,5	12,1	1,0
RS15	17 kDa	0,0	0,0	1,5	1,5	1,0
DDX21	87 kDa	0,0	0,0	1,5	1,5	1,0
EIF3B	92 kDa	4,2	7,4	17,6	18,2	1,0
ASCC2	86 kDa	0,0	0,0	0,7	0,8	1,0
CCD93	73 kDa	0,0	0,0	0,7	0,8	1,0
GSE1	136 kDa	0,0	0,0	0,7	0,8	1,0
EIF2B1	63 kDa	0,0	0,0	0,7	0,8	1,0
IPO8	120 kDa	0,0	0,0	0,7	0,8	1,0
RL23	15 kDa	0,0	0,0	0,7	0,8	1,0
RL7A	30 kDa	0,0	0,0	0,7	0,8	1,0
RLA1	12 kDa	0,0	0,0	0,7	0,8	1,0
SMHD1	226 kDa	0,0	0,0	0,7	0,8	1,0
SRP14	15 kDa	0,0	0,0	0,7	0,8	1,0

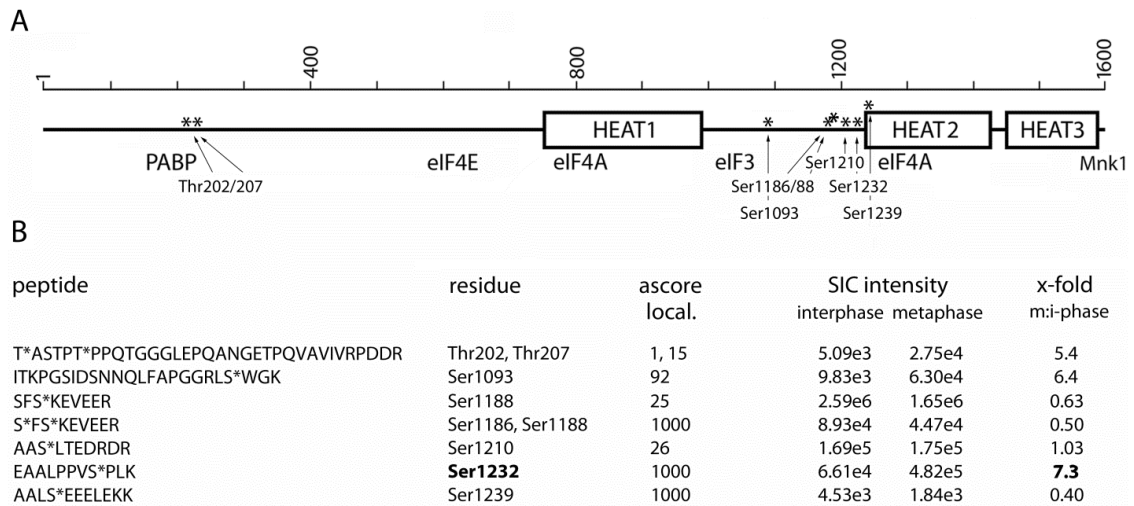
BA2L1	166 kDa	0,0	0,0	0,7	0,8	1,0
XPO7	124 kDa	0,0	0,0	0,7	0,8	1,0
EIF3J	29 kDa	0,0	1,8	4,4	4,5	1,0
RS19	16 kDa	0,0	0,9	4,4	4,5	1,0
IF4E	25 kDa	0,0	0,0	4,4	4,5	1,0
RS2	31 kDa	2,5	1,8	5,9	6,1	1,0
EIF3D	64 kDa	0,8	2,8	11,0	12,1	0,9
EIF3I	37 kDa	3,4	7,4	10,3	11,3	0,9
PABP4	71 kDa	1,7	0,0	5,1	6,1	0,8
EIF3M	43 kDa	0,0	0,0	2,9	3,8	0,8
EIF3E	52 kDa	0,0	0,9	8,1	10,6	0,8
<b>EIF3K</b>	25 kDa	0,0	0,0	2,2	3,0	<b>0,7</b>
<b>RS25</b>	14 kDa	0,8	0,9	3,7	5,3	<b>0,7</b>
<b>DDX50</b>	83 kDa	0,0	0,0	1,5	2,3	<b>0,6</b>
<b>RS17</b>	16 kDa	0,0	0,0	2,9	4,5	<b>0,6</b>
<b>RS13</b>	17 kDa	0,0	0,0	1,5	3,0	<b>0,5</b>
<b>ABCE1</b>	67 kDa	0,0	0,0	2,2	4,5	<b>0,5</b>
<b>RS9</b>	23 kDa	0,8	0,9	2,2	5,3	<b>0,4</b>
<b>PDCD4</b>	52 kDa	0,0	1,8	0,7	3,8	<b>0,2</b>
<b>ZA2G</b>	34 kDa	0,0	0,0	0,0	1,5	<b>N/A</b>

### 3.3.2.2 Phosphoproteomics of eIF4G in mitosis and interphase

For phosphoproteomics, we followed the procedure of phosphopeptide enrichment on TiO<sub>2</sub> columns (for more detail see Materials and Methods, Chapter 2.11).

Phosphopeptide abundance across metaphase and interphase samples were obtained by generating selected ion chromatograms (20 ppm window around the most abundant charge state of the precursor ion with seven point Boxcar smoothing) from raw liquid chromatography (LC)-MS data. To ensure the same phosphopeptide was being selected for quantitation across both samples, peptides had to match the retention time as indicated by database search results and accurate mass to within 20 parts per million (ppm). Once the corresponding peaks were identified, the peak height of manually selected ion chromatograms was used to calculate peptide abundance.

Using this approach we detected 9 sites in eIF4G that were phosphorylated in the interphase and metaphase (Fig. 17). Although the Mascot search engine can easily identify the proper peptide sequence, it fails to provide information about the presence or absence of site-determining ions, which localize the exact modified residue. The Ascore algorithm measures the probability of correct phosphorylation site localization



**Figure 17 Comparison of eIF4G1 phosphorylation in interphase and mitosis.**

(A) Schematic view of eIF4G. Structural domains, sites of interaction with main binding partners and phosphorylation sites that were identified in the phosphoproteomics experiment are indicated. (B) Summary of peptides identified by LC-MS/MS after TiO<sub>2</sub>-Enrichment of Phosphopeptides from Trypsin-Digested eIF4G1. Aminoacid sequence of the peptide is shown, and phosphorylated residue is indicated with a star. a. Ascore. 1000 - unambiguous, >20 - 99% localization, >15 - 90% localization, >3 80% localization. b. SIC - selected ion chromatogram of 2+ precursor ion (10 ppm window). Intensity value calculated at peak apex. c. Fold change in SIC intensities between metaphase and interphase is indicated.

based on the presence and intensity of site-determining ions in the MS/MS spectra (Beausoleil, Villen et al. 2006). Following submission of the identified peptides to the algorithm, six peptides out of nine had probabilities of correct localization of phosphorylation sites higher than 99%.

Identified phosphosites on eIF4G are clustered in two regions – one in the N-terminus (TT206/208) and another in the flexible interdomain linker that is located between HEAT-1 and HEAT-2 domains (Marintchev, Edmonds et al. 2009) (Fig. 17A). All of the phosphorylation sites that we describe were previously identified in high-throughput mass spectrometry studies (“PhosphoSitePlus, [www.phosphosite.org](http://www.phosphosite.org)” (Hornbeck, Kornhauser et al. 2012)). However, only two phosphosites, S1188 and S1232, were experimentally verified (Raught, Gingras et al. 2000).

