Relocalization of eIF4E by its binding partners upon stress

By

Rami Sukarieh

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Department of Biochemistry

McGill University

Montreal, Quebec, Canada

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ABSTRACT

Translation is the vital process by which the information contained in messenger RNAs (mRNA) is used to synthesise proteins. This is a highly regulated process that involves a complex machinery and tight control at every step. In eukaryotes, translation initiation is the rate-limiting step and the most tightly controlled. The translation of an mRNA is preceded by multiple post-transcription steps including, splicing, export and chemical modification including the addition of a 5'-end cap structure. The initiation factor eIF4E binds to this cap structure while in the nucleus, and initiates the translation by recruiting the ribosome after export to the cytoplasm. Controlling the availability of eIF4E within the various compartments of the cell has a direct effect on the efficiency of translation initiation and indirectly on the rate of proliferation of the cell. On the other hand, disturbing the level of eIF4E synthesis could lead to various pathologies especially tumour development. eIF4E requires multiple binding partners to fulfill its mission. The factor 4E-T is involved in eIF4E transport, while eIF4G enhances the binding of eIF4E to the cap structure. eIF4E can be inactivated upon sequestration by the 4E Binding Protein (4E-BP) who competes with eIF4G for binding of eIF4E.

The localization of eIF4E inside the cell is clearly critical for its normal function. It is known that many external stresses can influence cellular translation and this prompted us to investigate the effect of such stresses on the cellular localization of eIF4E and to study the role of eIF4E-binding partners under these conditions. The present work demonstrates that, during heat shock and oxidative stress, 4E-BP plays an essential role in controlling the localization of eIF4E to the stress response cytoplasmic foci, known as the stress granules (SGs). In addition, eIF4E is partially retained in the nucleus during heat shock but not in oxidative stress. These observations suggest that upon stress the cellular translation mechanism is delayed or even stopped by reducing the availability of eIF4E to the translation complex. On the other hand, we show that eIF4E nuclear translocation upon poliovirus infection is correlated with eIF4G cleavage. This translocation could favour the shutdown of host cell protein synthesis by reducing the cap dependent translation and preventing mRNA circularization.

In our study, we focused on the role of eIF4E as a key player for cellular survival under stressful conditions. Therefore, identifying reagents that induce the

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relocalization of eIF4E to the nucleus or to SGs could help in the development of anti-proliferative drugs.

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RESUME

La traduction est un processus vital par lequel l'information contenue dans l'ARN messager est utilisée pour la synthèse protéique. Chaque étape de ce procédé est strictement régulé grâce à l'implication d'une machinerie complexe hautement contrôlée. Chez les eucaryotes, l'initiation est l'étape limitante et la mieux contrôlée du processus de traduction. Surviennent ensuite, les étapes dites post-traductionnelles qui incluent, l'épissage, l'exportation nucléaire et les modifications biochimiques. Le facteur de transcription eIF4E lie la coiffe du messager en 5' au niveau du noyau, permettant son exportation au cytoplasme et l'initiation de la traduction grâce au recrutement des sous-unites ribosomales. Le contrôle d'eIF4E dans les différentes parties cellulaires est crucial pour l'efficacité traductionnelle, mais génère également un effet indirect sur la prolifération et division cellulaire. Aussi, un défaut de synthèse ou de niveau d'expression d'eIF4E amènent de nombreuses anomalies, spécifiquement lors du développement tumoral. eIF4E requiert de nombreux partenaires pour agir efficacement. Le facteur 4E-T est impliqué dans le transport d'eIF4E tandis que eIF4G favorise sa liaison a la coiffe. eIF4E est connu pour être inactive par séquestration de 4E-BP, principal compétiteur pour la liaison a la coiffe.

La localisation d'eIF4E au sein de la cellule est critique pour son bon fonctionnement. De nombreux facteurs de stress sont connus pour influencer la traduction cellulaire. Je me suis donc concentre à étudier les effets des facteurs de stress dans la localisation cellulaire d'eIF4E et le rôle respectif de chacun de ses partenaires dans ces conditions. J'ai ainsi démontré que durant un choc de chaleur et un stress oxydatif, 4E-BP joue un rôle essentiel dans le contrôle de la localisation d'eIF4E au niveau des granules de stress. De plus, eIF4E est partiellement retenu au noyau lors d'un stress de chaleur mais pas au cours d'un stress oxydatif. Ces observations suggèrent que lors d'un stress, la machinerie traductionnelle est retardée ou arrêtée par une baisse de la disponibilité d'eIF4E a ce complexe protéique. D'un autre coté, j'ai montré que la translocation nucléaire d'eIF4E au cours d'une infection au Poliovirus est corrélée avec le clivage d'eIF4G. Cette translocation pourrait favoriser l'arrêt de la synthèse protéique de la

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cellule hote en réduisant la traduction dépendant de la coiffe et en prévenant la circularisation du messager.

En conclusion, eIF4E est un élément clé dans la survie cellulaire associée aux conditions de stress. De plus, l'identification de réactifs pouvant induire la relocalisation d'eIF4E au noyau ou aux granules de stress aiderait au développement de composes anti-prolifératifs.

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I would like to thank Dr Nahum Sonenberg for opening the door for me to enter into a highly valuable scientific institution. He gave me an excellent start in his laboratory as well as highly professional advises and suggestions.

I would like to express the deepest appreciation to Dr. Jerry Pelletier for all his supervision. Without his guidance and persistent help this study would not have been possible. He taught me various keys for success mainly perseverance, love for science, and professionalism. But mostly he thought me the meaning of the sentence "I can do it".

Working in a laboratory cannot be accomplished without the technical assistance of multiple supporting staff. For that I would like to thank all the supporting staff in both laboratories. It was a great honour to work with highly dedicated group of technician and research assistant. Having said that, I would like to address a special thanks to Isabelle Harvey for all her assistance and help as well as her good humour and her joyful smile.

I had the opportunity to work with talented graduate students and postdoctoral fellow to whom I am very grateful for all the support, advice and help throughout all those years. I enjoyed working in a very cooperative environment sharing expertise and knowledge. I had the opportunity to collaborate with many students and was rewarded by co-authorships on various papers dealing with different projects. From Sonenberg's lab, I would like to thank Avak kahvejian, Liwei Rong, and mark Livingstone. From Pelletier's lab, I would like to thank Regina Cencic and Betty Lin. From Gallouzi's Lab I would like to thank Rachid Mazroui. I am grateful to other students whose assistance and comments were an essence for a continuous progress. Not to forget someone, I will just thank them all.

I have a special acknowledgement to Francis Robert not only for his outstanding knowledge in the field of translation and his expertise in laboratory tools, but mainly for his generosity in sharing his knowledge with whoever need it. I would like to thank Francis for being who he is.

At last but not least, I would like to thank my family and friends for their unlimited love, care and support. I could not have done it without their presence around me.

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DEDICATION

I would like to dedicate this work to my family. I hope I made them proud of me and compensated a small percentage of their sacrifices.

Inshalla b3eish ta ha2i2lon ahlemon.

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PREFACE

Chapter1

This chapter consist of a general introduction in relation to the topics covered in the following chapters. It represents a review of the recent literature in the subjects of translation and cellular perturbation.

Chapter2

Chapter 2 of this thesis includes the entire text and figures of a published manuscript of which I am the first author.

Sukarieh R, Sonenberg N, and Pelletier J. The eIF4E binding proteins (4E-BPs) are modifiers of cytoplasmic eIF4E relocalization during the heat shock response. *Am J Physiol Cell Physiol* 296:C1207-17, 2009.

All the experiments done in this manuscript were performed by me. The text was a joined effort with my co-supervisors Dr. Jerry Pelletier and Dr. Nahum Sonenberg. I would like to acknowledge the McGill imaging facility members for their help and advices. I would like to thank Dr. Imed Gallouzi for providing essential antibodies for the work as well as fruitful advices.

Chapter3

Chapter 3 of this thesis includes the entire text and figures of a manuscript in pres in the journal of general virology of which I am the first author.

Sukarieh R, Sonenberg N, and Pelletier J. Nuclear assortment of eIF4E coincides with shut-off of host protein synthesis upon poliovirus infection. *JGV in Press.*

All the experiments done in this manuscript were performed by me. The text was a joined effort with my co-supervisors Dr. Jerry Pelletier and Dr. Nahum Sonenberg. I would like to thank Dr. Regina Cencic and Dr. Barbara Herdy for sharing their knowledge in the field of virology. I would like to thank Isabel Harvey for technical assistance.

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Chapter4

This chapter offers a general discussion of the data presented in chapters 2 and 3 in addition to the development of the topic of interest throughout the last ten years and my contribution via three co-authorship publications.

Mazroui R, **Sukarieh R**, et al. Inhibition of Ribosome Recruitment Induces Stress Granule Formation Independent of eIF2α Phosphorylation. *Mol Biol Cell* 17:4212-19, 2006.

Rong L, Livingstone M, **Sukarieh R**, et al. Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. *RNA* 14:1318-27, 2008.

Lin CJ, **Sukarieh R**, and Pelletier J. Silibinin inhibits translation initiation – Implications for anticancer therapy. *Mol. Cancer Ther* 8:1606-12, 2009.

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CHAPTER1:

GENERAL INTRODUCTION

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1.1 Protein synthesis

Protein synthesis is a highly regulated process allowing for multiple levels of control. This process requires multiple factors: a messenger RNA (mRNA) that is exported from the nucleus, several transfer RNA species that read the message, ribosomes that serve as catalytic and organizational centers, a variety of proteins factors including (but not limited to) initiation factors, elongation factors and RNA binding proteins and energy. The information carried in the nucleotide sequence of the mRNA determines the order of alignment of amino acids to form polypeptides. The pool of the RNA/proteins factors assure the exactitude of the protein biosynthesis in term of quality control, energy saving, and response to internal and external cellular stimulus. Newly synthesized protein may undergo post-translational modifications which will influence its location and/or its function. In general, these processes are carried out with speed and precision. Levels of translation are regulated globally and for specific proteins.

1.1.1 RNA feature

RNAs play an essential role in transmitting the genetic information stored in the nucleotide sequences of the nuclear DNA into the site of the cytoplasmic protein biosynthesis. To further understand this mechanism, essential RNA features will be described.

The messenger RNA (mRNA) involved in the process of translation is transcribed in the nucleus by RNA Polymerase II (Fig.1). It will go through the process of maturation that comprises capping, splicing or introns removal, the generation of a 3' end polyadenylation, the transport to the cytoplasm where it will be translated into a polypeptide by the ribosome, and sometimes degradation by nonsense mediated decay (NMD). Along the mRNA journey, many proteins and RNAs bind to it and affect its function and shape, and influence its fate; As a result, the mRNA is directed towards degradation, storage into granules or translation.

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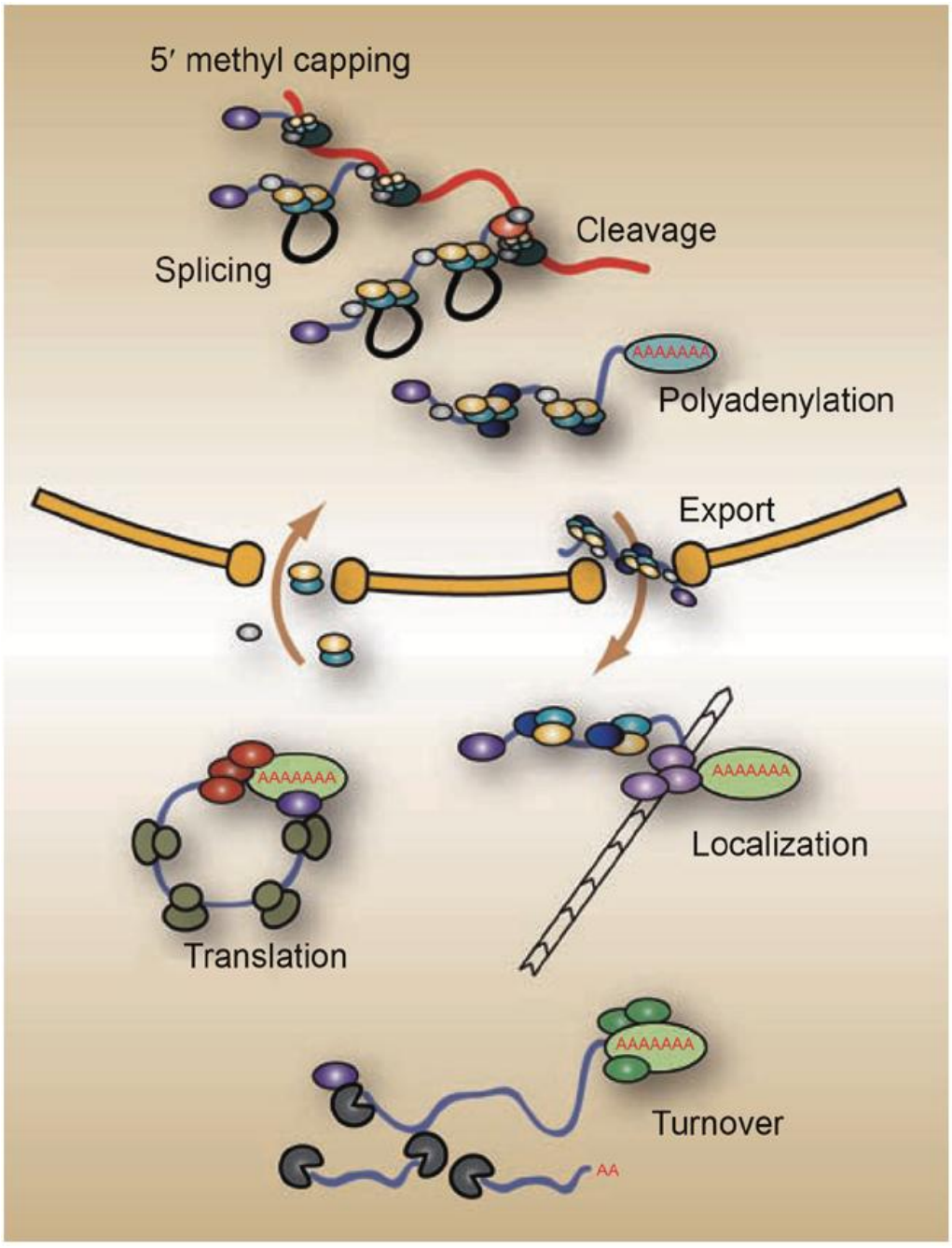

Figure 1: **mRNA life cycle:** The mRNA journey goes from transcription to translation while going through capping, splicing, polyadenylation, exportation, and degradation. Taken from (McKee and Silver 2007).

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Eukaryotic mRNA in the cytosol has several identifying characteristics. In general, it is always monocistronic, i.e encoding a single polypeptide. mRNAs are generally composed of four structures: the cap at the 5'end, two untranslated regions (UTR); the 5'UTR and the 3'UTR, one or multiple Open Reading Frames (ORFs), and the poly (A) tail at the 3'end (Fig.2).

The cap is added to the 5'end of the mRNA and consists of a 7-methylguanosine (m7G) at the 7'position bridged by a 5'-5' triphosphate to the messenger sequence (Shatkin 1976). In the nucleus, addition of the cap structure occurs while the RNA polymerase II transcribes the mRNA. The capping enzyme complex modifies the 5'-end of the nascent mRNA as the transcription complex moves along the DNA. The cap influences mRNA metabolism at multiple levels such as splicing, polyadenylation, export, stability and translation (Izaurralde, Lewis et al. 1994; Cooke and Alwine 1996) (Shatkin 1985; Izaurralde, Stepinski et al. 1992; Ishigaki, Li et al. 2001; von der Haar, Gross et al. 2004). The RNA polymerase transcribes the bulk of the gene including the ORF region as well as the 5'UTR and 3'UTR regions. The 5'UTR region of up to a few hundred nucleotides in length separates the cap from the translation initiation signal (a 3-nucleotide-long codon sequences, AUG). Usually, translation starts upon the detection of this initiation signal (marked as the first sequence), and stops when the termination codon is detected. The area limited by the initiation and termination codons is referred to as one ORF. In some cases, other starting sequences might be encountered and therefore different ORFs are translated. Those ORFs are known as upstream ORFs (uORF). The translation termination codon is followed by the 3'UTR. The 5' and 3'UTR hold regulatory elements that can control the mRNA translation efficiency, its cellular localization as well as its stability. The 5'UTR region may include secondary or tertiary RNA structures such as helices and loops known to hinder translation initiation rates (Koromilas, Lazaris-Karatzas et al. 1992). mRNAs can also contain elements in the 5'UTR regions known as internal ribosome entry site (IRES) which direct translation in a cap-independent manner (Pelletier and Sonenberg 1988) (see section 1.1.4). One example of cis-acting elements found in mRNA is the adenine-uridine rich elements (ARE) in the 3'UTR which influences the mRNA stability and translation by ARE-

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binding proteins (ARE-BPs) (Barreau, Paillard et al. 2005). The 3'end of most eukaryotic mRNAs is dominated by a poly (A) tail that varies in length from 100 to 200 adenosine monophosphates (Jacobson and Peltz 1996). The RNA polymerase continues transcribing the gene until a polyadenylation signal sequence is reached. Then, the nascent mRNA is cleaved 20 nucleotides downstream and the poly (A) synthesis takes place. The poly (A) tail is essential to mRNA stability and translation (Munroe and Jacobson 1990; Wilusz, Wormington et al. 2001; Kahvejian, Svitkin et al. 2005), and it plays a role in mRNA export (Huang and Carmichael 1996).

The translation of mRNAs is highly controlled and the rate-limiting step under various conditions occurs during initiation. The control of translation plays a role in the expression of genes in many processes including cell growth, proliferation and survival. In addition, it has been established that alterations in the translation rates can play a role in cellular transformation.

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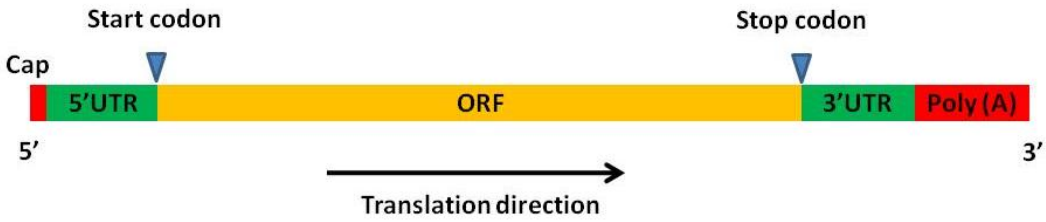

Figure 2: **RNA structures:** Linear structure of mRNA showing at the 5' end the cap structure followed by the 5'UTR region. On the 3'end, the mRNA structure shows the 3'UTR region followed by a poly (A) tail. The open reading frame ORF is delineated by the two UTR regions.