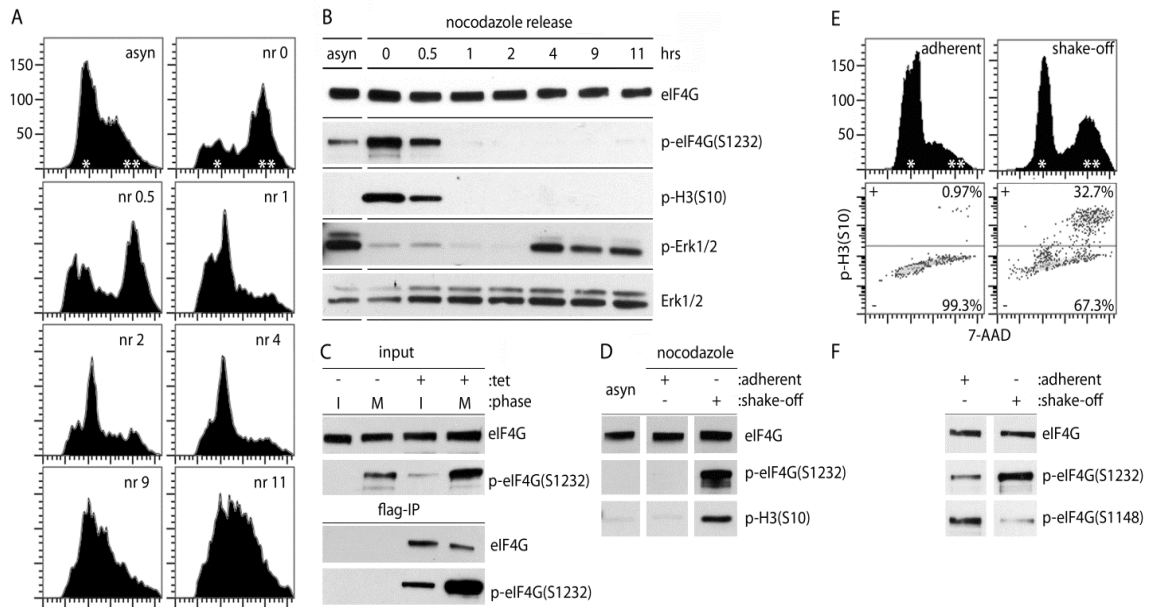
Four of the identified sites were upregulated in mitosis: T206/208 (5.4 fold), S1093 (6.4 fold) and S1232 (7.3 fold) (Fig. 17B). Sites TT206/208 and S1232 are proline-dependent, thus may be targeted by either MAPK or CDK kinases. Phosphosite S1093 fits the Aurora B consensus R/KxS/T, thus, it might be phosphorylated by this kinase in mitosis (Cheeseman, Anderson et al. 2002). We focused our studies on the S1232 phosphosite for several reasons. First, S1232 was identified as a major phosphosites on eIF4G (Raught, Gingras et al. 2000), arguing that this site is more likely to be functional

significant, since a higher percentage of eIF4G molecules would be affected by this phosphorylation. Second, only two phosphospecific eIF4G antibodies are commercially available, developed against S1148 and S1232. Phosphorylation of S1148 is not increased in mitosis (data not shown). Third, the S1232 phosphosite is located-at the border of the flexible linker and the structured HEAT2 domain, which starts at the 1235 residue, suggesting that S1232 phosphorylation may affect interactions of eIF4G with mRNA or other initiation factors (Marintchev and Wagner 2005).

### **3.3.2.3 eIF4G is phosphorylated at S1232 in mitosis**

To confirm our phosphoproteomic data we obtained commercially available antibodies to phosphor(p)-eIF4G(S1232). Immunoblot with this antibody recognizes a protein of ~180 kDa, which corresponds to the predicted molecular weight of eIF4G.

We used p-eIF4G(S1232) antibodies to investigate phosphorylation of eIF4G(S1232) during cell cycle progression (Fig. 18). HEK293 cells were arrested in mitosis with double thymidine block followed by nocodazole treatment (Fig. 18A). Flow cytometry analysis of 7-AAD-stained cells demonstrated that this treatment arrested most cells in the G2/M phase of the cell cycle (Fig. 18A; nr 0). After release from nocodazole block, the synchronized cells successfully completed mitosis, re-entered the G1 phase and further progressed through the cell cycle (Fig. 18A; nr 0.5 – nr 11). We



**Figure 18 eIF4G is phosphorylated at S1232 is during mitosis.**

(A, B) HEK293 cells were synchronized by double thymidine block and incubated for 12 hours in nocodazole-containing media to arrest cells in mitosis. Then cells were released from nocodazole (nr) and processed at the indicated time-points. Asynchronous cells (asyn) were used as a control. Cells were either fixed and stained with 7-AAD for FACS analysis of DNA content (A) or lysed and cell lysates were analyzed by immunoblotting (B). C. HEK293 myc-eIF4G-flag tet-inducible were either mock (tet-) or tet-induced (tet+) and either synchronized by double thymidine block (interphase, I) or arrested in mitosis with nocodazole (metaphase, M) as described in (A). Cells were lysed, myc-eIF4G-flag was purified on flag-sepharose (flag-IP) and analyzed by immunoblotting. (D-F) Mechanical shake-off of either nocodazole treated (D) or untreated (E,F) HeLa cells was performed to separate round mitotic cells from adherent cells in interphase. Cells were either lysed and cell lysates were analyzed by immunoblotting (D, F) or fixed and stained with 7-AAD and anti-H3-phospho APC-conjugated antibody and subjected to FACS analysis (E).

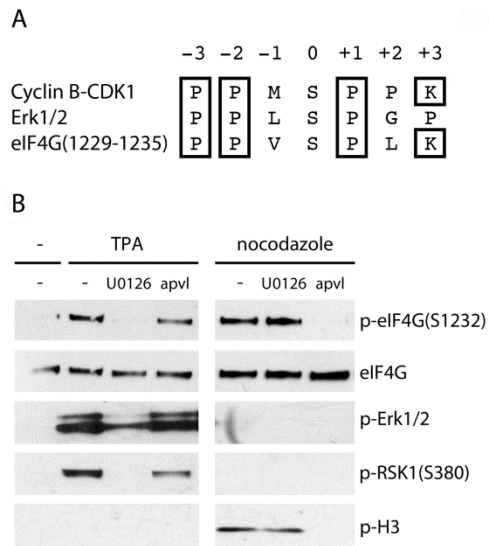
collected cultures at multiple intervals after nocodazole release and analyzed p-eIF4G(S1232) (Fig. 18B). S1232 phosphorylation is at its peak when cells are arrested in the prophase/prometaphase (Fig. 18B; nr 0), slightly diminishes when cells progress through mitosis (Fig. 18B; nr 0.5) and all but disappears when cells enter the G1 phase (Fig. 18B; nr 1) and progress through G1- and S-phase (Fig. 18B; nr 2-11). S1232 is phosphorylated to some extent in asynchronous cultures, probably because such cultures contain ~5% of mitotic cells at any given time. The S1232 phosphorylation pattern mimics that of histone H3(S10), which serves as a marker of mitosis (Goto, Tomono et al. 1999).