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1.1.2 General mechanism of translation

mRNA translation consists of a series of steps (Fig.3): initiation, elongation, termination and recycling (Mathews 2000). Initiation requires bringing together the 40S ribosomal subunit, an mRNA and a tRNA complex of the amino-terminal amino acid, all in the proper orientation. This is followed by association of the 60S subunit to form the 80S ribosome. During initiation, a ribosome is assembled at the initiation codon in the mRNA with a methionyl initiator tRNA bound in its peptidyl (P) site. In elongation, the aminoacyl tRNAs specified by the next codon of the mRNA is bound to the acceptor (A) site. Selection of the correct aminoacyl tRNA is enhanced by elongation factor EF-1. If the codon-anticodon interactions are correct the aminoacyl tRNA is placed in the (A) site and GTP hydrolysis results in a conformational change of EF1α. The first peptide bond will be formed and the mRNA-peptidyl tRNA complex will be translocated to the (P) site. After the tRNAs and mRNA are translocated such that the next codon is moved into the A site, the process is repeated and additional amino acids are added to the peptide. Termination takes place when a stop codon is encountered and the finished peptide is released from the ribosome. A chain-terminating UAG, UAA, or UGA codon in the (A) site promotes binding of release factor eRF. eRF is similar in size and shape to tRNA, recognizes and interacts with the stop codon. The peptide-tRNA linkage is cleaved by peptidyl transferase and the complete peptide is released from its tRNA and ribosome. In the final stage, eRF is released from the ribosome after GTP hydrolysis and the two ribosomal subunits are free to re-enter the protein synthesis process. This is referred to as recycling.

In eukaryotes, two principal pathways are available for attachment of the small ribosomal subunit to the 5' translated region (Fig.4). These pathways are termed cap-dependent and cap-independent or IRES-dependent (see section 1.1.4) translation initiation. The majority of eukaryotic transcripts are translated in a cap-dependent manner. In the cytoplasm, a cap-binding protein complex, so called eukaryotic Initiation

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Factor 4F (eIF4F) complex, binds to the cap structure which can mediate recruitment of the small subunit 40S of the ribosome to the 5' end of the mRNA.

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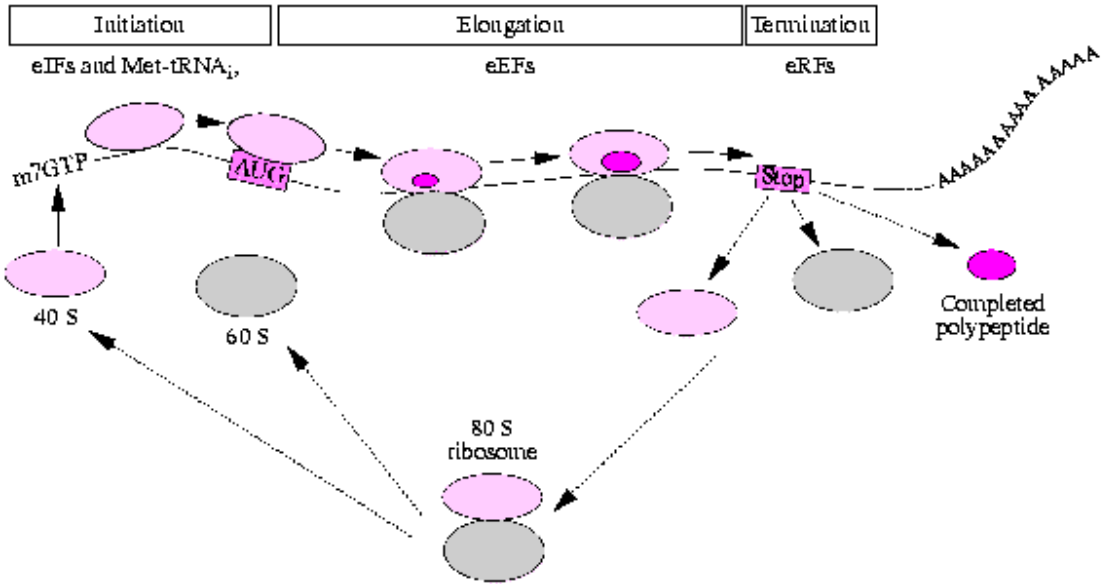

Figure 3: **Translation steps:** The small ribosomal subunit recognizes the mRNA in the presence of multiple proteins and RNAs. The mRNA is scanned for the start codon allowing the addition of the large ribosomal subunit. All this process is called the Initiation step. The Elongation step involves the synthesis of the polypeptide. This is followed by the Termination step once the termination codon is encountered, leading to the dissociation of the two ribosomal subunits. Once the ribosomal subunits are free, they could be re-used in the translation steps again, hence the name Recycling step. Taken from (Proud and Denton 1997).

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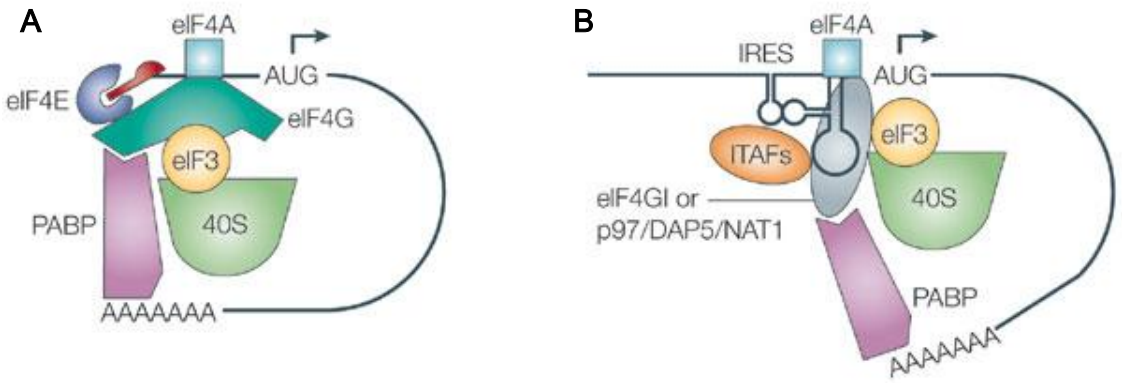

Figure 4: **Cap dependent vs IRES dependent translation. A)** The cap structure of the mRNA is recognized by eIF4E as part of the translation initiation complex. **B)** Translation initiation factors, excluding eIF4E, recognize the IRES element of the mRNA. Taken from (Holcik and Sonenberg 2005)

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1.1.3 Translation initiation mechanism/factors

For the cap-dependent translation initiation to take place, the cumulative function of multiple factors is required. The major complexes involved are the ternary complex and the eIF4F complex. eIF4F is composed of 3 proteins eIF4E, eIF4A and eIF4G (these 3 proteins will be discussed in more details in section 1.2).

The ternary complex is composed of eIF2, GTP and the initiator tRNA; Met-$tRNA_i^{Met}$ (Fig.5). eIF2 is a heterotrimer composed of α, β and γ subunits. The α-subunit contains the main target for phosphorylation, a serine at position 51, therefore considered the regulatory subunit. The ternary complex binds the 40S ribosomal subunit in the presence of eIF3, eIF1 and eIF1A to form the 43S pre-initiation complex (reviewed in (Kapp and Lorsch 2004)). The eIF3 complex consists of 13 protein subunits. It interacts with both the 40S ribosomal subunit and the central domain of eIF4G. This dual interaction is thought to play a role in enhancing the link between the mRNA and the ribosome during initiation (Fraser, Lee et al. 2004; Valasek, Nielsen et al. 2004). In parallel, the eIF4F complex recognizes the capped mRNA and unwinds structures in the 5'UTR with the help of two RNA chaperones eIF4B and eIF4H (Richter-Cook, Dever et al. 1998; Rogers, Richter et al. 1999). eIF4F, in conjunction with eIF3 and the poly (A) binding protein (PABP) bound to the 3′-poly (A) tail, loads the mRNA onto the 43S pre-initiation complex. PABP stimulates translation of poly (A) positive mRNAs (Sachs 2000). The amino-terminal region contains four conserved RNA recognition motifs (RRMs) (Adam, Nakagawa et al. 1986). PABP–eIF4G binding could mediate mRNA circularization (Fig.6), promoting the recycling of terminating ribosomes by bridging the two ends of an mRNA, a model reinforced by the interaction between PABP and the translation termination factor eRF3 (Uchida, Hoshino et al. 2002). A second model suggests that PABP stimulates 60S ribosomal subunit joining. The absence of the poly(A) tail led to a decrease in 60S ribosomal subunit joining (Munroe and Jacobson 1990). The 43S pre-initiation complex then scans the untranslated region in a 5' to 3' direction until the initiation codon is detected resulting in the formation of the 48S initiation complex.

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This scanning process is thought to require ATP hydrolysis. Establishment of proper codon-anticodon base pairing activates eIF5, a GTPase activating protein (GAP) that stimulates GTP hydrolysis. After GTP hydrolysis, eIF2·GDP releases the Met-$tRNA_i$ into the P site of the 40S subunit and then dissociates from the complex. eIF1, eIF1A, eIF3 and eIF5 will be released whereas eIF5B·GTP binds to the complex. The eIF5B-GTP allows the 60S subunit to join and form the 80S initiation complex. GTP is hydrolyzed which causes eIF5B release from the 80S ribosome. The ribosome is now ready to proceed with elongation (Fig.7).

The formation of the ternary complex is controlled by the assembly of eIF2 and GTP. eIF2B catalyzes the exchange between eIF2-GDP and eIF2-GTP with the latter being crucial for the formation of the ternary complex. The phosphorylation of the α-subunit of eIF2 at residue Ser51 inhibits the function of eIF2B, and therefore prevents the formation of the ternary complex. The phosphorylation of eIF2α reduces the translation of most mRNAs since the ternary complex is decisive for most mRNAs with few exceptions. In this latter case, these mRNAs, which encode proteins that function in the adaptation to stress, are enhanced upon eIF2α phosphorylation. (as reviewed in (Hinnebusch 2000)).

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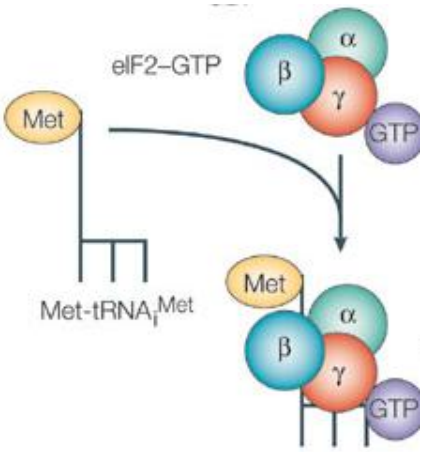

Figure 5: **Ternary complex.** Three members of the ternary complex: eIF2 (which is made of α, β and γ subunit), GTP and the initiator Met-tRNA$_i$. Taken from (Holcik and Sonenberg 2005)

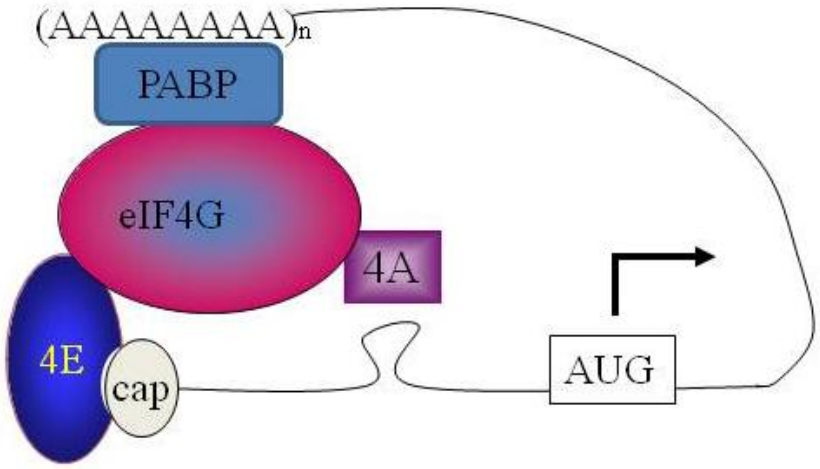

Figure 6: **Circularization of the mRNA.** Circularization of the mRNA requires the binding of eIF4E to the cap structure, the binding of PABP to the poly (A) tail and the binding of eIF4G to the two proteins.

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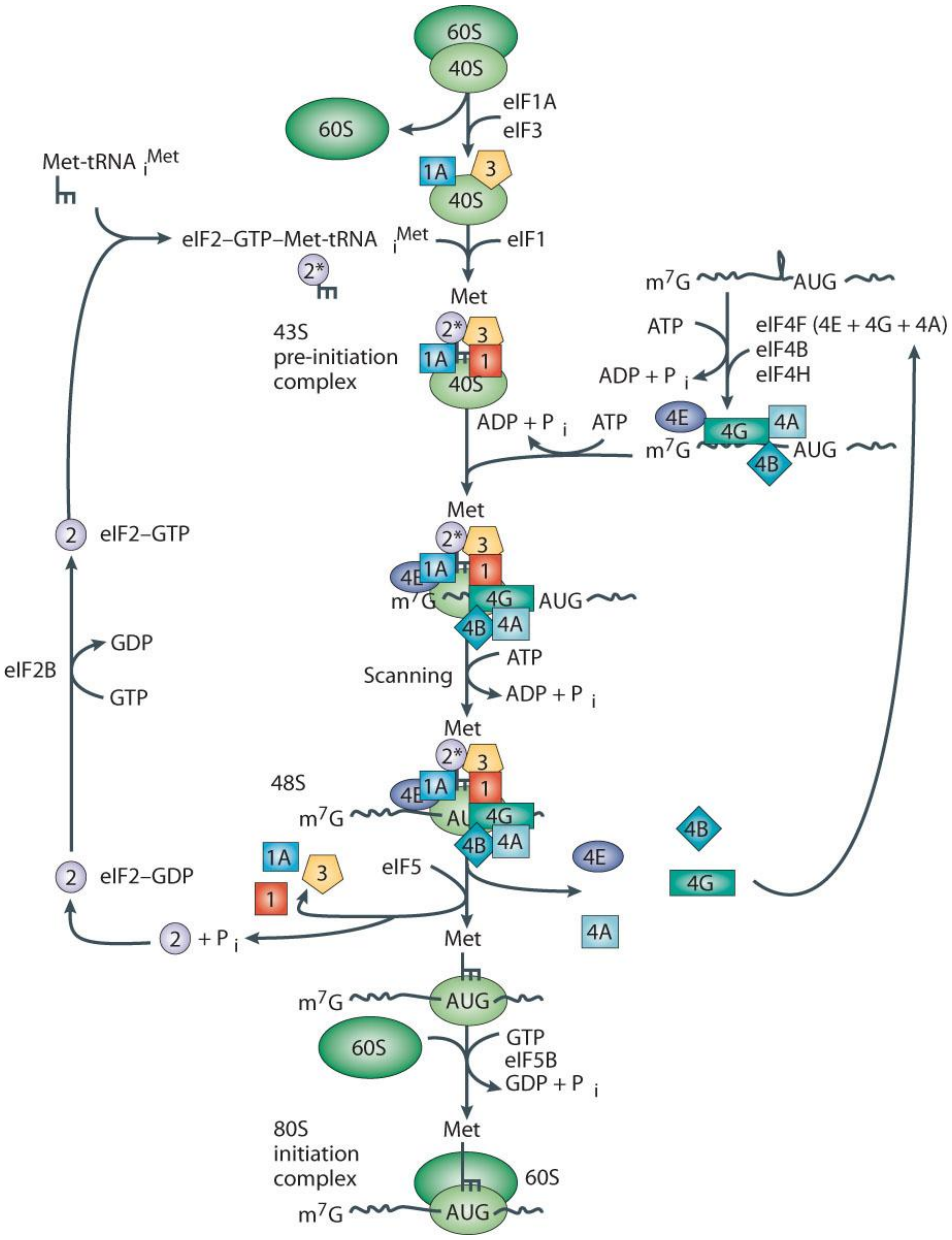

Figure 7: **Eukaryotic translation initiation.** Multiple proteins and RNAs are involved in the complex process of eukaryotic translation initiation. On one hand, the 40S ribosomal subunit binds multiple proteins and the ternary complex to form the 43S pre-initiation complex. On the other hand, eIF4F complex in addition to eIF4H and eIF4B bring the capped mRNA to the 43S pre-initiation complex forming the 48S complex. The 60S ribosomal subunit will join the 40S subunit to form the 80S ribosome, leaving behind the rest of the proteins to be recycled in a new translation initiation process. Taken from (Holcik and Sonenberg 2005).

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1.1.4 IRES dependent translation

Initiation on some cellular mRNAs is cap-independent and is mediated by binding of the ribosome to the IRES elements rather than the cap structure. Cellular IRESs range in size from 60 to 1000 nucleotides in length with a minimal active element around 100 nucleotides (Stoneley and Willis 2004). IRESs are complex RNA structural elements present in the 5'UTRs of certain genes whose protein products are involved in various processes including cell growth and cell death. The IRES elements promote a cap-independent translation initiation mechanism by providing elements to reduce ribosomal scanning from the 5' end of the mRNA. It is thought that initiation factors that can bind both the IRES and the ribosome recruit ribosomal subunits. Otherwise, IRES elements should bind directly the ribosome. The activity of all cellular IRESs depends on auxiliary proteins that are known as ITAFs (IRES *trans*-acting factors). These proteins are thought to act as RNA chaperones which remodel the structure of the IRES into an active configuration (as reviewed in (Sonenberg and Hinnebusch 2007)).

IRESs enhance translation initiation of some mRNAs involved in growth factors, protooncogenes, angiogenesis factors, and apoptosis regulators. This translation is induced under a variety of stress conditions, including hypoxia, serum deprivation, irradiation and apoptosis. Thus, cellular cap-independent translational control might regulate cellular responses in transient stress conditions that would otherwise lead to cell death. On the other hand, the same mechanism is used by viruses to evade the post-infection shutting-off of host protein synthesis (reviewed in (Holcik and Sonenberg 2005)).