To confirm the identity of the p-eIF4G(S1232) antibody-reactive signal, we used HEK293 cells with tet-inducible expression of ectopic myc-eIF4G-flag (Fig. 18C). Cells were mock- or tet-induced and treated with DMSO (I=interphase) or nocodazole-arrested (M=metaphase) (Fig. 18C). Immunoblot of total lysates revealed potent enhancement of p-eIF4G(S1232) signal in the metaphase samples. The increased levels of total and p-eIF4G(S1232) levels with the tet-induced samples are due to the presence of exogenous eIF4G (Fig. 18C). Flag-immunoprecipitation (IP) and immunoblot demonstrated that the p-eIF4G(S1232) antibody-reactive band is indeed eIF4G phosphorylated on S1232 (Fig. 18C).

To validate if phosphorylation of S1232 is influenced by the toxic effects of nocodazole, we performed the classic shake-off procedure to mechanically separate adherent (interphase) from rounded/detachable (mitotic) HeLa cells. First, we combined the shake-off procedure with nocodazole-arrest (Fig. 18D). Nocodazole arrest produced both round, mitotic and adherent, interphase cells in the cultures which were separated by shake off. Both populations of cells were incubated in the presence of nocodazole for the same amount of time. However, nocodazole-treated cells that remained adherent after shake-off lacked p-eIF4G(S1232) signal, whereas the detachable fraction exhibited abundant immunoreactivity (Fig. 18D). The p-H3(S10) marker confirmed the mitotic state of the shake-off fraction. Second, we performed the shake-off procedure in the absence of nocodazole (Fig. 18E). Shake-off of HeLa cell cultures produced a cell population enriched for mitotic cells [ $\sim 1/3$  of shake-off cells stained positive for p-H3(S10) (Fig. 18E)]. Partial enrichment of mitotic cells with the crude shake-off method revealed increased eIF4G(S1232) phosphorylation compared to the adherent, interphase sample (Fig. 18F). Phosphorylation of S1148 in eIF4G trended in the opposite direction (Fig. 18F). These assays confirm that phosphorylation of eIF4G(S1232) occurs specifically during mitosis and is not influenced by the presence of the microtubule-destabilizing agent nocodazole.

#### **3.3.2.4 Dual phosphorylation of eIF4G S1232 by Erk1/2 and CDK1/cyclin B1 in vivo**

Next we sought to determine which kinase phosphorylates S1232 in mitosis. The aa sequence context surrounding eIF4G(S1232) closely resembles the consensus for both MAP and CDK kinases (Songyang, Blechner et al. 1994) (Songyang, Lu et al. 1996) (Fig. 19A). We reported previously that S1232 phosphorylation responds to the phorbol ester TPA in serum-starved cells and is abolished upon Erk1/2 inhibition (Dobrikov, Dobrikova et al. 2011). To test whether Erk1/2 or CDK1 may be responsible for S1232 phosphorylation in mitosis, we nocodazole-arrested cells and then treated them for 15 min either with the selective CDK1 inhibitor aminopurvalanol (apvl), or with the MEK1 inhibitor UO126 (Fig. 19B). Apvl diminished p-eIF4G(S1232) signal substantially, in parallel with inhibition of p-H3(S10) (Fig. 19B). In contrast, treatment of nocodazole-arrested cells with UO126 had no effect on S1232 phosphorylation (Fig. 19B). However, when cells were pre-treated with apvl or UO126 and then stimulated with TPA for 15 min, only UO126 prevented S1232 phosphorylation (Fig. 19B). This data suggests that S1232 may be phosphorylated independently by (a) kinase(s) in the MEK1/Erk1/2 pathway and the CDK1 pathway, depending on the stage of the cell cycle and presence of mitogenic signals.

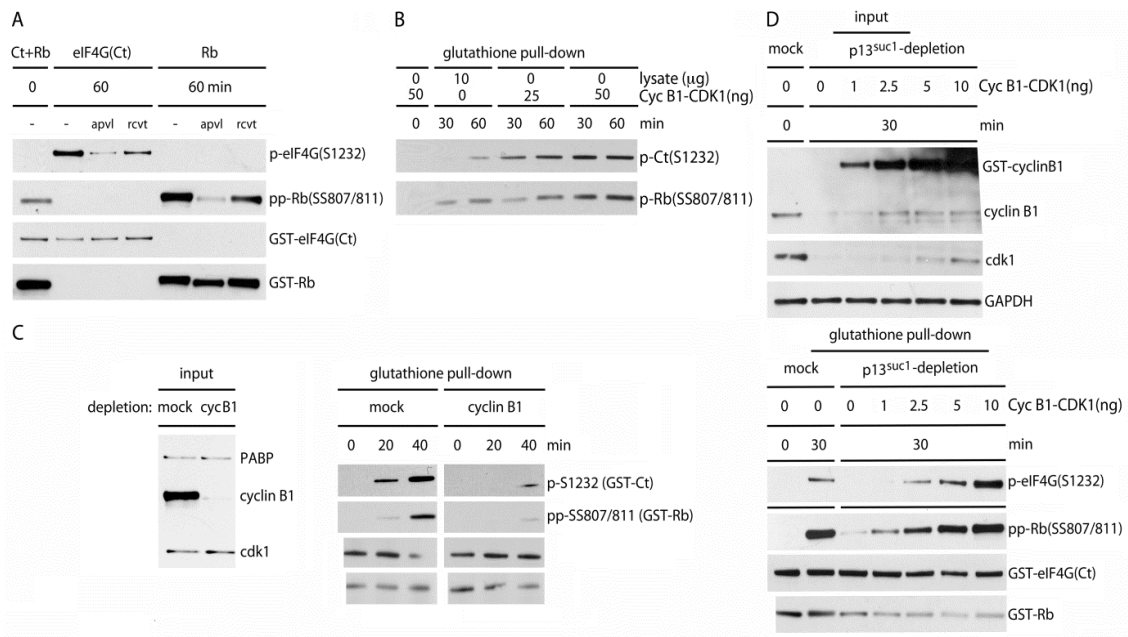


**Figure 19 CDK1-dependent phosphorylation of eIF4G phosphorylation at S1232 in vivo**

(A) Substrate consensus of CDK1-cyclin B1 and ERK1/2 kinases (Songyang, Blechner et al. 1994) (Songyang, Lu et al. 1996) compared with the eIF4G sequence (aa 1229 to 1235) around the proposed phosphosite at S1232. Squared letters in the eIF4G sequence indicate positions identical to the consensus. (B) CDK1-dependent phosphorylation of eIF4G1 at S1232 in response to nocodazole. Cells were either incubated with nocodazole for 18 hrs and then treated with CDK1 inhibitor aminopurvalanol (apvl) or MEK1 inhibitor UO126, or pre-treated with apvl or UO126 for 1 hour and then incubated with TPA for 15 minutes.

### 3.3.2.5 Cdk1/cyclin B1 phosphorylates eIF4G in vitro

Suppression of mitotic eIF4G S1232 phosphorylation with apvl suggests that S1232 lies downstream of the CDK1 kinase. However, it does not prove that CDK1 is directly involved in eIF4G S1232 phosphorylation. To establish a role for CDK1 in eIF4G phosphorylation, we first conducted experiments with extracts of nocodazole-arrested HEK293 cells to phosphorylate recombinant eIF4G in vitro. To this end, we generated recombinant, GST-tagged eIF4G-Ct, the C-terminal eIF4G fragment generated by cleavage with the enteroviral 2A protease (encompassing aa 682-1600 of eIF4G). It is used in place of the intact protein, due to the notorious instability and insolubility of recombinant full-length eIF4G. Incubation of recombinant eIF4G-Ct, which was generated in a bacterial system and was not phosphorylated at S1232, in 'mitotic' cell extracts produced p-Ct(S1232) (Fig. 20A). The reaction was sensitive to CDK1 inhibitors (Fig. 20A). Phosphorylation of a recombinant, GST-tagged C-terminal fragment of retinoblastoma protein (Rb) at S807/811 was used as a positive control to probe for CDK1 function (Lees, Buchkovich et al. 1991) (Fig. 20A). To test if CDK1 is capable of phosphorylating recombinant eIF4G-Ct at S1232 in vitro in the absence of cell lysate, recombinant CDK1/cyclin B1 was incubated with recombinant GST eIF4G-Ct and GST-Rb. In vitro phosphorylation of eIF4G-Ct by CDK1/cyclin B1 at S1232 was time and dose-dependent and correlated closely with phosphorylation of Rb (Fig. 20B).



**Figure 20 CDK1/cyclin B1 phosphorylates eIF4G1 at S1232 in vitro.**

(A) Recombinant GST-tagged C-terminal fragment of eIF4G (GST-eIF4G Ct) and recombinant C-terminal fragment of Rb (GST-Rb) were incubated with mitotic cell extract from nocodazole-treated cells plus CDK1 inhibitors aminopurvalanol (apvl) and roscovitine (rsct) and then isolated using glutathione sepharose. (B) GST-eIF4G Ct and GST-Rb was incubated with either mitotic lysates as in (A) or with recombinant CDK1-cyclin B1. (C,D) Depletion of CDK1 and/or cyclin B1 from mitotic lysate abolishes *in vitro* phosphorylation of eIF4G at S1232. (C) Cyclin B1 was depleted from mitotic lysate with anti-cyclin B1 antibody. Mock-depleted and cyclin B1-depleted lysates (left panel) were used to phosphorylate GST-Ct and GST-Rb for indicated time intervals (right panel). (D) p13<sup>suc1</sup> sepharose was used to deplete CDK1/cyclin B1 from mitotic lysates. Lysates were reconstituted with increasing amounts of recombinant CDK1-cyclin B1 (top panel) and used to phosphorylate GST-eIF4G Ct and GST-Rb (bottom panel).

To further confirm our observations, we tested if immunodepletion of CDK1/cyclin B1 with anti-cyclin B1 antibody affects eIF4G(S1232) phosphorylation. We were not able to co-deplete CDK1 with cyclin B1 IP, possibly because the antibody is in excess and recognizes the epitope within the CDK1 binding site. However, depletion of cyclin B1 alone was sufficient to reduce eIF4G(S1232) phosphorylation substantially (Fig. 20C). This result supports dependence of S1232 phosphorylation on CDK1, since cyclins are essential co-factors of CDK kinases. Finally, we depleted HEK293 cell lysates of all CDKs by incubating nocodazole-arrested lysates with the yeast p13suc1 protein, followed by reconstitution with recombinant CDK1/cyclin B1 (Fig. 20D). Addition of 5-10 ng of recombinant CDK1/cyclin B1 restored eIF4G(S1232) phosphorylation to endogenous levels, although reconstitution at this level provided an excess of cyclin B1 and did not completely restore CDK1 levels (Fig. 20D; top panel). Taken together, our observations suggest that CDK1/cyclin B1 phosphorylates eIF4G directly at S1232 during mitosis.

#### **3.3.2.6 Over-expression of the C-terminal domain of eIF4G with a S1232A mutation fails to stimulate c-myc IRES-dependent translation in mitosis**

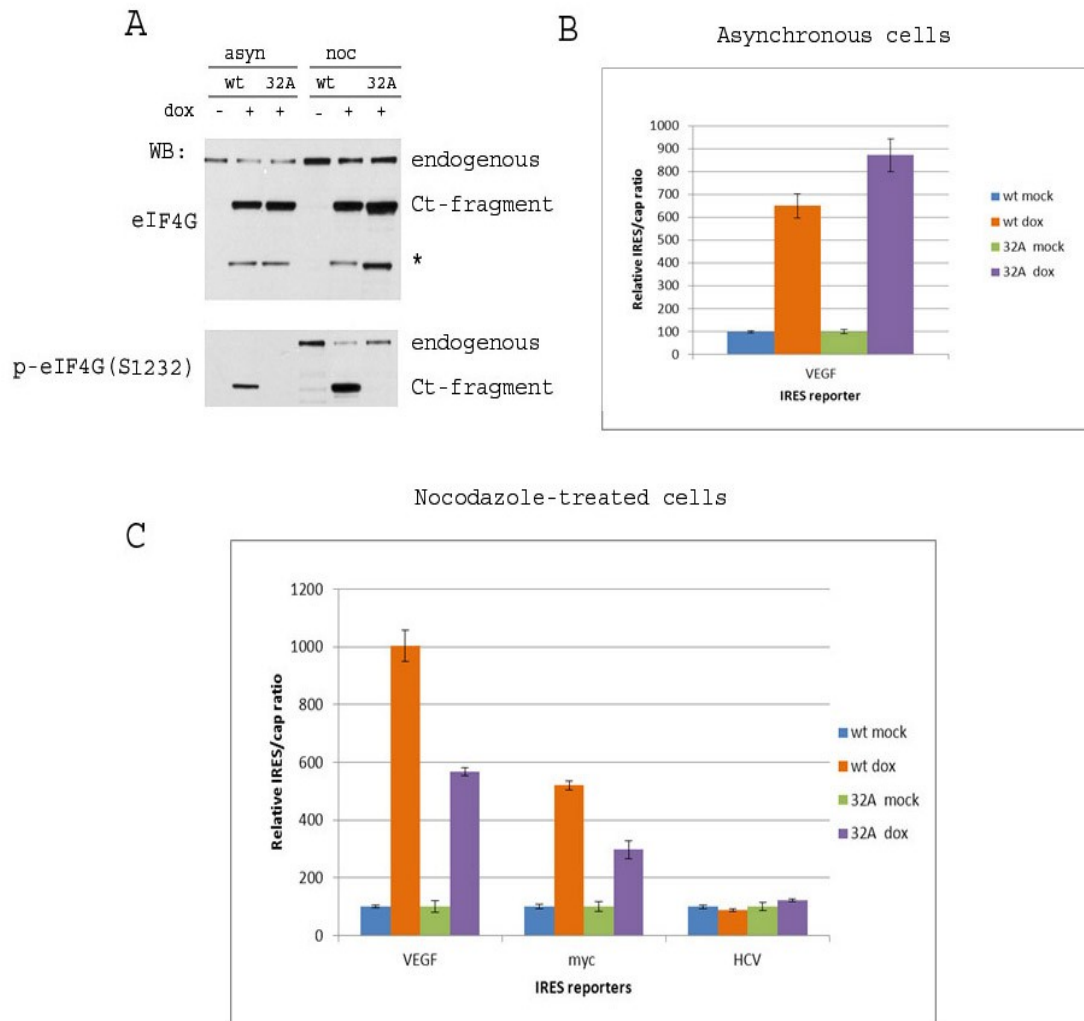
Next, we aimed to test the functional significance of S1232 phosphorylation in mitosis. Comparison of wt and S1232A full-length over-expressed eIF4G showed that mutation of S1232 to Ala does not affect the binding of eIF4E, eIF4A, PABP or eIF3 to eIF4G (data not shown). This result was expected, since the interaction patterns of these proteins