1.2 eIF4F complex

eIF4F is a 3-subunit complex composed of eIF4E, eIF4A and eIF4G. eIF4E is the cap-binding protein and is therefore essential for the start of cap-dependent translation initiation (reviewed in (Gingras, Raught et al. 1999)). eIF4A is a member of the DEA(D/H)-box RNA helicase family, a diverse group of proteins that couples ATP

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hydrolysis to RNA binding and duplex separation (Rogers, Komar et al. 2002). eIF4G serves as a scaffold for the coordinated assembly of the translation initiation complex, leading to the attachment of the template mRNA to the translation machinery at the ribosome (Prevot, Darlix et al. 2003).

1.2.1 eIF4E

eIF4E polypeptide is a 24-kD polypeptide that plays an essential role within the eIF4F complex. It is involved in the mRNA-ribosome binding step of eukaryotic protein synthesis. eIF4E is conserved across kingdoms (Fig.8): eIF4E proteins from plants, *Drosophila*, and *Aplysia*, among others, share striking homology to mammalian eIF4E. Yeast (*Saccharomyces cerevisae*) and human eIF4E are 32% identical (Altmann, Muller et al. 1989). eIF4E is best known for its function in the initiation of protein synthesis on capped mRNAs in the cytoplasm. However, recent studies have highlighted that eIF4E has nuclear functions. In the nucleus, the role of eIF4E is to promote the export of some mRNAs such as cyclinD1 and ornithine decarboxylase (ODC) without affecting the export of GAPDH or actin mRNA (Rousseau, Kaspar et al. 1996; Topisirovic, Capili et al. 2002; Topisirovic, Culjkovic et al. 2003). The specificity of eIF4E binding to cyclin D1 mRNA in the nucleus was due to the presence of a 100 nt-sequence in the 3'UTR (Culjkovic, Topisirovic et al. 2005). It was shown that depletion of eIF4E from cell-free extracts drastically reduces translation of capped mRNA whereas full activity is restored by addition of bacterially expressed or purified native eIF4E (Svitkin, Ovchinnikov et al. 1996). This role has been revisited recently where it was shown that inhibition of eIF4E most profoundly influences the protein expression of potent regulators of cell growth and survival as compared to housekeeping proteins such as β-actin (Graff, Konicek et al. 2007). eIF4E mRNA levels are regulated by c-myc, a transcription factor that binds to the promoter region of eIF4E (Jones, Branda et al. 1996; Lin, Cencic et al. 2008). Growth factors stimulation of fibroblasts leads to an increase in c-myc levels that correlate with increased eIF4E mRNA levels (Rosenwald, Rhoads et al. 1993).

Although the majority of eIF4E is cytoplasmic, a significant fraction (12-33%) is also present in the nucleus and localizes with splicing factors into speckles (Lejbkowicz,

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Goyer et al. 1992; Dostie, Lejbkowicz et al. 2000). The nuclear import of eIF4E is mediated by 4E-T, a transporter that binds eIF4E through a conserved motif Tyr-*X*-*X*-*X*-*X*-Leu-Φ (where *X* is variable and Φ is a hydrophobic amino acid) that is also shared by eIF4E-Binding Protein (4E-BP) and eIF4G (Dostie, Ferraiuolo et al. 2000). 4E-T is a ubiquitously expressed protein with nucleocytoplasmic shuttling activity (Dostie, Ferraiuolo et al. 2000). It has one nuclear localization signal (NLS) and two export signals (NES). At steady state, 4E-T is cytoplasmic and concentrated in cytoplasmic foci called P-bodies. P-bodies are cytoplasmic RNA granules that contain components of the 5′–3′ mRNA decay machinery, the nonsense-mediated decay pathway, and the RNA-induced silencing complex (Anderson and Kedersha 2006). 4E-T imports eIF4E into the nucleus by the importin α/β pathway (Dostie, Ferraiuolo et al. 2000) and it is required for eIF4E localization to P-bodies (Ferraiuolo, Basak et al. 2005). In mammals, eIF4E is phosphorylated on Ser209 upon activation by either the p44/42 or p38 MAPKs. The best candidate for the eIF4E kinase is mitogen-activated protein kinase (MAPK)-interacting protein kinase 1 (MNK1) (Fukunaga and Hunter 1997). MNK1 interacts directly with eIF4G, bringing it in close proximity to eIF4E (Pyronnet, Imataka et al. 1999). The effect of eIF4E phosphorylation on translation is subject to controversy. In some cases, eIF4E phosphorylation is linked to an increase in the translation rates (reviewed in (Gingras, Raught et al. 1999; Silva and Wendel 2008)) or for certain viral proteins (Walsh and Mohr 2004), or inversely correlated with translation as it occurs during arsenite stress (Wang, Flynn et al. 1998).

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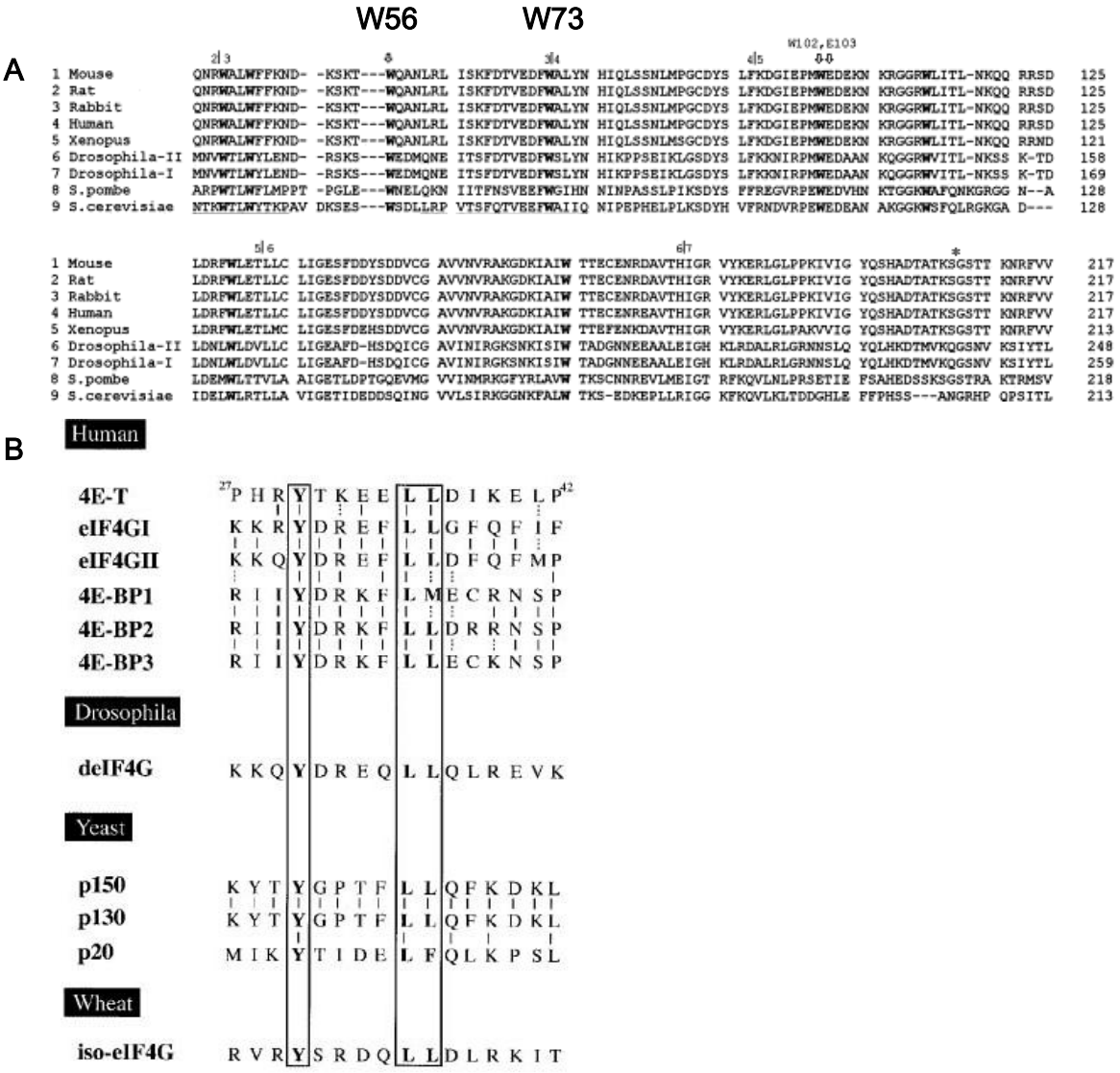

Figure 8: **eIF4E**

A. eIF4E sequence alignment between various species is showing conserved residues in various positions in the polypeptide. The tryptophan W56 involved in the binding of eIF4E to the cap structure is well conserved across species. Similarly, the tryptophan W73 involved in the binding of eIF4E with eIF4G, 4E-T and 4E-BP Taken from (McKendrick, Pain et al. 1999).

B. Amino acid sequence alignment of the eIF4E binding proteins showing the consensus sequence for binding to eIF4E. Taken from (Dostie, Ferraiuolo et al. 2000).

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1.2.2 eIF4A

There are three eIF4A family members: eIF4AI, eIF4AII, and eIF4AIII. eIF4AI and eIF4AII show 90-95% similarity at the amino acid level. They are involved in translation and appear to have the same biological activity *in vitro* (Yoder-Hill, Pause et al. 1993). eIF4AIII is 65% similar to the other isoforms and is implicated in nonsense-mediated decay (Ferraiuolo, Lee et al. 2004; Shibuya, Tange et al. 2004). The eIF4A isoforms are members of the DDX protein family. These and related DEXD/H (where X is any amino acid) box proteins are characterized by seven highly conserved amino acid sequence motifs implicated in RNA remodeling. These proteins are involved in virtually all aspects of cellular RNA metabolism, including ribosome biogenesis, splicing, translation, and mRNA degradation. The helicase activity of eIF4A is thought to unwind local secondary structure in the 5′ UTR of mRNAs to facilitate cap-dependent ribosome recruitment (Pause, Methot et al. 1994; Svitkin, Pause et al. 2001). eIF4A helicase activity is inhibited when associated with the transformation suppressor programmed cell death protein 4 (Pdcd4). Pdcd4 physically associates with eIF4A resulting in inhibition of translation (Yang, Jansen et al. 2003).

1.2.3 eIF4G

The eIF4G family is composed of three large scaffolding proteins (eIF4GI, eIF4GII and p97) (Fig.9). eIF4GI and eIF4GII appear to be functionally equivalent, bind to the same translation factors, and display translation stimulatory activity in vitro and in vivo (Gradi, Imataka et al. 1998). Structurally, eIF4GI and eIF4GII are thought to have three domains: the N-terminal domain is the binding site for eIF4E and PABP, the middle domain is the binding site for eIF3 and eIF4A, and the terminal domain is the binding site for eIF4A and a docking sequence for Mnk1 (Imataka and Sonenberg 1997; Morino, Imataka et al. 2000). p97 which is homologous to the carboxyl two-thirds of eIF4G, can interact with eIF3, eIF4A and MNK1, but lacks the amino-terminal binding site for eIF4E

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and PABP. Also, in its C-terminal region, p97 does not contain an eIF4A binding site (Imataka and Sonenberg 1997).

eIF4G has an important role in translation initiation. Its serves as a docking site for other initiation factors to assemble at the 5'cap of an mRNA and promote its recruitment by the small ribosomal subunit. It can bind to eIF4E, poly (A) binding protein (PABP), eIF4A, eIF3, and MNK. eIF4G-eIF4E interaction was shown to enhance the affinity of eIF4E for the cap structure (Haghighat and Sonenberg 1997; von Der Haar, Ball et al. 2000) and increase the activity of eIF4A (Pause, Methot et al. 1994; Korneeva, First et al. 2005; Oberer, Marintchev et al. 2005). In addition, increased binding of eIF4E to eIF4G favours translation of mRNAs encoding cell-cycle and growth-regulated proteins such as c-Myc, cyclin D and fibroblast growth factor (De Benedetti and Harris 1999). Furthermore, the eIF4G-PABP interaction is implicated in enhancing the formation of 48S and ribosome recycling through mRNA circularization (Sachs 2000; Kahvejian, Svitkin et al. 2005). The high content of Pro, Glu, Ser and Thr residues (PEST sequences) in the N-terminus of eIF4G may also contribute to the instability of eIF4G (reviewed in (Morley, Curtis et al. 1997).

eIF4G is subject to cleavage by viral proteases as well as some cellular caspases. Both eIF4GI and eIF4GII are targets for caspase-3-mediated degradation during apoptosis (Morley 2001). Caspase-3 cleaves eIF4Gs at two positions between amino acids 492 and 493 and between amino acids 1136 and 1137, leaving behind three breakdown products (Clemens, Bushell et al. 1998). These products are involved in the IRES dependent translation of some apoptosis related proteins such as the anti-apoptotic X-chromosome-linked inhibitor of apoptosis (XIAP) (as reviewed in (Holcik and Sonenberg 2005)). In contrast to the cleavage of eIF4Gs during poliovirus, the caspase cleaves eIF4GI and eIF4GII at the same kinetics (Marissen, Gradi et al. 2000). (The effect of poliovirus on eIF4Gs is further explained in section 1.4.2 and chapter 3).

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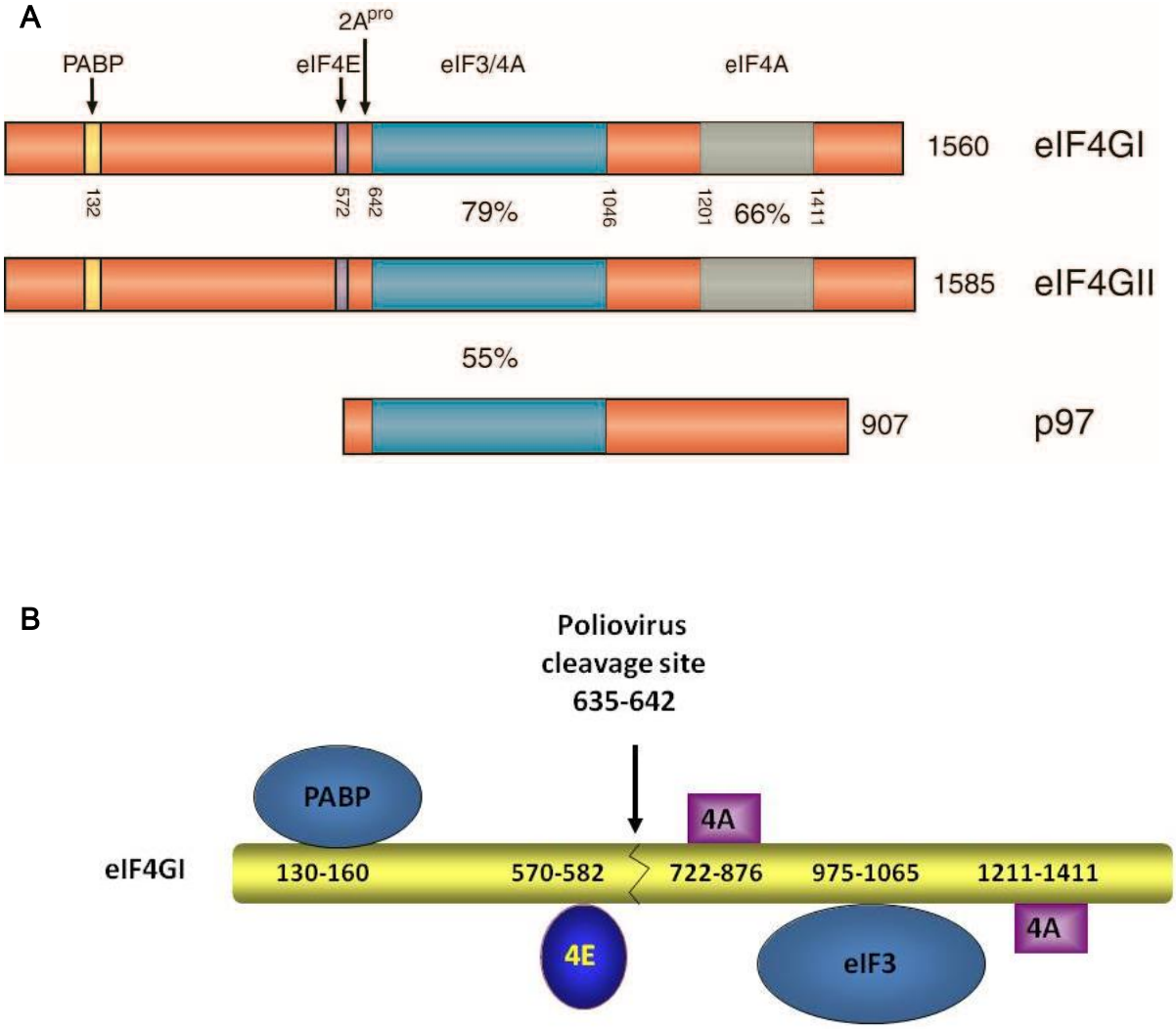

Figure 9: **eIF4G**

A. Comparison between eIF4GI, eIF4GII and p97 domain shows various binding sites for eIF4G binding partners. Taken from (Gingras, Raught et al. 1999).

B. Representation of eIF4GI showing the poliovirus cleavage site separating the protein into an N-terminus and a C-terminus. The N-terminus holds the binding site of eIF4E and PABP, whereas the C-terminus holds the binding site of eIF4A and eIF3. Adapted from (Jackson 2005).

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1.3 Translation initiation signalling control

Translation initiation, as shown above, is a very complex mechanism which involves multiple factors and sequential steps in different compartments of the cell. This mechanism is subject to different control pathways including the mTOR, the MAPK, and the eIF2-kinase pathways.

1.3.1 mTOR pathway

The cap-dependent mRNA translation is an important downstream target of the mTOR signaling pathway (Fig. 10). This signaling pathway is activated by growth factors, mitogens and hormones via the PI3K/AKT upstream kinase of mTOR (as reviewed in (Hay and Sonenberg 2004)). The serine-threonine kinase mammalian target of rapamycin, mTOR, can be part of 2 different complexes mTORC1 and mTORC2. mTORC1 consists of mLST8 (mammalian lethal with SEC13 protein 8, also known as G protein beta subunit-like protein (GβL)), regulatory-associated protein of TOR (raptor), and mTOR. Whereas the mTORC2 consists of mLST8, rapamycin-insensitive companion of mTOR (rictor; also known as mammalian AVO3), and mTOR (Wullschleger, Loewith et al. 2006). As the name indicates, mTOR is the target of Rapamycin. Rapamycin was originally isolated from a strain of the soil bacterium, *Streptomyces hygroscopicus*. It acts by forming an inhibitory complex with its intracellular receptor, the FK506-binding protein, FKBP12, which binds a region in the C terminus of TOR proteins termed FRB (FKB12-rapamycin binding), thereby inhibiting TOR activity (Chen, Zheng et al. 1995; Choi, Chen et al. 1996). AKT, also known as protein kinase B (PKB), is a positive upstream regulator of mTORC1, connects the phosphatidyl-inositol 3-kinase (PI3K) pathway to mTORC1 via tuberous sclerosis complex 1 (TSC1), TSC2, and Ras homolog enriched in brain (Rheb) (Hay and Sonenberg 2004).