with eIF4G do not change upon cell cycle arrest in mitosis. It is possible that S1232 phosphorylation affects interaction of eIF4G with some novel binding partners or binding to mRNA. Moreover, it was shown that interaction of eIF4G with eIF4A is highly dynamic (Marintchev, Edmonds et al. 2009), thus our co-immunoprecipitation techniques may not be able to faithfully recapitulate fine-tuning of such interactions.

Instead, we decided to functionally probe the significance of eIF4G phosphorylation at S1232. To this end we assessed the effect of overexpressed C-terminal fragment of eIF4G (eIF4G-Ct) with the S1232A mutation on translation of several IRES-containing reporters. eIF4G-Ct encompasses aa 682-1600 of eIF4G and corresponds to the fragment that is generated by cleavage with the poliovirus 2A protease. It interacts with eIF4A and eIF3 and binds directly and facilitates translation of poliovirus mRNA, which lacks the canonical cap structure and initiates translation via an IRES. A class of mRNAs is still translated in poliovirus-infected cells where eIF4G is cleaved, suggesting that eIF4G-Ct can support the translation of certain cellular IRES-containing mRNAs (Johannes and Sarnow 1998). Indeed, several mRNAs, such as c-myc and VEGF, upon inhibition of cap-dependent translation can initiate translation in a cap-independent manner via binding of RNA binding proteins and/or translation initiation factors directly to their 5'UTR (Stoneley, Paulin et al. 1998) (Stein, Itin et al. 1998). Indeed, over-expression of wild-type eIF4G-Ct stimulates c-myc and VEGFA IRES

driven translation of RNA reporters both in vitro and in vivo (Hundsdoerfer, Thoma et al. 2005) (Kaiser, Dobrikova et al. 2008).

To test if the S1232A mutation impairs eIF4G-Ct function, we created a stable cell line that upon induction with doxycycline (dox) over-expresses either wt or S1232A mutant eIF4G-Ct. Upon treatment of cells with nocodazole, wt eIF4G-Ct is efficiently phosphorylated on S1232 and this phosphorylation is absent with S1232A mutant eIF4G-Ct (figure 21A). First, we compared the stimulation of the VEGF IRES reporter in wt and S1232A eIF4G-Ct dox-induced cell lines that were not synchronized. We transfected cells with two types of reporters: uncapped Renilla luciferase reporter which contains the IRES in the 5'UTR and m7G-capped Firefly luciferase reporter under control of the b-globin leader, which serves as a transfection control. We showed before that translation at m7G-capped b-globin leader driven reporters is not affected by ectopic eIF4G-Ct expression (Kaiser, Dobrikova et al. 2008). Thus, the ratio of Renilla to Firefly luciferase serves as a readout of IRES-dependent translation. In asynchronous cells wt and S1232A proteins stimulate VEGF-driven translation to the same extent, to about 7 and 8-fold, respectively. This is expected, since in asynchronous cells, only a small amount of wt eIF4G-Ct is phosphorylated (in 5% of cells that are mitotic). However, when cells were synchronized, the S1232A eIF4G-Ct stimulation of c-myc and VEGF IRES translation was about 50% of that of a wt protein.



**Figure 21 C-terminal fragment of eIF4G with S1232A mutation fails to stimulate myc IRES-dependent translation**

(A) HEK-293<sup>eIF4G<sup>Ct</sup></sup> cells were un-induced (dox -) or dox-induced (dox +) and were either mock-treated (asyn) or synchronized with 5nM thymidine for 16 hours and then treated for 13 hours with 100nM nocodazole (noc) Cells were lysates were submitted to immunoblotting with antibodies to total eIF4G and phosphospecific antibody to S1232. Endogenous eIF4G and over-expressed C-terminal (Ct) fragment are indicated, \*-demarks degradation product of Ct. (B, C) Cells were synchronized as in (A) and 7 hours prior to lysis were transfected with indicated RNA reporters. RLuc activity (VEGF, myc and HCV IRES) and Fluc activity (capped b-globin) were measured and RLuc/Fluc ratio was calculated and normalized to mock-induced cells for each cell line. (B) asynchronous cells, (C) nocodazole-treated cells. Standard error is indicated.

This data indicates that S1232 phosphorylation is important for the stimulation of IRES-dependent translation by eIF4G-Ct. The hepatitis C virus IRES (HCV) serves as a negative control here, since the HCV IRES binds directly to the small ribosomal subunit and does not require eIF4G for its translation.

### **3.3.2.7 Creation of knock-down/rescue eIF4G stable inducible cell lines**

Experiments with over-expressed mutated eIF4G-Ct were important to demonstrate the functional difference between wt and S1232A proteins. However, these experiments can recapitulate the physiological situation only to an extent. First, eIF4G-Ct lacks PABP and eIF4E binding sites, and second, translation is always measured in the presence of endogenous eIF4G, which is phosphorylated in mitotic cells.

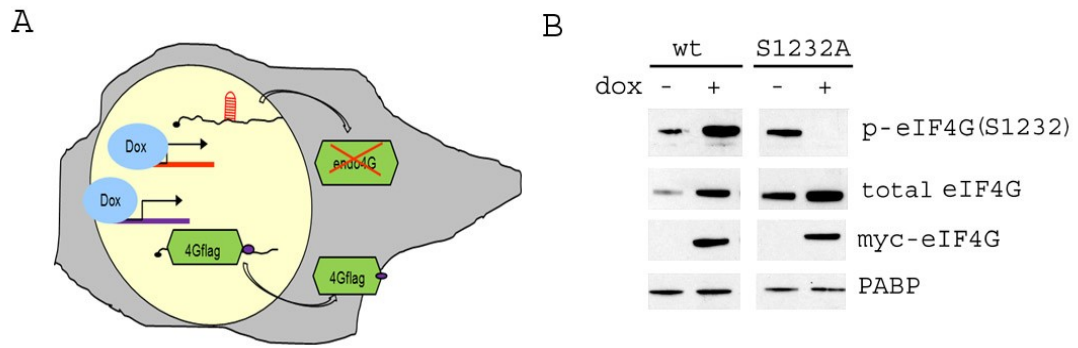
To assess the function of S1232A phosphorylation in more relevant conditions, we devised a knock-out/rescue strategy to replace endogenous eIF4G with the over-expressed S1232A version. We created a stable inducible cell line that upon induction with doxycycline would express both tagged eIF4G variants and shRNA that targets endogenous eIF4G for degradation (figure 22A). To generate a stable inducible eIF4G knock-down cell line, we transfected HEK293 Flpn T-Rex cells with a construct encoding a shRNA that is directed to the 3'UTR of endogenous eIF4G. Stable clones were selected and analyzed for the efficiency of the eIF4G knock-down. Next, these cells were used to

stably incorporate a tagged eIF4G (isoform B) that is resistant to shRNA-mediated knock-down. We confirmed that after 48 hours of induction, endogenous eIF4G is replaced with the S1232A phosphomutant variant, since despite the presence of high quantities of total eIF4G in these cells, no signal was detected with the S1232-phosphospecific antibody (figure 22B). We are conducting further experiments to test if these cell lines have defects in mitotic translation, either globally or for select mRNAs.

### **3.3.3 Discussion**

In this study we assessed the phosphorylation status of eIF4G in mitosis and investigated the role of CDK/cyclin B1-mediated phosphorylation of eIF4G at S1232 on IRES-dependent translation in mitosis. We collaborated with the Duke Proteomics facility to perform phosphoproteomics of purified tagged full length eIF4G, that was over-expressed in either nocodazole-arrested (mitosis) or thymidine blocked (interphase) HEK293 cells. We also identified several proteins that are enriched in eIF4G precipitates from mitotic cells. Several of them are RNA-binding proteins, such as SRSF3 and DDX3X, which have a previously described role in translation. (Fitzgerald and Semler 2011) (Geissler, Scholze et al. 2011). It remains to be determined which factors dictate changes in eIF4G interactions upon mitotic entry.

This is the first study to identify an eIF4G phosphorylation event in a targeted small-scale phosphoproteomic analysis. Earlier studies of eIF4G phosphorylation



**Figure 22 Creation of inducible stable knock-down/rescue cell lines for eIF4G**

(A) Strategy to replace endogenous eIF4G with tagged over-expressed variant. See text for details. Briefly, treatment of stable cell lines with dox induces expression of artificial miRNA targeting endogenous eIF4G and resistant tagged full-length eIF4G, either wt or S1232A mutant (B) Stable cell lines from (A) were uninduced (dox -) or dox-induced (dox +) and treated with TPA for 15 minutes (to stimulate S1232 phosphorylation). Cell lysates were submitted to immunoblotting with antibodies to total eIF4G, phosphospecific antibody to S1232, and anti-myc antibody that specifically recognizes tagged over-expressed eIF4G.

demonstrated the presence of at least 10 phosphosites in eIF4G, however, the identity of only three of them was determined (S1148, S1188, S1232) (Raught, Gingras et al. 2000).

We identified 9 phosphosites, out of which 4 were up-regulated in mitosis (more than 2-fold), four were down-regulated, and 1 was unchanged in relative intensity of the peptide between two samples. This method only allows comparison of phosphopeptide intensities between two samples, thus we cannot explicitly predict if the low/high intensity of the phosphopeptide identified in mass spectrometry correlates with overall low/high level of phosphorylation of this site. However, we focused our efforts on the S1232 phosphosite, since it seems to be one of the major phosphosites in eIF4G, as determined by radioactive labeling (Raught, Gingras et al. 2000).

Next, we confirmed phosphorylation of S1232 in nocodazole-arrested HEK293 cells. Phosphorylation was at maximum in mitosis and rapidly decreased when cells entered the cell cycle. Moreover, phosphorylation of S1232 was enriched in untreated mitotic cells, isolated by the mechanical shake-off procedure. This confirms that phosphorylation of S1232 is specific to mitosis, rather than to nocodazole treatment.

The amino acid sequence around S1232 is very similar to the consensus of CDK and MAPK kinases (figure 19). Utilizing inhibitors of the CDK1 and MAPK pathways,

we demonstrated that S1232 phosphorylation is mediated by Erk1/2 upon phorbol ester stimulation and by CDK1 in mitosis. In vitro kinase assays utilizing CDK1-depleted extracts reconstituted with recombinant CDK1/cyclin B1 confirmed that CDK1/cyclin B1 can directly phosphorylate eIF4G at S1232.

Dual phosphorylation of proteins by different classes of kinases with similar consensus is a widely recognized phenomenon. For instance, caspase 9 is also phosphorylated by CDK1/cyclin B1 in mitosis and by Erk1/2 during G1/S transition (Allan and Clarke 2007). Interestingly, although we saw up-regulation of Erk1/2 activity in G1 phase, we did not detect a corresponding increase in S1232 phosphorylation. Phorbol esters are very potent activators of Erk1/2, thus, it is plausible that the effects of TPA treatment may be exaggerated compared to the cell cycle-dependent stimulation of Erk1/2 in the G1 phase.

Interestingly, identical modification of eIF4G in response to two different stimuli suggests that phosphorylation of eIF4G may play similar roles in response to growth signaling and during mitosis. These data are in accordance with finding that mTORC1, the major growth signaling regulated kinase, is also active during mitosis.

These results are counterintuitive, since protein synthesis is activated during mitogenic stimulation and inhibited in mitosis. However, it has been shown that despite general down regulation of mitotic translation, certain mRNAs, such as c-myc and ODC are actively recruited to ribosomes (Pyronnet, Pradayrol et al. 2000). Since these mRNAs are capable of translation via IRESs, their translation escapes the mechanism of translation down-regulation. Indeed, eIF4G was shown to be required for the translation of c-myc RNA (Spriggs, Cobbold et al. 2009), thus, it is plausible that phosphorylation of eIF4G is required for translational of certain mRNAs in mitosis.

As follows, we aimed to investigate whether phosphorylation of eIF4G is functionally relevant for the translation of c-myc RNA. Previously, we reported that over-expression of the C-terminal fragment of eIF4G stimulates myc IRES dependent translation (Kaiser, Dobrikova et al 2008). We tested if abolishing S1232 phosphorylation affects translation of the myc IRES reporter. Interestingly, in interphase cells, S1232A eIF4G-Ct stimulated myc IRES translation to the same extent as the wild type protein, probably due to the inherently low levels of phosphorylation of wild type eIF4G in asynchronous cells. However, in mitotic cells, S1232A eIF4G-Ct failed to stimulate myc IRES translation as potently as the wild-type protein, suggesting that this phosphorylation is required for the full activity of eIF4G.

Preliminary experiments with over-expression of S1232A eIF4G Ct illuminated the potentially important role of this phosphorylation for mitotic translation of certain mRNAs. However, results of these experiments possess two inherent flaws: (i) since it was not possible to highly over-express full length protein, we utilized a fragment of eIF4G that lacks both PABP and eIF4E binding sites; (ii) over-expressed cells still contain endogenous eIF4G that is fully phosphorylated in mitotic cells.

To address these issues we created stable inducible cell lines in which endogenous eIF4G was efficiently replaced with over-expressed tagged wt or S1232A phosphomutant eIF4G. The next experiments I plan to conduct will focus on the translation efficiencies of these cells in mitosis.

## **4. Conclusions and future directions**

In this dissertation, I studied the role of eIF4G in translation control with viral infection, mitogenic stimulation and cell division. The main function of eIF4G is to bring multiple translation initiation factors together and to locate the small ribosomal subunit to the vicinity of the start codon; thus, I focused my studies on the regulation of eIF4G interactions with its binding partners.