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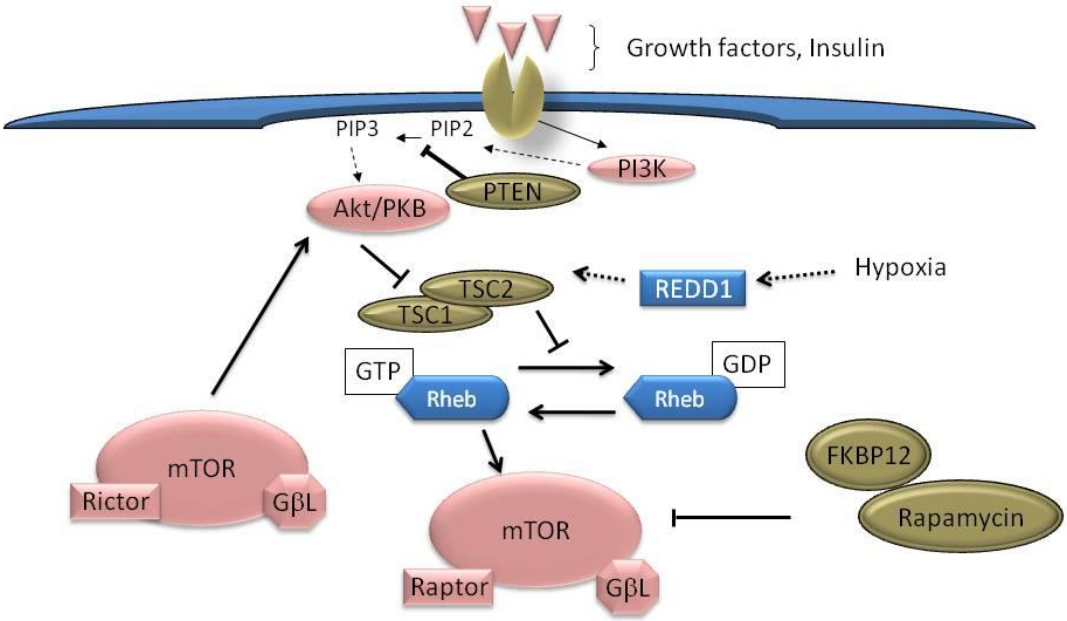

Figure 10: **mTOR pathway.** mTOR is the downstream target of PI3K and AKT. These kinases are activated by various stimuli such as growth factors and mitogens. Environmental conditions as well as compounds were shown to inhibit the activity of the mTOR pathway leading to a general shutdown of translation.

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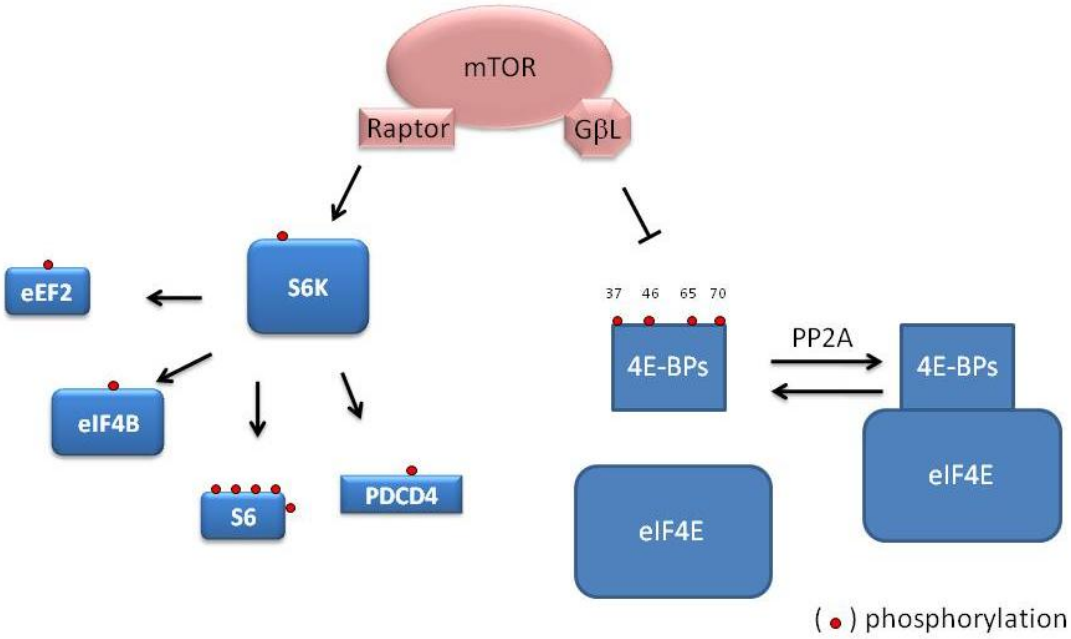

Figure 11: **mTOR effectors 4E-BPs and S6Ks.** mTOR induces the activation of S6K and deactivation of 4EBPs upon phosphorylation. The phopho-S6K is responsible for the activation of multiple targets involved in the activation of translation. Whereas, the phosphorylation of 4EBPs liberates the binding inhibition on eIF4E and therefore induces translation initiation.

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The downstream effectors of mTOR are S6-Kinase (S6K) and 4E-BPs. Hormones and mitogens activate mTOR and so S6K and 4EBPs, whereas reducing the amino acids levels and the energy would prevent mTOR function. (Fig.11).

In mammals, there are three 4E-BP members. 4E-BP1 and 4E-BP2 are expressed in most tissues, whereas 4E-BP3 expression is more restricted (Poulin, Gingras et al. 1998; Tsukiyama-Kohara, Poulin et al. 2001). The mammalian 4E-BP1 and 4E-BP2 were identified in a screen using radiolabelled recombinant eIF4E protein to probe a human placenta expression library (Pause, Belsham et al. 1994). 4E-BP3 was isolated from a prostate cancer cell line cDNA library (Poulin, Gingras et al. 1998). All three isoforms contain a conserved eIF4E-binding motif Tyr-*X*-*X*-*X*-*X*-Leu-ϕ (where *X* is variable and ϕ is a hydrophobic amino acid) and phosphorylation sites critical to the regulation of cap-dependent translation. 4E-BPs and eIF4G share the same eIF4E-binding motif. Hypophosphorylated 4E-BPs bind tightly to eIF4E, whereas the hyperphosphorylated forms interact weakly (Gingras, Gygi et al. 1999). The phosphorylation of 4E-BP1 upon stimulation (growth factors, mitogens and hormones) occurs at multiple sites and proceeds in a hierarchical manner (first Thr37 and Thr46, then Thr70 and last Ser65) (Gingras, Gygi et al. 1999; Gingras, Raught et al. 2001). Treatment with the inhibitor of the mTOR, rapamycin, inhibits 4E-BP phosphorylation, increases the interaction between eIF4E and 4E-BP, and consequently inhibits cap-dependent translation (Gingras, Raught et al. 2001). Mammalian cells contain two ribosomal protein S6 kinases (S6K1 and S6K2) encoded by two separate genes (Shima, Pende et al. 1998). Both S6Ks are downstream targets of mTOR (reviewed in (Hay and Sonenberg 2004)). Both proteins are phosphorylated and all of the phosphorylation sites are conserved between the two proteins. Once activated, S6K1 can increase mRNA translation and so control the cellular growth (Montagne, Stewart et al. 1999; Radimerski, Montagne et al. 2002). It was shown by (Holz, Ballif et al. 2005) that S6K1 is bound to eIF3 in an inactive state. Following an activating signal, such as insulin stimulation, mTOR/raptor is recruited to eIF3, leading to phosphorylation of S6K1 on T389. S6K1 can influence the phosphorylation state of various target proteins involved in translation such as ribosomal protein S6, eIF4B, eukaryotic elongation factor 2 kinase, insulin receptor substrate-1 (IRS-1) and PDCD4

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(reviewed in (Hay and Sonenberg 2004). The effect of phosphorylation on these biological targets varies between activation (S6) or degradation (PDCD4) (Dorrello, Peschiaroli et al. 2006).

1.3.2 MAPK pathway

Mitogen-activated protein kinase (MAPK) pathways (Fig. 12) are intermediate players between environmental signals detected on the cell membrane and the nucleus through a series of phosphorylation steps leading to the activation of transcription factors, which in turn regulate gene expression. In mammalian systems, there are three subgroups of MAPKs: ERKs (extracellular signal-regulated kinases), SAPK/JNK (stress-activated protein kinase/ jun N-terminal kinase) and p38 MAPKs. These kinase pathways are involved in the cellular response to cytotoxins and environmental stresses, but can also be modulated during the processes of apoptosis, transformation, development, immune activation, inflammation and ischemic injury. The stress kinases (SAPK/JNK and p38) respond to various stimuli, including UV radiation, oxidative stress, protein synthesis inhibitors, heat shock, cytokines and osmotic shock. Following their activation, stress kinases can relocalize to the nucleus, where they phosphorylate and activate their target transcription factors. However, not all stress kinase targets are nuclear since these kinases regulate translational as well as transcriptional processes (reviewed in (Pearce and Humphrey 2001)).

The MAPK pathway affects the translation initiation mechanism either by modifying the protein level of the initiation complex or their phosphorylation state. Mitogenic stimulation has a rapid induction on the expression of c-Myc protein (reviewed in (Grandori, Cowley et al. 2000)). An increase in the level of Myc was shown to increase the level of eIF4E, eIF4A and eIF4G (Lin, Cencic et al. 2008). MnK1 and MnK2 are targets of both the ERK and the p38 MAPK pathway. Both MnKs have been shown to phosphorylate eIF4E on Ser209 (Waskiewicz, Flynn et al. 1997; Waskiewicz, Johnson et al. 1999). The consequence of eIF4E phosphorylation on translation has been a subject of debate. Stimulating protein synthesis and cell proliferation via hormones, growth factors and mitogens promote eIF4E phosphorylation (Gingras, Raught et al. 1999). On

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the other hand, manipulation of MnKs levels *in vivo* has been shown to have no impact on translation. eIF4E phosphorylation does not appear to modulate cap-dependent translation *in vitro* (McKendrick, Morley et al. 2001), nor does it affect the eIF4E:eIF4G interaction (Knauf, Tschopp et al. 2001). In parallel, some studies reported an increase affinity of the phosphorylated eIF4E for the cap structure compared to its dephosphorylated counterpart, however opposite results were obtained by other groups (reviewed in (Scheper and Proud 2002)). It was shown that deletion of both MNK kinases abolishes phosphorylation of eIF4E, but does not have an effect on development or health of adult animals (Ueda, Watanabe-Fukunaga et al. 2004). In cases of transformation and tumorigenesis, mutation of the Ser209 phosphorylation site blocks eIF4E's ability to transform rodent fibroblast (Topisirovic, Ruiz-Gutierrez et al. 2004) or contribute to tumour development in mice (Wendel, Silva et al. 2007).

On the other hand, both the p38 and the ERK1/2 MAPK pathway have been shown to regulate eIF4E activity by promoting the phosphorylation of 4E-BPs. For instance, UV mediated the phosphorylation of 4E-BP1 by the target of p38, MsK1 (Liu, Zhang et al. 2002). In addition, the Ras/MAPK was shown to induce the phosphorylation of eIF4B which was suggested to increase its binding to eIF3 and therefore its function (Shahbazian, Roux et al. 2006).

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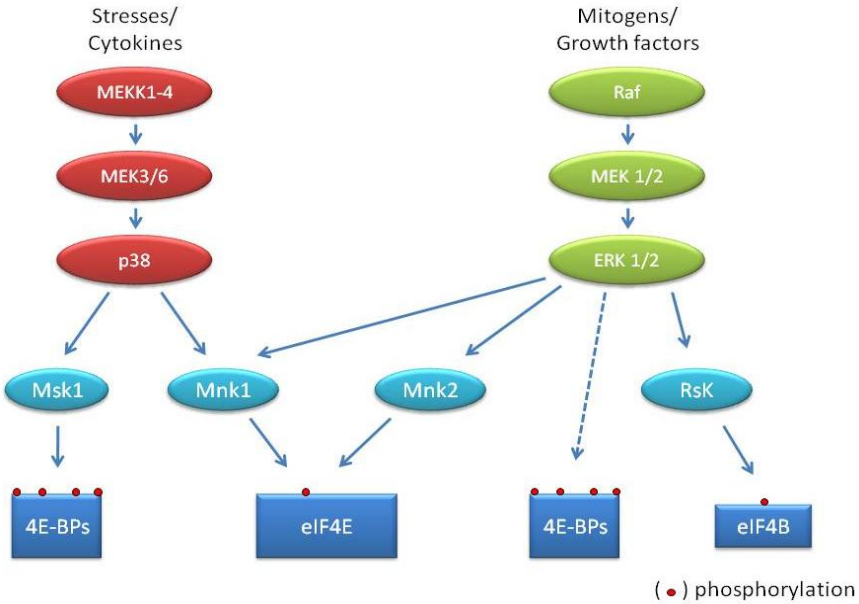

Figure 12: **MAPK pathway signalling to translation initiation factors.** The MAPK pathway influences the translation initiation proteins by affecting the phosphorylation state of eIF4E, 4EBPs and eIF4B.

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1.3.3 eIF2-kinases pathway

As mentioned in section (1.1.3), eIF2 is a crucial translation initiation factor for the formation of the ternary complex. Once eIF2 is phosphorylated on Serine 51 of its α-subunit, the ternary complex will not be formed and translation is generally inhibited. Various upstream kinases that hold similarities in their catalytic domain are responsible for the phosphorylation of eIF2. These kinases are the effect of multiple external or internal challenges and they are heme-regulated inhibitor kinase (HRI), protein kinase RNA (PKR), PKR-like endoplasmic reticulum kinase (PERK), and general control non-derepressible-2 (GCN2) (Fig.13) (reviewed in (Kapp and Lorsch 2004)). These kinases can respond to various regulatory mechanisms because their effectors domains are variable. HRI is activated under low haem level or upon arsenite, osmotic or heat shock treatment (reviewed in (Chen 2000)). Amino acid starvation and UV irradiation induce GCN2 (Kimball 2001; Jiang and Wek 2005), whereas PKR is activated by double stranded RNA (Kaufman 2000). PERK is activated by ER stress (reviewed in (Ron 2002).

As proposed by (Hinnebusch 2000), the inhibition of the ternary complex formation results in a global protein synthesis inhibition whereas it is beneficial for the translation of selected proteins whose mRNAs are rich with multiple uORFs. An example is the mRNA of GCN4 which comprise 4 short uORFs: 1 to 4. Under normal conditions, the 40S ribosomal subunit binds to the ternary complex and scans the mRNA from the cap structure end of the mRNA. The ribosomes translate the uORF1 and the 40S subunit is free to bind another ternary complex and translate the other uORF2 through 4. These conditions will not favor the translation of the GCN4 ORF. Upon amino acid starvation, the ternary complex becomes scarce. Therefore, the 40S subunit cannot translate uORF2 to 4. After bypassing uORF4, the minimal amount of ternary complex will be available to bind the ribosome and translation of the GCN4 ORF can take place.

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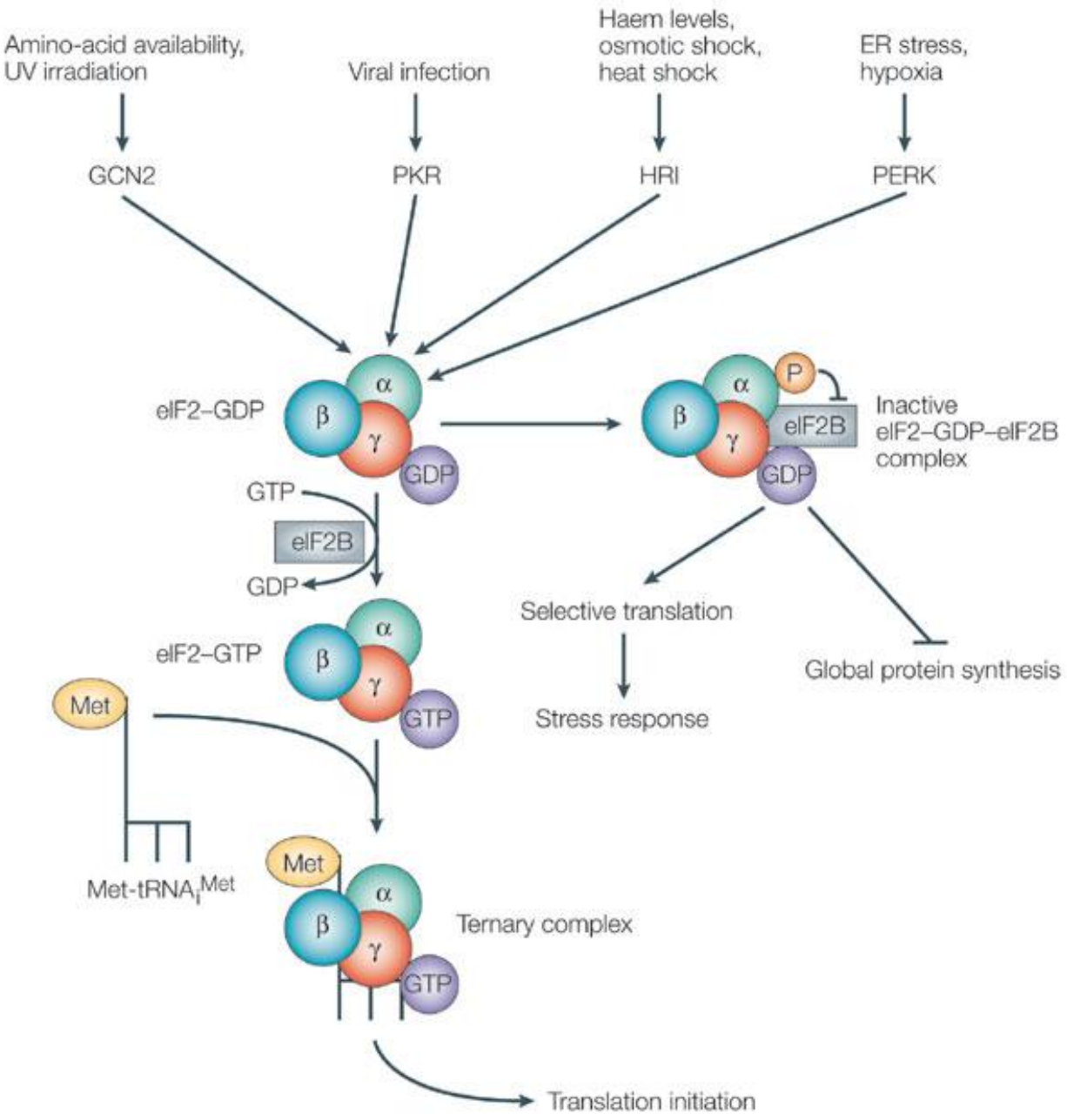

Figure 13: **eIF2-kinases pathway.** Various stimuli activate different eIF2-kinases. Once eIF2 is phosphorylated, the ternary complex formation will be inhibited which will lead to a global protein synthesis inhibition. Taken from (Holcik and Sonenberg 2005)

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1.4 Translational perturbation

The homeostasis of a translation mechanism depends on the functional equilibrium between all the factors involved in its function. In the context of an internal or an external perturbation, the translation initiation mechanism is the major target. In this section, I will describe the effect of various external perturbations on the translation initiation mechanism.