### ***4.1 Insights on PABP gained from studying HSV-1 infection***

We discovered that in HSV-1 infected cells, PABPC1 dissociates from eIF4G and relocates to the nucleus (Dobrikova, Shveygert et al 2010). Although later studies confirmed our findings, several questions remain to be elucidated. The main question is, of course, if re-localization of PABPC to the nucleus is beneficial or deleterious to the virus.

It is possible that PABPC re-distribution to the nucleus is a part of the antiviral strategy. For instance, Burgess et al have shown that several types of stress, such as gamma-irradiation and oxidative stress also lead to PABPC accumulation in the nucleus (Burgess, Richardson et al. 2011). Since HSV-1 infection also induces the oxidative stress

response, it is plausible that this causes PABP re-localization to the nucleus (Salaun, MacDonald et al 2010).

On the other hand, multiple lines of evidence suggest that the virus may purposefully re-direct PABPC to the nucleus to block host cell gene expression. Both KSHV (gamma herpes virus) and HSV-1 (alpha herpes virus), encode unrelated proteins, SOX and ICP27, respectively, which can re-distribute PABPC to the nucleus (Lee, Glaunsinger, 2009) (Dobrikova, Shveygert 2010). Moreover, PABPC is required for SOX-mediated host protein synthesis shutoff and its relocalization may contribute to this process. Furthermore, PABPC accumulation in the nucleus induces mRNA hyperadenylation (unusually long polyA tails), blocking mRNA export (Kumar and Glaunsinger, 2010). In line with these discoveries, Borah et al has shown that KSHV encodes highly abundant long noncoding polyadenylated nuclear (PAN) RNA, which in turn may bind the excess of nuclear accumulated PABPC and stimulate the export of viral mRNAs (Borah, Darricarrere et al. 2011). Interestingly, no gene encoding similar long noncoding RNA was found in the HSV-1 genome. It remains to be determined how HSV-1 protects its mRNAs from the toxic effects of PABPC compartmentalization in the nucleus.

Another unanswered question is how HSV-1 mRNAs successfully translate in the absence of cytoplasmic PABP. One hypothesis is that some viral proteins can substitute for its function, bridging 5' and 3' ends of the mRNA independent of PABP. For instance, the rotaviral non-structural protein 3 (NSP3) binds both to the 3'UTR of the viral mRNA and to eIF4G, circularizing viral templates. Although proteomics analysis of eIF4G-interacting proteins from HSV-1infected cells was performed and ICP6 was identified as an eIF4G binding partner, no evidence exists that this interaction can promote mRNA circularization (Walsh and Mohr, 2006).

On the other hand, 20% of total PABPC is still present in the cytoplasm of infected cells. It is possible that this reduced amount of PABPC is sufficient to sustain viral translation, especially considering that competition with cellular mRNAs is absent due to blocked transcription and splicing. Importantly, we also documented that in HSV-1 infected cells less PABPC is bound by the PABP inhibitor Paip2 (Dobrikova, Shveygert et al 2010). Upon binding to Paip2, PABP dissociates both from eIF4G and the polyA-tail (Khaleghpour, Kahvejian et al. 2001) (Karim, Svitkin et al. 2006). Since Paip2 binds to PABP with ~60 times higher affinity than eIF4G (Karim, Svitkin et al. 2006), it should outcompete eIF4G for binding to PABP in infected cells, restoring the PABP-Paip2 complex. However, we reported that some PABP still binds eIF4G even in the presence of free Paip2. One possible explanation for this is that HSV-1 acts on Paip2 to

block its inhibitory function and provides PABP that is available for translation. Although Paip2 is an important regulator of PABP function, only one study so far has demonstrated how Paip2 function may be regulated. Yoshida et al reported that Paip2 can directly bind to the E3 ubiquitin ligase EDD and when PABP levels drop, Paip2 that is not bound to PABP is quickly degraded by the proteasome (Yoshida, Yoshida et al. 2006). However, upon HSV-1 infection, levels of Paip2 remain unaltered, indicating that the pathway of proteosomal degradation is suppressed. Thus, it seems obvious to perform a comprehensive study of Paip2 interactions in mock and HSV-1 infected cells to search for the possible effectors of Paip2 function.

## ***4.2 Role of Mnk kinases in translation***

In chapter 3.2 we reported that MAPK phosphorylation of the Mnk1 active site leads to Mnk1 conformational changes, allowing it to bind eIF4G and subsequently phosphorylate eIF4E (Shveygert, Kaiser et al, 2010). We also investigate interactions of eIF4G with the nuclear isoform of Mnk1 (Mnk1b) and Mnk2, another member of Mnk family of kinases. Moreover, we demonstrated that Mnk1 dissociates from eIF4G after the phosphorylation reaction is complete. Our study opens up several intriguing questions, some of which are mentioned below:

1. How many proteins that are relevant for translation are phosphorylated by Mnk1? Does phosphorylation of these substrates require Mnk1-eIF4G binding? Can Mnk1 phosphorylate eIF4G?
2. Do Mnk1 and Mnk2 have isoform specific functions?
3. What is the role of the Mnk1b nuclear isoform?

Even though eIF4E is the best-characterized Mnk1 substrate, several other proteins pertinent to translation were reported to be phosphorylated by this kinase. For instance, upon stress induction, Mnk1 phosphorylates heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), which is a shuttling RNA binding protein that plays a myriad of roles in the mRNA post-transcriptional regulation, including mRNA maturation, export, stability and translation. Phosphorylation of hnRNP A1 leads to its relocalization to the cytoplasm and accumulation in stress granules, which are important cellular complexes that contain translationally repressed mRNAs (Guil, Long et al. 2006). However, direct interaction of Mnk1 with hnRNP A1 has not been demonstrated. Moreover, hnRNP A1 lacks the characteristic acidic-aromatic region that is required for Mnk1 binding to eIF4G (Morino, Imataka et al, 2000). Since hnRNP A1 binds mRNAs, it is plausible that Mnk1 phosphorylates this substrate while bound to eIF4G. Alternatively, unidentified protein may act as a scaffold to bring Mnk1 into the vicinity of hnRNP A1. Determining all Mnk1 interactions, for instance, via immunoprecipitation

followed by mass spectrometry or utilizing yeast two-hybrid seems like an attainable and important goal.

It would also be interesting to determine whether Mnk1 can phosphorylate eIF4G. It has been recently reported that Mnk2 inhibits phosphorylation of eIF4G on S1148. However, the mechanistic details of this inhibition are unclear, since even kinase-dead Mnk2 is capable of blocking the phosphorylation (Hu, Katz et al 2011). One explanation is that the kinase-dead mutant of Mnk2 may also bind strongly to eIF4G, displacing the putative kinase for S1148. It would be interesting to determine whether Mnk1 or Mnk2 are capable of directly phosphorylating eIF4G.

It is also unclear whether Mnk1 and Mnk2 have isoform specific functions. Mnk1 needs to be activated to bind eIF4G and phosphorylate eIF4E (Scheper, Morrice et al. 2001). Mnk2, on the contrary, is active even in unstimulated cells and its interaction with eIF4G does not increase upon MAPK activation (Scheper, Morrice et al. 2001) (Shveygert, Dobrikova et al 2010). Furthermore, Mnk2 contains multiple other phosphorylation sites which may also be positively or negatively regulated by yet unidentified signaling pathways. Although Mnk1 and Mnk2 bind to the same region in eIF4G, they are present in the cell in much lower quantities than eIF4G, indicating that in physiological conditions they do not compete for eIF4G binding (Nagaraj, Wisniewski

et al. 2011). It would be of interest to determine the upstream activators of Mnk2, as well as additional downstream targets.

Finally, we reported that the nuclear isoform of Mnk1, Mnk1b, binds strongly and constitutively to eIF4G. This kinase is also constitutively active, thus, does not require MAPK activation to phosphorylate eIF4E (O'Loughlen, Gonzalez et al. 2004). Although, Mnk1b is physically separated from the bulk of cytoplasmic eIF4E and eIF4G by the nuclear envelope, traces of both eIF4E and eIF4G were detected in the nucleus (Rong, Livingstone et al. 2008) (McKendrick, Thompson et al. 2001). So far, the nuclear function of these proteins has not been defined in a comprehensive manner, albeit it was shown that eIF4G associates with the nuclear cap-binding complex (CBC) and may facilitate exchange of nuclear CBC to eIF4E. Phosphorylation of eIF4E, in turn, was proposed to be important for the nucleo-cytoplasmic transport of growth-promoting mRNAs, including cyclin D1 (Topisirovic, Ruiz-Gutierrez et al. 2004). Thus, it would be interesting to determine if Mnk1b may phosphorylate eIF4E in the nucleus.

### ***4.3 Regulation of mitotic translation***

In Chapter 3.3 we investigated phosphorylation of eIF4G and reported that CDK1/cyclin B1 is responsible for the increased phosphorylation of eIF4G at S1232 in mitosis. Our preliminary data suggests that phosphorylation of S1232 may be required for the

stimulation of c-myc IRES-dependent translation by eIF4G. Additional experiments are required to fit these findings into the broad picture of translational regulation during the cell cycle.

First, it is necessary to investigate how this phosphorylation event affects the structure of eIF4G. S1232 is located at the border of the flexible linker and the structured HEAT-2 domain. No recognized eIF4G interaction partner binds in this region. Flexible regions of the proteins often have regulatory functions and are subject to post-translational modifications. If we think of structured regions as a rigid skeleton, then the flexible regions appear as hinges that provide plasticity and modulate conformational changes of the protein. For instance, eIF4A has two binding sites on eIF4G, and eIF4A-eIF4G interaction is continuously re-arranged depending on the ATP binding/hydrolysis cycle of the helicase during 5'UTR unwinding (Marintchev, Edmonds et al, 2009). Thus, phosphorylation of the flexible region may change the three-dimensional structure of the protein. Moreover, our group has previously demonstrated that phosphorylation of eIF4G(S1186) affects the binding of Mnk1 to eIF4G despite the binding region being localized in the HEAT-3 domain (1400-1600aa). Thus, further biochemical studies are required to address precisely how eIF4G phosphorylation affects intramolecular interactions within the protein, as well formation of eIF4F complex.

Next, it is important to define whether CDK1 binds directly to eIF4G and whether it can phosphorylate other sites on eIF4G or other translation initiation factors. One important goal for this study is to determine whether mitotic phosphorylation of eIF4G affects mitotic translation. We have already developed S1232A eIF4G knock-down/rescue cell lines and demonstrated that we can effectively replace wild type endogenous protein with mutated counterparts. If we want to study the effect of eIF4G phosphosites on the function of the protein, it seems crucial to perform the rescue experiments with eIF4G that is mutated on all phosphorylation sites that are upregulated in mitosis. These cell lines can be further analyzed for cell division defects or induction of apoptosis, etc.

Another exciting direction would be to analyze novel proteins that bind to eIF4G in a cell cycle dependent manner, regardless of whether eIF4G phosphorylation controls their binding to eIF4G. Since at least several of these proteins are known regulators of translation for specific mRNAs, it would be of interest to determine whether their binding to translation initiation apparatus in mitosis may be relevant to overall inhibition or selective mRNAs activation of translation.

Finally, the global question that everyone who works on mitotic translation should keep in mind is: what is the scope of selective translation and does it play functional significance in the correct progression of events during cellular division?

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## Biography

Mayya Shveygert was born to Irina and Vitaliy Schweigert on September 6, 1984 in Novosibirsk, Russia. In 2006 she received a Honors Specialist Diploma (5 year degree) in Molecular Biology from Novosibirsk State University, Novosibirsk, Russia.

In the same year Mayya enrolled in the Graduate school at Duke University and worked on her Ph.D. thesis in the laboratory of Dr. Matthias Gromeier. While at Novosibirsk State University, she met a fellow scientist, her future husband, Pavel Nikitin. In 2011 they welcomed their first daughter, Maria Nikitina. While in graduate school, Mayya has received multiple fellowships and awards, and published 6 peer-reviewed manuscripts. Mayya is looking forward to her future life in California!!!

### PUBLICATIONS

- Shveygert M, Bradrick S, Kaiser C, Gromeier M. Regulation of eIF4E phosphorylation by MAPK occurs through modulation of Mnk1-eIF4G interaction Mol. Cell. Biol. 2010 Nov;30(21):5160-7
- Lawson S, Dobrikova E, Shveygert M, Gromeier M p38-alpha MAPK depletion and attenuation of signal transduction to translation machinery by miR-124 and -128 in neurons Mol. Cell. Biol. In revision

- Goetz C, Dobrikova E, Shveygert M, Dobrikov M, Gromeier M. Oncolytic poliovirus against malignant glioma. *Future Virology*, Sep 2011, Vol. 6, No. 9, Pages 1045-1058.
- Dobrikov M, Dobrikova E, Shveygert M, Gromeier M. Phosphorylation of eIF4G1 by PKC $\alpha$  Regulates Interaction with Mnk1. *Mol. Cell. Biol.* 2011 Jul;31(14):2947-59.
- Vikhrova MA, Shveygert MV, Khrapov EA, Filipenko ML, Gileva IP, Morozova VV, Tikunova NV. Selection of naturally occurring autoantibodies to interleukin-18 from phage display library. *Hum Antibodies*. 2010;19(2-3):71-8.
- Dobrikova E\*, Shveygert M\*, Walters R, Gromeier M. Herpes simplex virus proteins ICP27 and UL47 associate with polyadenylate-binding protein and control its subcellular distribution. *J Virol*. 2010 Jan;84(1):270-9. (\*Equal contribution)
- Kaiser C, Dobrikova EY, Bradrick SS, Shveygert M, Herbert JT, Gromeier M. Activation of cap-independent translation by variant eukaryotic initiation factor 4G in vivo. *RNA* 2008 Oct;14(10):2170-82.
- Bradrick SS, Dobrikova EY, Kaiser C, Shveygert M, Gromeier M. Poly(A)-binding protein is differentially required for translation mediated by viral internal ribosomal entry site. *RNA* 2007 Sep;13(9):1582-93.

#### AWARDS AND FELLOWSHIPS

- Department of Molecular Genetics and Microbiology Chairman's Travel Award, Duke University (2012)
- Selected poster prize, Symposium on RNA biology, Durham, NC USA (2009)
- Best student oral presentation award, MGM retreat, Durham, NC USA (2009)
- Conference travel fellowship from Duke University Graduate School (2008, 2012)