1.4.1 Cellular response to stress

Cells manage to resist stressful conditions that hinder cellular growth and induce apoptosis. This resistance is accomplished either via the expression of stress related genes (Pearce and Humphrey 2001), or global translation reduction which goes hand-in-hand with a switch to the selective translation of protein required for cell survival (Mathews 2000). In both cases, the ultimate goal of the stressed cell is to save energy and prevent further damage (Mathews 2000). One key element to the cellular response is the phosphorylation of the α-subunit of eIF2 at residue Ser51. Even though, eIF2α phosphorylation reduces the availability of the ternary complex and so inhibits the translation of most mRNAs, it enhances the translation of targeted mRNAs such as GCN4 (see section 1.3.3) (Hinnebusch 2000).

Environmental stress is associated with the formation of cytoplasmic foci called Stress Granules (SG). In times of stress, their composition may be remodelled to exclude mRNA transcripts whose products are required for survival and the current evidence indicates that they are not static entities, but rather proteins continuously flux in and out of SGs. First described in heat shock plant and animal cells, SGs also occur in cells exposed to a number of other environmental stresses, including oxidative stress, UV irradiation, ER stress, viral infection, and osmotic shock (reviewed in (Pelletier, Mazroui et al. 2008)). SGs contain transcripts encoding housekeeping genes but exclude those encoding stress-induced genes such as *HSP70*. Stress granules have been observed in yeast (such as *Saccharomyces pombe*), protozoa (*Trypanosoma brucei)* and metazoa (such as *Homo sapiens* and *Caenorhabditis elegans*). They have also been observed in

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plants and in chloroplasts, suggesting that they may be assembled in prokaryotes as well (reviewed in (Anderson and Kedersha 2009)). SGs are composed of stalled 48S complexes containing mRNAs, translation initiation factors (such as eIF4E, eIF4G, eIF4A, eIF4B, eIF3 and PABP), and the small ribosomal subunit (not the large subunit) (Anderson and Kedersha 2002). Furthermore, SGs contain RNA-binding proteins linked to translational silencing or mRNA stability such as TIA-1 (T cell internal antigen-1) and TIAR (TIA-1-related) (Kedersha, Gupta et al. 1999) and argonaute (Leung, Calabrese et al. 2006). RNA decay-associated SG components include the Argonaute proteins, tristetraprolin (TTP) and BRF1 (Stoecklin, Stubbs et al. 2004). Other RNA-binding proteins not involved in translation nor in decay are G3BP (Ras-GTPase-activating protein SH3-domain-binding protein) (Tourriere, Chebli et al. 2003) and caprin (Solomon, Xu et al. 2007).

The use of murine embryo fibroblasts (MEFs) expressing the non-phosphorylatable eIF2α[S51A] mutant, obtained from 'knock-in' ($eIF2\alpha^{S51A/S51A}$) mice (Scheuner, Song et al. 2001), has revealed that SG formation in response to arsenite, heat shock, puromycin, G3BP expression plasmid tranfection, is eIF2α(P) dependent (Kedersha, Stoecklin et al. 2005). SGs contain components of the small ribosomal subunits, mRNA, RNA binding proteins, and translation initiation factors. Another mechanism of SG induction is independent of eIF2α phosphorylation. This mechanism involves inhibiting the activity of the translation initiation factor eIF4A or eIF4G (Mazroui, Sukarieh et al. 2006). Hippuristanol, a natural product that selectively inhibits eIF4A RNA binding (Bordeleau, Mori et al. 2006). Pateamine and Silvestrol, other natural products that inhibit eukaryotic translation by acting as a chemical inducer of dimerization - stimulating the non-sequence selective binding of eIF4A to RNA (Bordeleau, Matthews et al. 2005; Bordeleau, Cencic et al. 2006; Cencic, Carrier et al. 2009), block ribosome recruitment and induce SG formation (Mazroui, Sukarieh et al. 2006; Cencic, Carrier et al. 2009). In addition, infection of cells with poliovirus, which shuts off host protein synthesis by cleaving the eIF4GI and eIF4GII subunit of eIF4F, or knocking down expression levels of eIF4AI by siRNA, were sufficient to induce SG formation (Mazroui, Sukarieh et al. 2006).

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1.4.2 Translation inhibition upon poliovirus infection

The positive-stranded viral mRNA (such as the poliovirus) is translated via an internal ribosome entry site (IRES), an RNA sequence in the 5' noncoding region of the viral RNA. Ribosomal recruitment is required for this mechanism without the need of an intact eIF4F complex (Pelletier and Sonenberg 1988).

Virus-encoded protease 2A cleaves the eIF4G component of eIF4F at a specific site within the 220-kD protein (Etchison, Milburn et al. 1982). Infection of cells by poliovirus results in a shut-off of host cell protein synthesis which is preceded by the cleavage of eIF4GI. Cleavage of eIF4GI results in the inhibition of cap-dependent translation. Whereas poliovirus translation is not affected by eIF4GI cleavage since the truncated form of eIF4G can stimulate translation of the poliovirus proteins (Borman, Kirchweger et al. 1997). Cleavage of eIF4GI alone cannot explain the shutoff of host protein synthesis, because after infection in the presence of inhibitors of virus replication, eIF4GI is cleaved, yet host protein synthesis is only partially inhibited. Thus, cleavage of both eIF4GI and eIF4GII appears to be required for the shutoff of host protein synthesis after poliovirus infection (Gradi, Svitkin et al. 1998). The proteolytic cleavage of eIF4G separates the cap-binding activity from the 40S-subunit-binding activity and this effectively inhibits cap-dependent translation. The N-terminal cleavage product contains the binding site for eIF4E, PABP, and the C-terminal product harbours the binding sites for eIF3 and eIF4A. Furthermore, PABP is proteolyzed during poliovirus infection by viral 3C proteases (Joachims, Van Breugel et al. 1999).

1.4.3 Oncogenesis

eIF4E was shown to cause malignant transformation when overexpressed in cultured rodent cells (Lazaris-Karatzas, Montine et al. 1990). Overexpression of eIF4E enhances the translation of many highly structured mRNAs, which are involved in cell growth, proliferation and survival (reviewed in (Mamane, Petroulakis et al. 2004)). An increase in the eIF4E levels was shown to be associated with a large number of cancers (colon, breast, bladder, lung, prostate, cervix, gastrointestinal tract, head and neck,

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Hodgkin's lymphomas and neuroblastomas). Furthermore, two groups independently demonstrated that eIF4E promotes lymphomagenesis in cooperation with Myc (Ruggero, Montanaro et al. 2004; Wendel, De Stanchina et al. 2004). In parallel, short interfering RNA (siRNA) knockdown of eIF4E inhibits the growth of several cell lines, including those of head and neck squamous carcinoma cells (Oridate, Kim et al. 2005). On the other hand, Graff and colleagues showed that the administration of eIF4E-specific antisense oligonucleotides (ASOs) reduced eIF4E expression in human tumour xenografts, and as a result significantly suppressing tumour growth. These results suggested that cancers may be more susceptible to eIF4E inhibition than normal tissues (Graff, Konicek et al. 2007). In parallel, 4E-BPs are capable of controlling transformation by inhibiting the cell growth of eIF4E-overexpressing cells or cells transformed by *ras* or *src* genes (Rousseau, Gingras et al. 1996). Enhancement of the eIF4E/4E-BP interaction, through the expression of a constitutively active 4E-BP mutant (all phosphorylation sites mutated to Ala), leads to a decrease in cell size and inhibition of cell cycle progression in a manner that mimics rapamycin treatment (Fingar, Salama et al. 2002). This 4E-BP mutant also slows G1 progression, blocks c-*myc*-induced transformation and prevents tumor growth (Avdulov, Li et al. 2004; Lynch, Fitzgerald et al. 2004). Reducing eIF4E expression in tumours would inhibit cell proliferation, suppress tumour-related angiogenesis, repress metastasis, and increase apoptosis by selectively repressing the expression of potent growth and survival factors (e.g., cyclin D1, c-myc, ODC, survivin, BCL-2, VEGF, FGF-2) (Graff, Konicek et al. 2007). The reduction of eIF4E would have a minimal effect on total protein synthesis and may therefore minimally affect normal tissue function (as reviewed in (De Benedetti and Graff 2004)).

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CHAPTER2:

The eIF4E binding proteins (4E-BPs) are modifiers of cytoplasmic eIF4E relocalization during the heat shock response.

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2.1 ABSTRACT

Stress granules (SGs) arise as a consequence of cellular stress, contain stalled translation preinitiation complexes, and are associated with cell survival during environmental insults. SGs are dynamic entities with proteins relocating into and out of them during stress. Among the repertoire of proteins present in SGs is eIF4E, a translation factor required for cap-dependent translation and which regulates a rate-limiting step for protein synthesis. Herein, we demonstrate that localization of eIF4E to SGs is dependent on the presence of a family of repressor proteins, eIF4E-binding proteins (4E-BPs). Our results demonstrate that 4E-BPs regulate the SG localization of eIF4E.

2.2 INTRODUCTION

In response to environmental stress, eukaryotic cells reprogram their gene expression profile by evoking transcriptional and post-transcriptional regulatory mechanisms. An early phase of this response involves a change in mRNA metabolism that is associated with the formation of cellular aggregates, known as stress granules (SGs) (for a review, see (Anderson and Kedersha 2006; Pelletier, Mazroui et al. 2008)). SG formation occurs upon exposure of cells to a number of stresses, including heat shock, UV irradiation, ER stress, oxidative stress, viral infection and osmotic stress. Non-translating mRNAs, eukaryotic translation factors, and other RNA binding proteins become deposited in SGs, and these appear to act as temporary storage sites until the elimination of the stress inducing stimulus [for a review, see (Pelletier, Mazroui et al. 2008)]. Inhibitors of translation that block initiation or cause disassembly of polysomes (e.g.-puromycin) stimulate SG formation, whereas those that stabilize polysomes induce SG disassembly. Fluorescence recovery after photobleaching (FRAP) has demonstrated the shuttling of several proteins (GFP-TIA1, GFP-PABP, YFP-eIF4E, EGFP-Ago2) in and out of SGs (Kedersha, Cho et al. 2000; Leung, Calabrese et al. 2006; Pelletier, Mazroui et al. 2008). Labile mRNAs are also stabilized during stress (Laroia, Cuesta et al. 1999; Bollig, Winzen et al. 2002). Taken together these results have led to the

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suggestion that SGs constitute dynamic entities where mRNAs assemble for quality control before being redirected for translation, degradation, or storage (Kedersha, Stoecklin et al. 2005). Most eukaryotic cellular mRNA translation is thought to occur by a cap-dependent process, which is effected by the eukaryotic initiation factor (eIF) 4F complex [for a review, see (Gingras, Raught et al. 1999)]. eIF4F is comprised of three subunits: (i) eIF4E, the cap-binding protein which binds to the cap structure; (ii) eIF4A, a DEAD-box RNA helicase thought to unwind local RNA structure and facilitate access of the 43S ribosomal complex to the mRNA; and (iii) eIF4G, a modular scaffold that binds eIF4E and eIF4A and recruits ribosomes to mRNA via its interactions with eIF3. All eIF4F subunits localize to SGs under stress (Kedersha and Anderson 2002; Kedersha, Chen et al. 2002; Kimball, Horetsky et al. 2003; Kim, Back et al. 2005; Dang, Kedersha et al. 2006; Mazroui, Sukarieh et al. 2006). The activity of eIF4E is regulated by a family of translation initiation repressors - referred to as eIF4E-binding proteins (4E-BPs). eIF4E shuttles between the active eIF4F complex and the inhibitory 4E-BP/eIF4E heterodimer with 4E-BPs and eIF4G competing for a common binding site on eIF4E(Gingras, Gygi et al. 1999). In mammals, there are three 4E-BP members, of which 4E-BP1 and 4E-BP2 are expressed in most tissues, whereas 4E-BP3 expression is more restricted (Poulin, Gingras et al. 1998; Tsukiyama-Kohara, Poulin et al. 2001). The binding of eIF4E to 4E-BP1 (the best characterized 4E-BP) is regulated by phosphorylation of 4E-BP1 by mTOR (mammalian target of rapamycin) [for a review, see (Hay and Sonenberg 2004)]. Increased phosphorylation of 4E-BP1 decreases its affinity for eIF4E, eventually leading to its dissociation and allowing binding of eIF4G.

Although the majority of eIF4E is cytoplasmic, a significant fraction (12-33%) is also present in the nucleus and localizes with splicing factors into speckles (Lejbkowicz, Goyer et al. 1992; Dostie, Lejbkowicz et al. 2000). The nuclear import of eIF4E is mediated by 4E-T, a transporter that binds eIF4E through a conserved motif that is also shared by 4E-BP and eIF4G (Dostie, Ferraiuolo et al. 2000). In addition, a sizeable portion of 4E-BP1 (~30%) is present in the nucleus and regulates eIF4E nuclear localization under stress and in response to mTOR activity (Rong, Livingstone et al. 2008). In the current study, we report that 4E-BPs regulate the localization of eIF4E to SGs. We find that localization of eIF4E to SGs is dependent on the presence of 4E-BPs,

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although 4E-BPs do not localize to SGs. In addition, we demonstrate a stimulus-dependent regulation of eIF4E localization to SGs that is 4E-BP dependent. These results highlight 4E-BPs as key regulators of eIF4E localization to SGs.

2.3 RESULTS

4E-BPs affect eIF4E localization to SGs.

We have previously reported that a significant fraction of 4E-BP1 resides in the nucleus and regulates eIF4E nuclear localization (Rong, Livingstone et al. 2008). This prompted us to assess whether 4E-BP could impact other events affecting sub-cellular targeting of eIF4E – notably the re-localization of eIF4E to SGs during stress. To this end, wild type (wt) and 4E-BP1$^{-/-}$/4E-BP2$^{-/-}$ mouse embryonic fibroblasts (MEFs) (referred to as DKO henceforth) were used. Although MEFs do not generally yield SGs that are as clear and distinct as in other cell types (Kedersha, Stoecklin et al. 2005), we were limited in the choice of our cells due to our desire to use defined isogenic lines. MEFs were heat stressed and analyzed by double-label immunofluorescence microscopy to score for eIF4E and G3BP or HuR (as markers for SG formation) subcellular localization (Kedersha, Stoecklin et al. 2005). Under standard growth conditions, endogenous eIF4E is localized primarily in the cytoplasm of both wt and DKO MEFs, with a minor amount (10-15%) of eIF4E present in the nuclei of wt MEFs (Figs. 2.1A, B). G3BP localization is diffuse and present in the cytoplasm and nuclei of both wt and DKO MEFs, as previously reported (Kedersha, Stoecklin et al. 2005) (Fig. 2.1 A). When wt and DKO MEFs were subjected to heat stress, SG formation was readily apparent (Figs. 2.1C, D). In wt MEFs, re-localization of eIF4E to the nucleus is evident (Fig. 2.1C). [The difficulty in observing eIF4E in SGs of wt MEFs appears to be a cell type phenomenon since in heat-shocked HeLa cells, relocalization of eIF4E to SGs (and nuclei) was readily apparent (See online manuscript (Sukarieh, Sonenberg et al. 2009)]. When DKO cells were exposed to 44^{o}C, there appeared to be an increase in the number of eIF4E-staining SGs (compare Fig. 2.1D to 2.1C and Fig. 2.2A). The levels of eIF4E in the nuclei of DKO cells was also reduced compared to heat-shocked wt MEFs (compare Fig. 2.1D to 2.1C and Fig. 2A). A significant difference in the total amount of SGs as

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well as in eIF4E-positive SGs per cell between wt and DKO MEFs was observed (Fig, 2.2B). The number of SGs was smaller (~2-fold) in wt MEFs relative to DKO MEFs (Fig. 2.2B) whereas the number of eIF4E-positive SGs was ~3-fold smaller.

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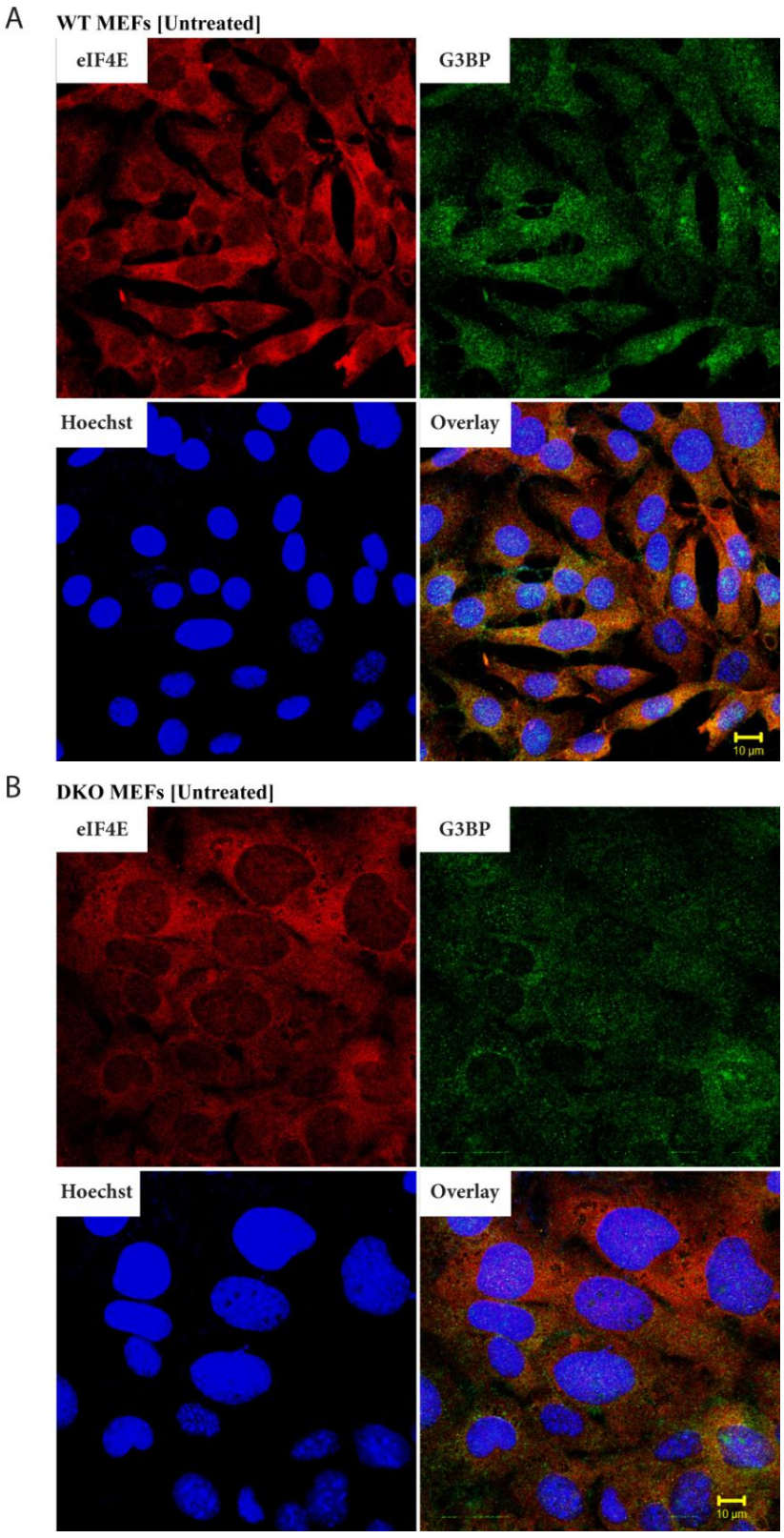

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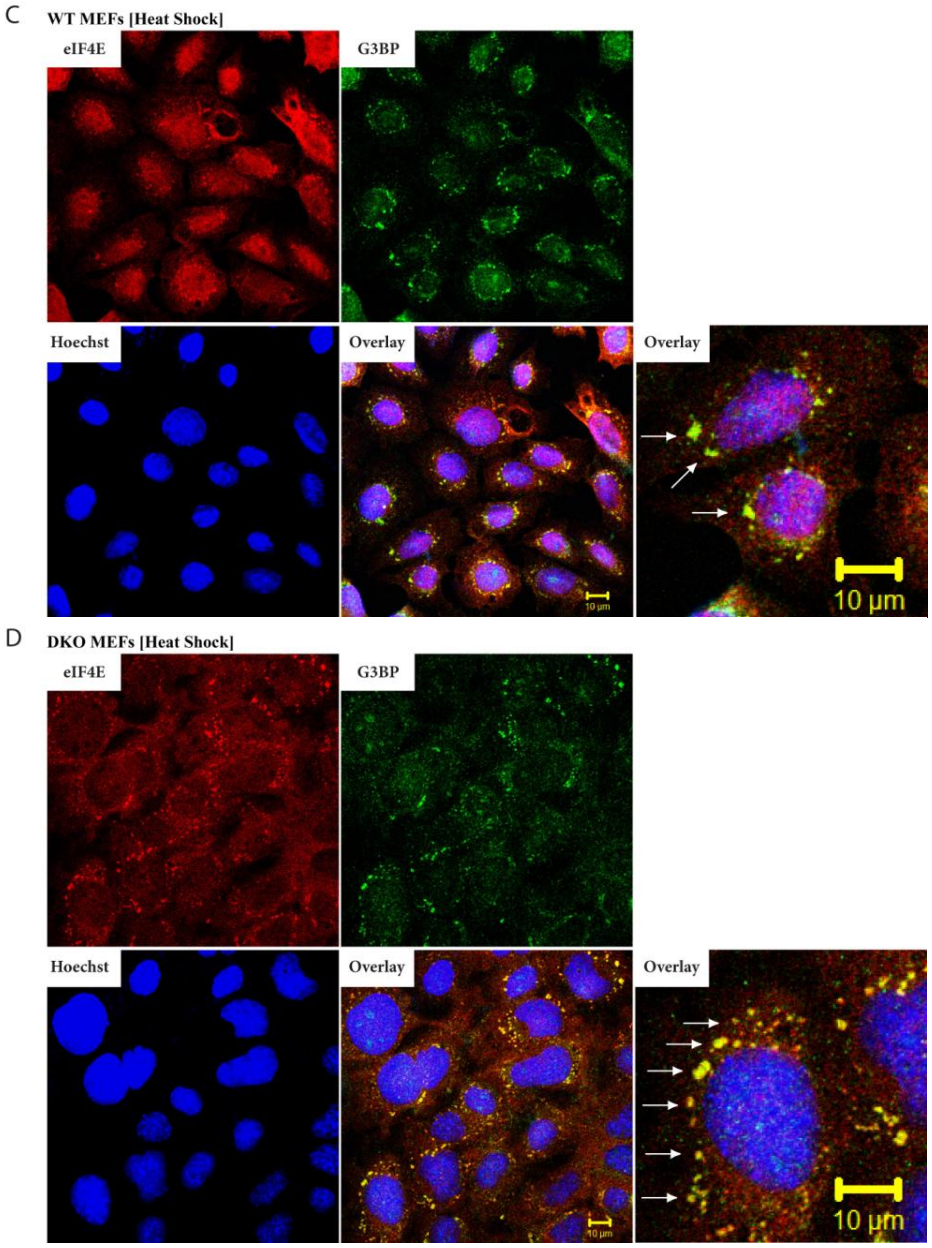

Figure 2.1: **Distribution of eIF4E in wt and DKO MEFs upon heat stress.** Untreated (**A, B**) or heat stressed (**C, D**) wt (**A, C**) and DKO (**B, D**) MEFs were fixed and stained for eIF4E and G3BP using a mouse monoclonal antibody and AlexaFluor594 conjugated secondary antibody (red) and a rabbit polyclonal antibody and AlexaFluor488 conjugated secondary antibody (green), respectively. White arrows highlight some eIF4E positive stress granules. Hoechst was used as nuclear marker. Images were taken from a 63X objective of a Zeiss LSM 510 confocal microscope.

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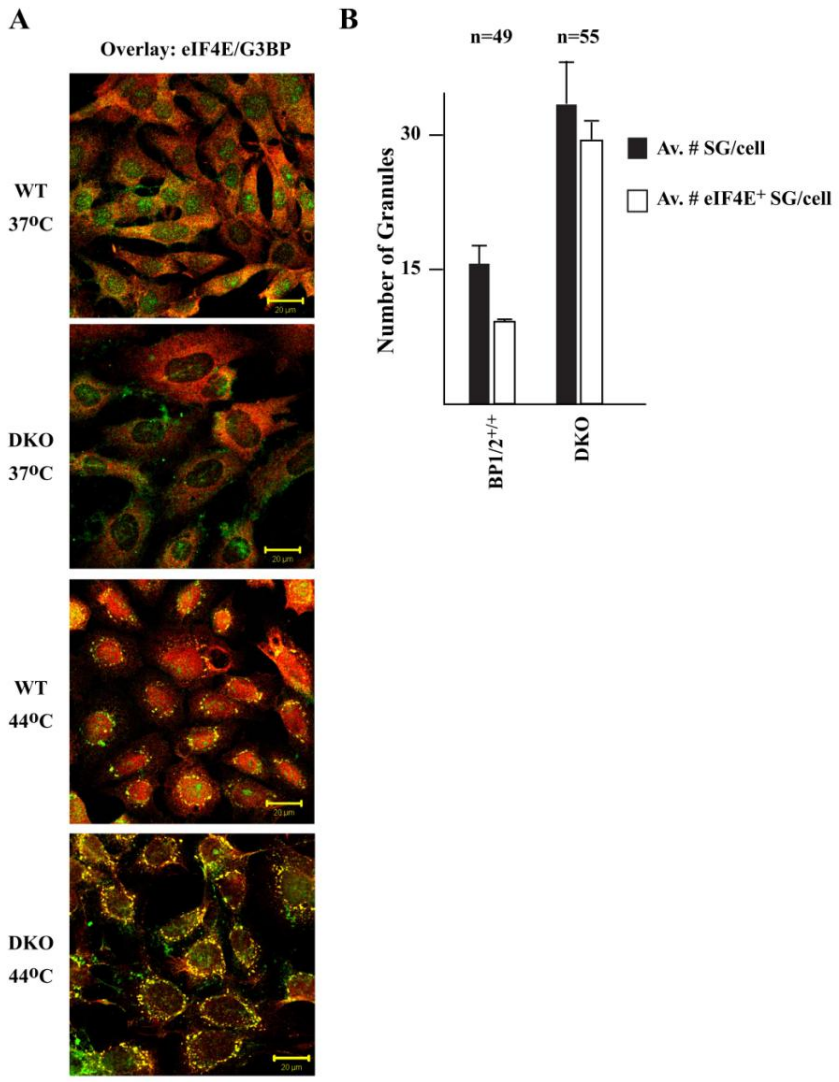

Figure 2.2: **DKO MEFs contain more SGs than wt MEFs. A.** Double overlay exposure of untreated or heat shocked wt and DKO cells fixed and stained for eIF4E (red) and G3BP (green). **B.** Quantitation of the number of SGs and $eIF4E^{+}$-SGs in heat shocked ($44^{o}C$/30 min) wt and DKO cells. n, represents the number of cells that were inspected in this experiment.

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We next examined the conditions used to induce SGs. SG formation was apparent in wt MEFs and DKOs as early as 10 min after applying heat shock, and did not increase in size 20 min after heat shock (see online manuscript (Sukarieh, Sonenberg et al. 2009)). We also monitored the effect of varying the temperature of the heat shock on SG formation (Fig. 2.3). In this experiment, cells were exposed for 30 min to 42^{o}C or 43^{o}C and the presence of SGs scored for. We note that both wt MEFs and DKOs showed the presence of SGs at 43^{o}C, but only DKOs showed SG formation at 42^{o}C (Fig. 2.3). Re-introduction of 4E-BP1 into DKO cells prevented the formation of SGs at 42^{o}C (see online manuscript (Sukarieh, Sonenberg et al. 2009)). These results indicate that DKO MEFs are more sensitive than wt MEFs at forming SGs. There was no difference in cell viability, as judged by the percent of cells staining for Annexin-PI after 4 hrs exposure to 42^{o}C, between WT and DKO MEFs (R.S., data not shown).

We also evaluated the reversibility of SG formation in wt and DKO MEFs that had been exposed to 44^{o}C for 30 min (see online manuscript (Sukarieh, Sonenberg et al. 2009)-Supp. Fig. 4A, B). For both wt and DKO MEFs, SGs disappeared by 4 hrs (see online manuscript (Sukarieh, Sonenberg et al. 2009) Supp. Fig. 4B), although they appeared to resolve from DKO cells slightly faster than from MEFs (compare the 1-2 h post-HS treatment between DKO and wt MEFs). We observed no differences in cell viability between wt and DKO MEFs 6 hrs following exposure of cells to 44^{o}C for 30 min (see online manuscript (Sukarieh, Sonenberg et al. 2009) Supp. Fig. 5).

To determine whether the increased SG localization of eIF4E in DKO cells was due to the absence of 4E-BPs, we re-introduced GFP-4E-BP1 into DKO cells by transient transfection. For these studies, we utilized a mutant of 4E-BP1, 4E-BP(5A), in which 5 phosphorylation sites (Thr37, Thr 46, Ser65, Thr70, and Ser83) are converted to alanines and which is no longer regulated by mTOR. Introducing GFP-4E-BP(5A) into DKO MEFs increased the amount of eIF4E localizing to the nucleus (Fig. 2.4A, compare the eIF4E nuclear staining pattern of GFP^{+} cells to GFP^{-} staining cells). The presence of 4E-BP(5A) in DKO cells exposed to heat shock caused an enrichment of nuclear eIF4E and a reduction in the number of eIF4E-positive SGs (Fig. 2.4B). The SGs also stained positive for G3BP (data not shown). This altered localization of eIF4E is dependent upon binding

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of eIF4E and 4E-BP since removal of the eIF4E binding site on 4E-BP1, 4E-BP(5A)Δ4E, prevented detectable relocalization of endogenous eIF4E from the cytoplasm to the nucleus (Figs. 2.4C, D). These results suggest that localization of eIF4E to SGs is dependent on its ability to exit the inhibitory 4E-BP: eIF4E complex.

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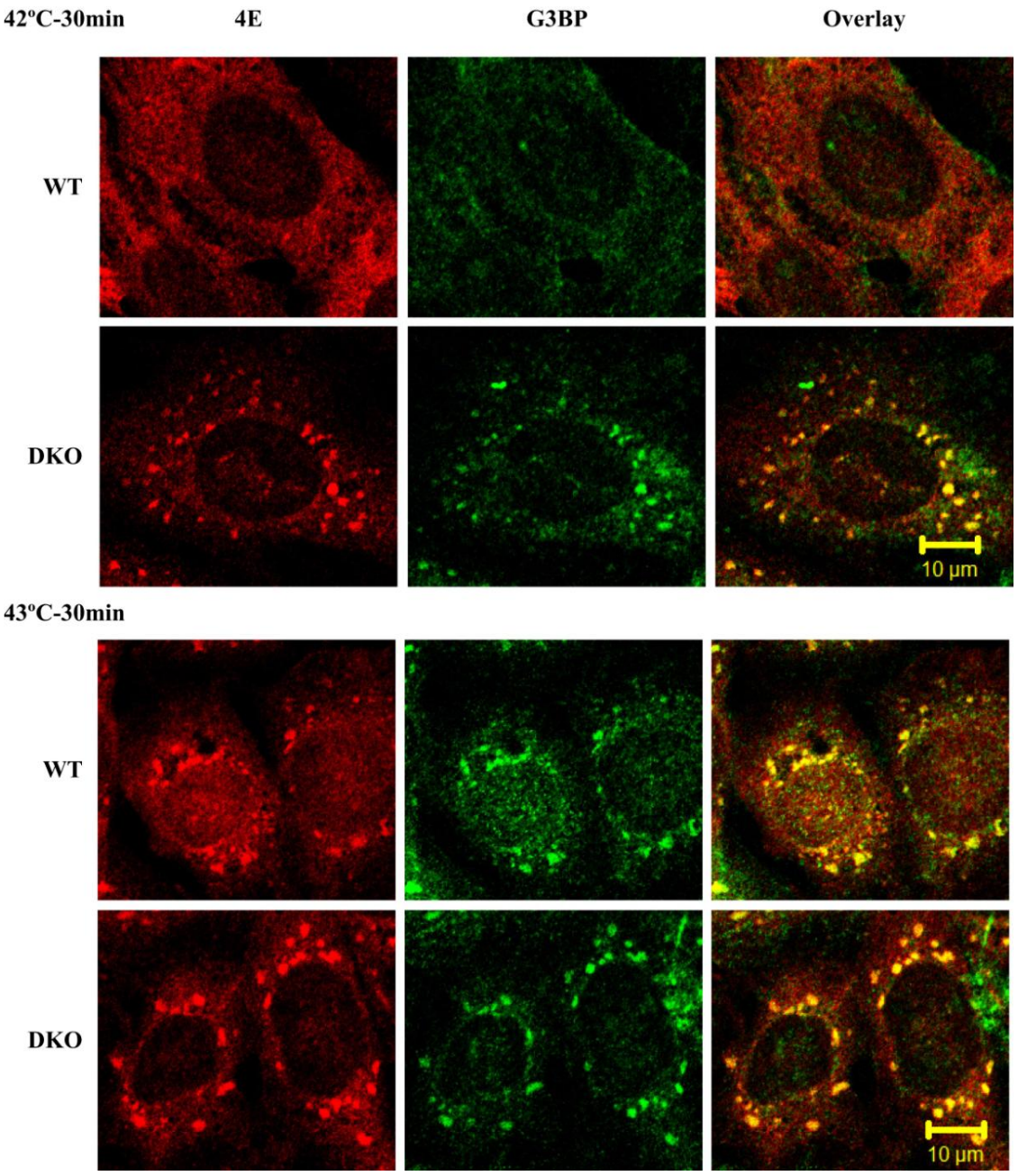

Figure 2.3: **SGs form at a lower temperature in DKO MEFs.** Wild-type and DKO MEFs were exposed to 42°C or 43 ^{0}C for 30 min, fixed, and stained for the presence of eIF4E and G3BP.

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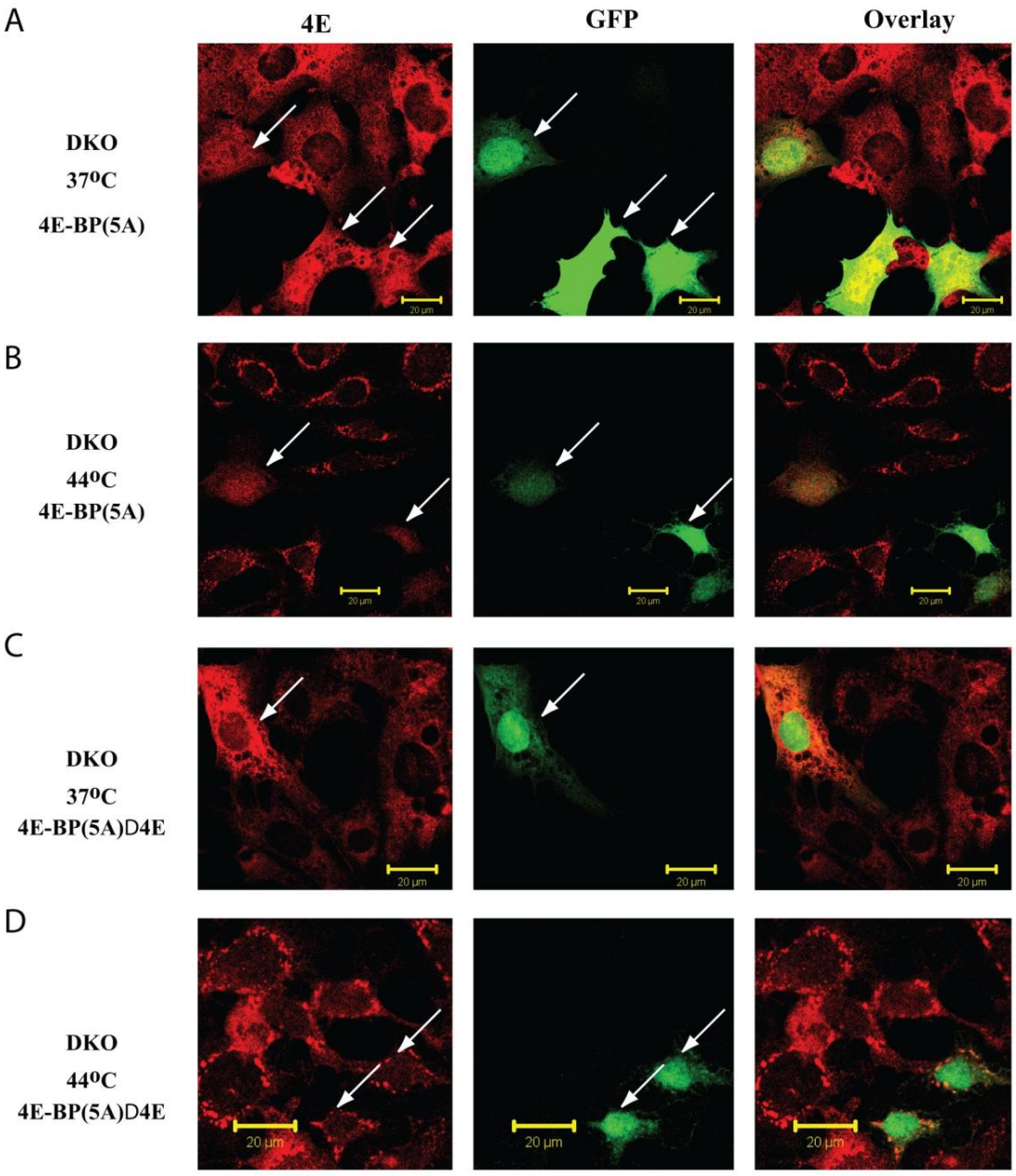

Figure 2.4: **Relocalization of eIF4E in MEFs cells in the presence of 4E-BP(5A) or 4E-BP(5A)D4E mutants .** WT and DKO MEF cells were transiently transfected either with MSCV-GFP-4E-BP(5A) **(A, B)** or MSCV-GFP-4E-BP(5A)D4E **(C, D)**. Cells were fixed and stained for eIF4E (red). GFP fluorescence was visualized as a marker of positively transfected cells.

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Rapamycin is a chemical suppressor of eIF4E localization to SGs.

We have previously shown that inhibiting mTOR activity with rapamycin increases nuclear retention of eIF4E (Rong, Livingstone et al. 2008). We wished to examine whether rapamycin would affect eIF4E localization to SGs (Fig. 2.5). As previously reported, rapamycin causes an enrichment of eIF4E in the nuclei of WT MEFs (Fig. 2.5A). Heat stress caused eIF4E to relocalize into SGs in wt MEFs whereas pre-exposure to rapamycin dampened this response by causing retention of eIF4E in the nucleus and reducing the formation of eIF4E-containing SGs (Fig. 2.5A). Rapamycin did not affect the formation of heat-induced SGs, as judged by the ability of HuR (Fig. 2.5C) or G3BP (see online manuscript (Sukarieh, Sonenberg et al. 2009) Supp. Fig. 6) to localize to SGs in wt or DKO cells. Exposure of DKO cells to rapamycin did not visibly alter the sub-cellular distribution of eIF4E in cells at 37^{o}C or heat shocked at 44^{o}C (Fig. 2.5B). 4E-BP phosphorylation was inhibited by rapamycin (Fig. 2.5D, compare lane 2 to 1) but not by heat-shock treatment (compare lane 3 to 1), as previously reported (Scheper, Mulder et al. 1997). Combined treatment of Rap and heat shock caused an even higher level of hypophosphorylated 4E-BP (compare lane 4 to 1-3).

4E-BP1 does not localize to SGs.

To investigate whether 4E-BP1 localizes to SGs under stress conditions, we analyzed the subcellular localization of 4E-BP1 under heat shock conditions. For these experiments we utilized HeLa cells since they exhibit more distinct SGs. At 37^{o}C, eIF4E is predominantly cytoplasmic, whereas there is 4E-BP1 in the nucleus (Fig. 2.6, top panel). Exposure of HeLa cells to 44^{o}C resulted in the formation of SGs containing eIF4E and G3BP, but not 4E-BP1 (Fig. 2.6).

Because both 4E-BP1 and eIF4GI compete for a common binding site on eIF4E, we examined whether the localization of eIF4G to SGs is affected by the presence of 4E-BP1. To investigate this, wt and DKO MEF cells were heat shocked and stained for eIF4G (Fig. 2.7A, B). As previously reported, eIF4GI localized to SGs upon heat shock (Kedersha, Stoecklin et al. 2005). The absence of 4E-BP did not impact on eIF4GI distribution to SGs upon heat shock (Fig. 2.7A,B). Thus, although 4E-BP1 plays a crucial

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role in the localization of eIF4E in the nucleus, it is not present in SGs and does not affect eIF4G localization.

Oxidative stress causes eIF4E localization to SGs regardless of the presence or absence of 4E-BP1/2.

Exposure of cells to oxidative stress (arsenite) has previously been shown to induce the localization of eIF4E to SGs (Kedersha, Stoecklin et al. 2005). We then asked whether the absence of 4E-BPs would similarly allow an increase in the localization of eIF4E to SGs under oxidative stress. WT and DKO MEFs were exposed to arsenite and analyzed by double-label immunofluorescence microscopy using anti-eIF4E and anti-G3BP. Inducing oxidative stress caused eIF4E to re-localize to SGs in both WT and DKO MEFs to similar extents (Fig. 2.8A, B). These results suggest that unlike heat shock, the presence or absence of 4E-BP1/2 does not impact on the ability of eIF4E to localize to SGs, when these are induced to form by oxidative stress. Unlike for heat shock, there was no change in 4E-BP1 phosphorylation status in WT MEFs exposed to arsenite (R.S., data not shown). These findings suggest that 4E-BPs do not play a role in the localization of eIF4E to SGs upon arsenite treatment.

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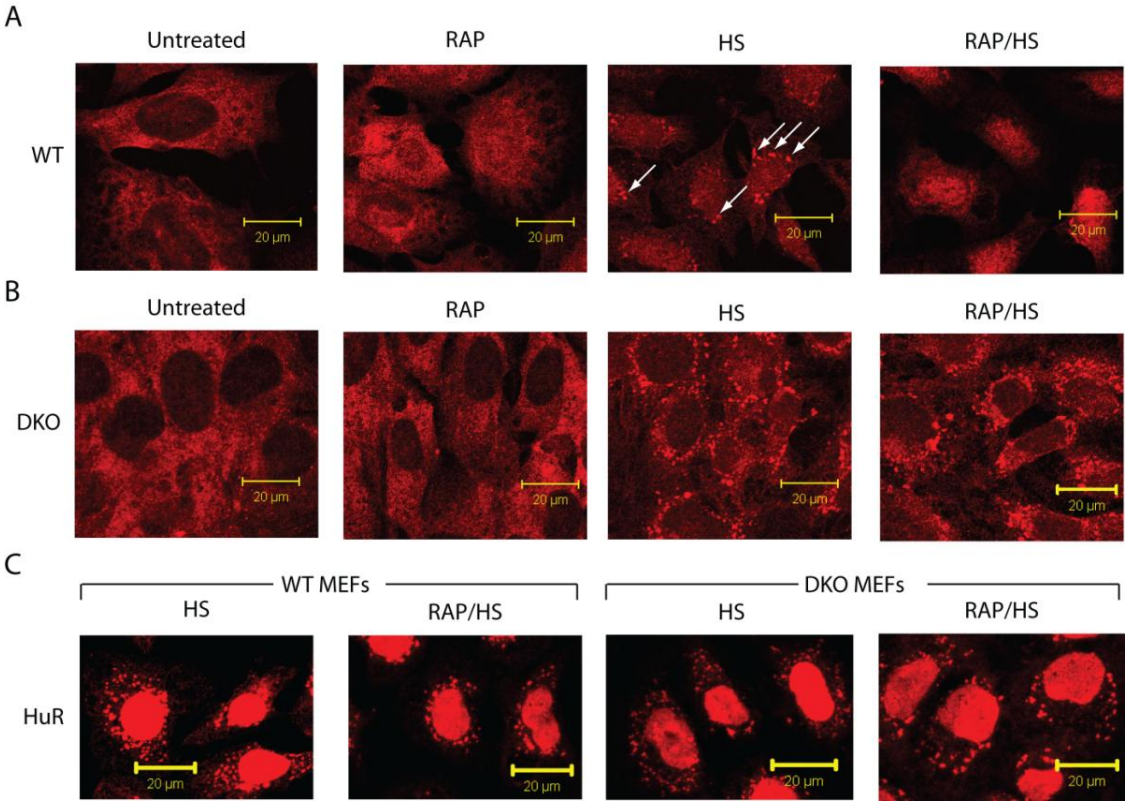

Figure 2.5: **Rapamycin alters the subcellular localization of eIF4E to SGs in wt, but not DKO, MEFs.** WT **(A)** or DKO **(B)** MEF cells were exposed to vehicle (untreated) or to RAP (20 nM for 1hr) or heat shock (HS: 44^{o}C/30 min) or RAP treatment followed by a heat shock (RAP/HS). Cells were fixed and stained for eIF4E. **C.** WT and DKO cells were heat shocked or pre-treated with RAP then heat shocked, fixed and stained for HuR. **D.** Western blot of 4E-BP1 from WT and DKO MEFs treated with Rapamycin (RAP: 20 nM for 1hr) or heat shock (HS: 44^{o}C/30 min) or RAP treatment followed by a heat shock (RAP/HS). β-Actin is used as a loading control.

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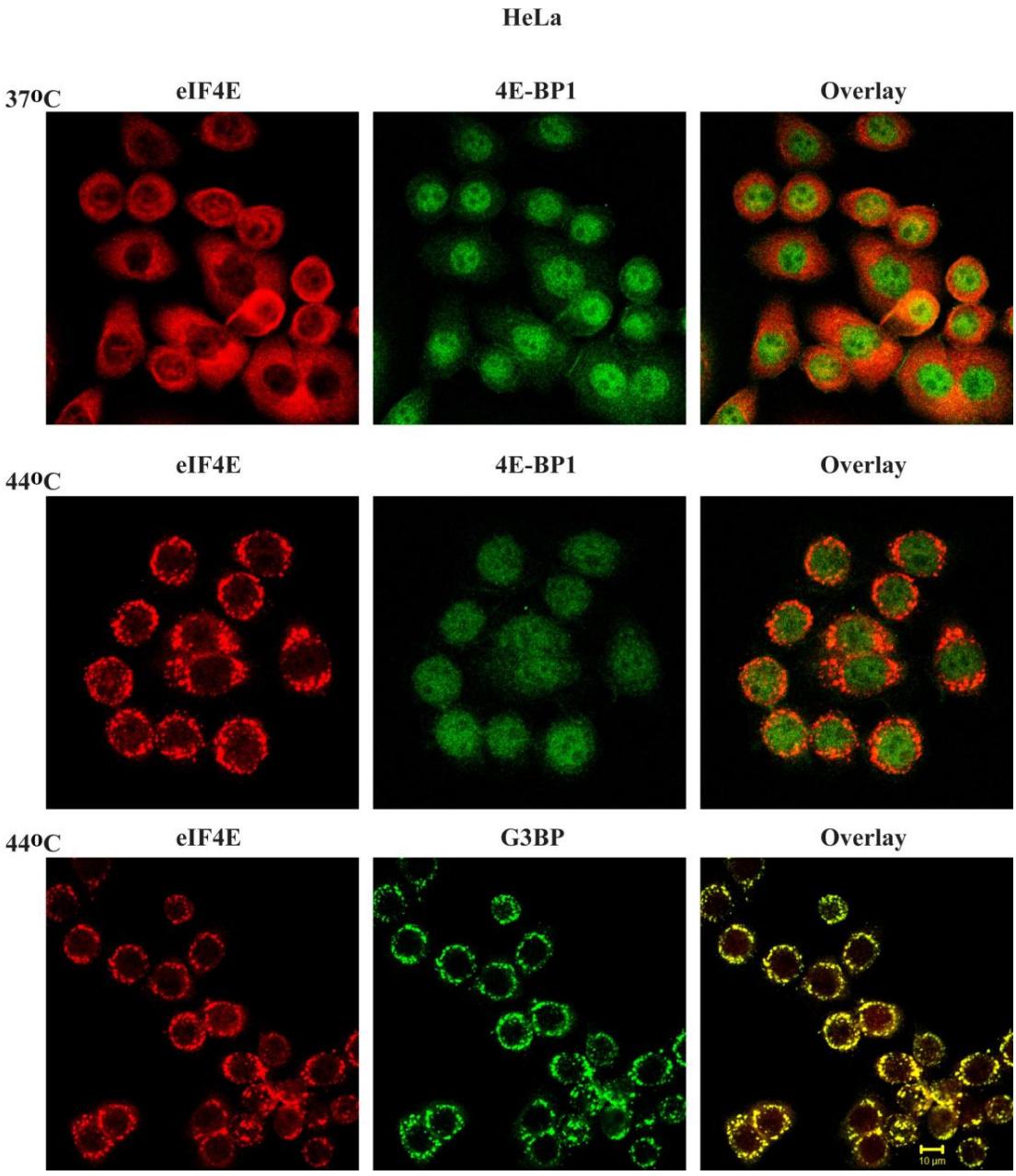

Figure 2.6: **4E-BP1 is not recruited to SGs upon heat shock.** HeLa cells were either maintained at 37°C or heat shocked (44°C/30 min), fixed, stained for eIF4E, 4EBP1, or G3BP.

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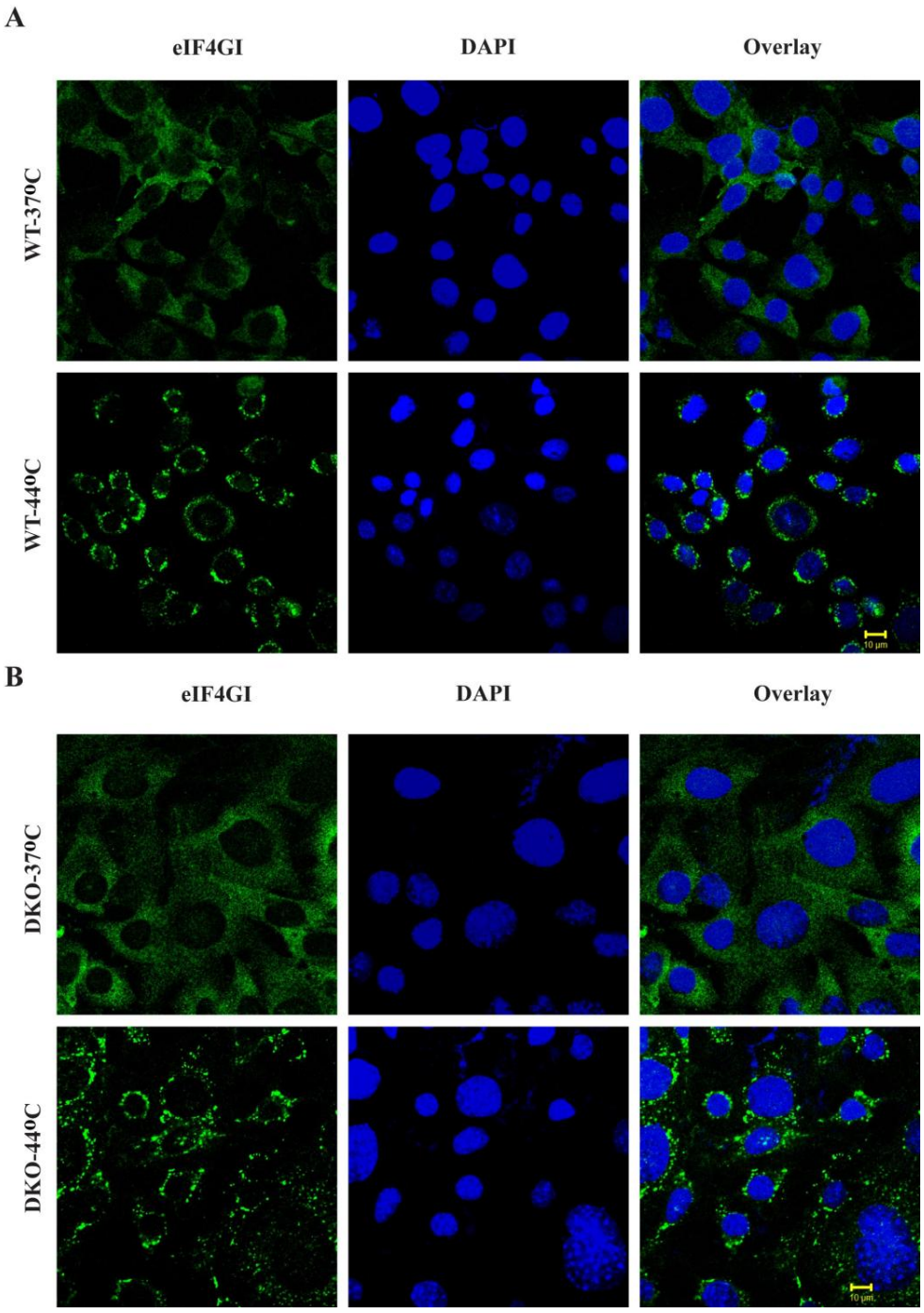

Figure 2.7: **eIF4GI recruitment to SGs is not affected by the presence or absence of 4E-BP1/2.** WT **(A)** or DKO **(B)** MEFs were maintained at 37^{o}C or exposed to 44^{o}C/30 min., fixed, and stained for eIF4GI and DAPI (nuclear marker).

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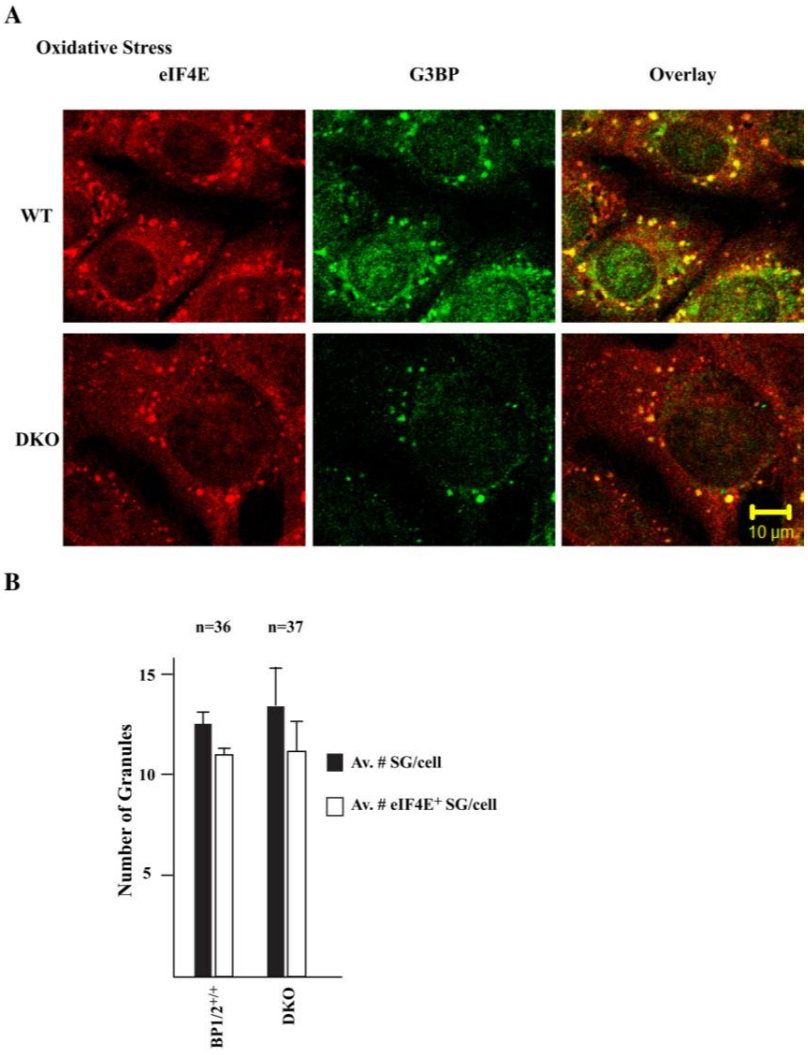

Figure 2.8: **Relocalization of eIF4E in MEFs cells upon arsenite treatment. A.** WT or DKO MEFs were exposed to arsenite (0.5mM for 1hr) after which time cells were fixed and stained for eIF4E and G3BP. **B.** Quantitation of the number SGs and eIF4E^{+}-SGs in arsenite-treated wt and DKO cells. n, represents the number of cells that were inspected in this experiment.

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2.4 DISCUSSION

Nuclear sequestration of eIF4E has been previously documented to be regulated by 4E-BPs (Rong, Livingstone et al. 2008). Attenuation of mTOR by serum starvation or rapamycin treatment causes increased nuclear sequestration of eIF4E. In addition, loss of nuclear 4E-BP1, in c-Ha-Ras expressing MEFs ablates starvation-induced nuclear accumulation of eIF4E (Rong, Livingstone et al. 2008). As well, ectopic expression of eIF4E-transporter causes mRNP remodelling by triggering the movement of eIF4E into P-bodies, an event associated with inhibition of cap-dependent protein synthesis (Lee, Cho et al. 2008). Our data extend these results by providing evidence that 4E-BP1 influences the cytoplasmic routing of eIF4E to SGs during heat shock stress. This is suggested by the increased presence of eIF4E in SGs of heat shocked DKO MEFs, relative to wt MEFs (Fig. 2.1) and reintroduction of a non-phosphorylateable 4E-BP1 mutant into DKO cells which diminished the amount of eIF4E localizing to SGs (Fig. 2.4). We interpret the ability of rapamycin to alter eIF4E localization upon heat shock to be a consequence of reduced 4E-BP phosphorylation, which prevents release of eIF4E from the nucleus to the cytoplasm (Fig. 2.5). Our results do not distinguish whether 4E-BPs directly or indirectly contribute to eIF4E accumulation in SGs, as could occur if SG accumulation were mediated by other competing factors in the absence of 4E-BPs. However, there does appear to be a strong correlation between 4E-BP phosphorylation status and eIF4E re-localization.

Our data is consistent with previous suggestions that 4E-BPs regulate the localization of eIF4E (Rong, Livingstone et al. 2008). Nuclear import of eIF4E is mediated by 4E-T via the importin α/β pathway (Dostie, Ferraiuolo et al. 2000). In the nucleus, eIF4E colocalizes with splicing factors (Dostie, Lejbkowicz et al. 2000). Binding of eIF4E in the nucleus to 4E-BP could regulate the amount of eIF4E present in the cytoplasm and hence the amount of eIF4E available for shuttling to SGs upon HS stress. The idea that cytoplasmic availability of eIF4E is regulated by 4E-BP is supported by data demonstrating that 4E-BP(5A), which is constitutively bound to eIF4E decreases the number of eIF4E-positive SGs in HS treated DKO cells (Fig. 2.4). A 4E-BP1(5A)

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mutant lacking the eIF4E binding site failed to alter the amount of eIF4E-positive SGs in DKO cells (Fig. 2.4). Consistent with this, inhibition of mTOR by rapamycin retained eIF4E into the nucleus and decreased the number of $eIF4E^{+}$-SGs in wt MEFs (Fig. 2.5).

The formation of SGs is considered to be one of the cellular hallmarks that ensures protection of the cell against stress-induced damage that might otherwise preclude recovery. SGs occur in cells exposed to a number of other environmental stresses, including oxidative stress, UV irradiation, ER stress, viral infection, and osmotic shock (Nover, Scharf et al. 1983; Arrigo, Suhan et al. 1988; Collier, Heuser et al. 1988; Nover, Scharf et al. 1989; Kedersha, Gupta et al. 1999; Gallouzi, Brennan et al. 2000). Since the pathways leading to the formation of SGs remain undefined, it is possible that different stimuli utilize different pathways and indeed this is reinforced by our findings that eIF4E localization to SGs appears more dependent on 4E-BP phosphorylation status in MEFs that are heat stressed, compared to MEFs that have been exposed to oxidative stress (Fig. 2.8) and where 4E-BP1 phosphorylation status is unchanged. Both heat stress and arsenite treatment were shown to induce the phosphorylation of eIF2α through the kinase activity of the heme-regulated inhibitor HRI (Lu, Han et al. 2001; McEwen, Kedersha et al. 2005). Although both heat stress and arsenite treatment were shown to enhance the eIF4E:4E-BP1 interaction (Feigenblum and Schneider 1996; Vries, Flynn et al. 1997; Patel, McLeod et al. 2002), we did not detect hypophosphorylation of 4E-BP1 upon arsenite-treatment (data not shown). Patel et al. (Patel, McLeod et al. 2002) used CHO cells in their study to document increased association between eIF4E and 4E-BP, we used MEFs in our current study.

Taken together we have provided evidence that 4E-BP1 regulates eIF4E localization to SGs. Hence, not only is eIF4E nuclear localization affected by 4E-BP1, but the amount of cytoplasmic eIF4E impacts on how much eIF4E is available to shuttle to SGs. Our results do not rule out the possibility that cytoplasmic sequestration of eIF4E by 4E-BPs also contributes to the differences observed in eIF4E SG localization between wt and DKO MEFs. As first noted by Nover and co-workers (Nover, Scharf et al. 1989), the heat shock response is associated with a shift in the pattern and intracellular distribution of mRNAs. They reported that mRNAs encoding heat shock proteins were predominantly present in polysomes of heat shocked cells, whereas nontranslating

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mRNAs were associated with SGs (Nover, Scharf et al. 1989). This led to the proposal that SGs act as storage sites for untranslated mRNAs that are retrieved upon elimination of the stress-inducing stimulus. If eIF4E is involved in trafficking mRNA to SGs via the cap structure then altering eIF4E levels could impact on how quickly SG formation occurs or how quickly protection is afforded.

2.5 MARERIALS AND METHODS

General Methods and Cell Maintenance.

Immortalized WT and 4E-BP1/2$^{+/+}$4E-BP1/2$^{-/-}$ MEFs were established by passing primary cells for more than 16 generations and experiments performed using cells between passage 30 and 40. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Plasmids MSCV-GFP-eIF4E-BP(5A), and MSCV-GFP-eIF4E-BP(5A) Δ4EBS were transiently transfected using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). Heat shock treatments were conducted by floating cell chambers in a water bath at the indicated temperatures and time. Cells were treated with 100 nM rapamycin (Calbiochem) or 0.5 mM sodium arsenite (Sigma-Aldrich) for 1 hour in a 37^{o}C/ 5% CO_2 incubator.

Plasmid Constructs

MSCV constructs (eIF4E-BP1, eIF4E-BP(5A) and eIF4E-BP5A-Δ4E) were made by subcloning XhoI / EcoRI fragments harbouring 4E-BPs into the same restriction sites in MSCV-GFP. eIF4E-BP(5A) and eIF4E-BP5A-Δ4E were described previously (Gingras, Gygi et al. 1999). To generate cell lines that stably express 4E-BP1 protein, MSCV constructs were transiently transfected into Phoenix-293T packaging cell line. After 48 hours, virus-containing medium was filtered (0.45 μm), collected and used to infect MEFs in the presence of 5 μg/ml polybrene (Sigma-Aldrich). The infection was repeated the next day. Fourty eight hours after the second infection, cells were FACS sorted for GFP+ cells.

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Antibodies

Anti-eIF4E antibody (monoclonal) was a gift from S. Kimball (Kimball, Horetsky et al. 2003). Rabbit polyclonal anti-G3BP and monoclonal ant-HuR antibodies have been previously described (Gallouzi, Parker et al. 1998; Gallouzi, Brennan et al. 2000). Anti-eIF4G1 and anti-4E-BP1 antibodies were obtained from Cell Signaling Technology (Danvers, MA).

Fluorescence Microscopy

Cells used for immunofluorescence were seeded in 4 well slide chambers at 20,000 cells per well and grown overnight. After treatment, cells were fixed using 4% formaldehyde in PBS, permeabilized with 4% formaldehyde/0.1%Triton X-100 in PBS, and blocked with 50%FBS/ 6%milk/3%BSA/0.1%Triton X-100/0.05%NaN_3 in PBS [modified from (Dostie, Ferraiuolo et al. 2000)]. Cells were then interrogated with anti-eIF4E (1:100), anti-G3BP (1:1000), anti-HuR (1:2000), anti-4E-BP1 (1:100), or anti-eIF4G1 antibodies (1:100) for 16 hrs at 4^{o}C. Cells were washed with PBS and incubated with secondary anti-rabbit and anti-mouse antibodies conjugated to Alexa fluor 488 or 594. Nuclei were stained either with Hoechst 33324 (Molecular Probes), or DAPI (Molecular Probes, D-1306) diluted at 1:2000. The immunofluorescence acquisition was performed more than 5 times, and the most representative results are shown. Images were taken from a 63X objective of a Zeiss LSM 510 confocal microscope.

The cell scoring module from MetaMorph (Molecular devices) was used to quantify the amount of SGs. For this purpose, we used the DAPI stained images to mask for the nuclear area. Then the software created an overlay with the images obtained with the anti-eIF4E and the anti-G3BP antibodies. This overlay image was used to quantitate the eIF4E-positive SGs. In addition, we use the G3BP images to quantitate the total number of SGs. The stress granules diameter range was set between 0.8 and 5μm, the software is capable of limiting the appropriate particles that fit within this range. By thresholding the background to 500 in a gray scale, the nonspecific background particles will be cancelled. The outcome will be the total number of stress granules or the total number of eIF4E positive stress granules.

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CHAPTER3

Nuclear assortment of eIF4E coincides with shut-off of host protein synthesis upon poliovirus infection

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3.1 ABSTRACT

Eukaryotic initiation factor (eIF) 4E is a component of the cap-binding protein complex, eIF4F, which recognizes the m^7GpppN cap structure of cellular mRNAs to facilitate translation initiation. eIF4E is assembled into the eIF4F complex via its interaction with eIF4G, an event that is under Akt/mTOR regulation. The eIF4E:eIF4G interaction is competed by eIF4E binding partners, such as eIF4E-Binding Proteins and eIF4E-Transporter. Cleavage of eIF4G occurs upon poliovirus infection and is thought to be responsible for the shut-off of host cell protein synthesis observed early in infection. Here we document re-assortment of eIF4E to the nucleus occurs concomitantly with cleavage of eIF4G upon poliovirus infection. This event is not dependent upon viral replication, but is dependent on eIF4G cleavage. We postulate that eIF4E nuclear re-assortment may contribute to the shut-off of host protein synthesis that is a hallmark of poliovirus infection by perturbing the circular status of actively translating mRNAs.

3.2 INTRODUCTION

Binding of eukaryotic initiation factor (eIF) 4E to the 5' mRNA cap structure [m^7GpppN; where N is any nucleotide] is thought to be the rate limiting step of cap-dependent translation [as reviewed in (Gingras, Raught et al. 1999)]. eIF4E is one of three subunits of the cap-binding protein complex eIF4F, which also includes eIF4A and eIF4G. eIF4A is an ATP-dependent RNA helicase required for unwinding of secondary structure in the 5'-untranslated region of mRNAs, in preparation for ribosome recruitment. eIF4G is a large scaffolding protein containing binding sites for eIF4E, eIF4A, and eIF3 - the latter likely mediating interactions between eIF4F and the small (40S) ribosomal subunit. A domain at the amino terminus of eIF4G also interacts with the poly (A)-binding protein (PABP) in mammals, plants, and yeast. This interaction is thought to mediate mRNA circularization during translation (Imataka, Gradi et al. 1998).

The assembly of eIF4E into the eIF4F complex is regulated by a family of three proteins called eIF4E-Binding Proteins (4E-BPs), which compete with eIF4G for binding to eIF4E. Once bound to eIF4E, 4E-BPs prevent the formation of the eIF4F complex and

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inhibit translation (Haghighat, Mader et al. 1995). We have recently shown that 4E-BPs control the sub-cellular localization of eIF4E upon stress (Rong, Livingstone et al. 2008; Sukarieh, Sonenberg et al. 2009). In the presence of 4E-BPs, a proportion of eIF4E is retained in the nucleus and its assembly into heat-shock-induced stress granules (SGs) is reduced. Although 4E-BPs and eIF4G compete for the same binding site on eIF4E, the knockout of 4E-BP1 and 4E-BP2 does not influence the localization of eIF4G to SGs but does allow increased eIF4E localization to SGs (Sukarieh, Sonenberg et al. 2009). These observations suggest that eIF4E binding partners play a general role in controlling the localization of eIF4E.

Two eIF4G isoforms, eIF4GI and eIF4GII (henceforth referred to collectively as eIF4G), have been indentified in eukaryotic cells. eIF4GI and eIF4GII are 46% identical, have similar biochemical activities, and are functionally interchangeable (Gradi, Imataka et al. 1998; Gradi, Svitkin et al. 1998; Imataka, Gradi et al. 1998). Infection of cells by poliovirus results in shut-off of host cell protein synthesis, which is preceded by the cleavage of eIF4GI, then eIF4GII (Gradi, Svitkin et al. 1998). Analysis of the kinetics of shut-off have shown that cleavage of both eIF4GI and eIF4GII appears to be required for the shut-off of host protein synthesis after poliovirus infection (Gradi, Svitkin et al. 1998).

Here we show that the subcellular localization of eIF4E is altered upon poliovirus infection, with eIF4E redistributing to the nucleus following infection and correlating with eIF4GI and eIF4GII cleavage. Nuclear sequestration of proteins is not a general phenomenon as the subcellular localization of PABP is not altered by poliovirus infection. eIF4E re-assignment to the nucleus occurs independent of poliovirus replication, but is dependent on viral gene expression and cleavage of eIF4G. Thus, as described for the cleavage of eIF4GI and eIF4GII, nuclear sequestration of eIF4E is an early signature of poliovirus infection.

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3.3 RESULTS

eIF4E nuclear re-assortment in poliovirus infected cells coincides with inhibition of host protein synthesis and cleavage of eIF4GI and eIF4GII.

We have previously demonstrated that eIF4E subcellular localization is influenced by the presence or absence of 4E-BP1 and 4E-BP2 (Rong, Livingstone et al. 2008; Sukarieh, Sonenberg et al. 2009). Since eIF4G family members are direct binding partners of eIF4E, we sought to investigate whether eIF4G cleavage during poliovirus infection would also influence sub-cellular localization of eIF4E. To address this, we infected HeLa cells with poliovirus type 1 Mahoney and monitored the subcellular distribution of eIF4E by immunofluorescence as a function of time post-adsorption (Fig. 3.1A-B). Analysis of uninfected HeLa cells indicates that the majority of eIF4E is present in the cytoplasm (Fig. 3.1A-B). However ~ 1.5 h post-adsorption of poliovirus, there was clear enrichment in the amount of eIF4E present in the nucleus and by 2.5 hrs, a significant proportion of eIF4E was relocalized to the nucleus (the average cytoplasmic/nuclear eIF4E ratio dropped by ~2.7 fold) (Fig. 3.1A-C).

To determine if the redistribution of eIF4E to the nucleus coincided with host protein synthesis shut-off, and cleavage of the eIF4G isoforms, we performed metabolic [^{35}S]methionine labeling and Western blot analysis on extracts prepared at various time points post-infection (Fig. 3.2). These experiments were performed concomitantly with those presented in Fig. 1. By 2.5 hrs post-infection of HeLa cells, the expected shut-off of host protein synthesis was observed (Fig. 3.2A) (Gradi, Svitkin et al. 1998). In parallel, cleavage of eIF4GI was complete by 2 hrs P.I. whereas eIF4GII was completely cleaved by 2.5 hrs P.I. (Fig. 3.2), as previously reported (Gradi, Svitkin et al. 1998).

eIF4E is retained into the nucleus despite altered nucleo-cytoplasmic distribution of hnRNPA1.

We next asked whether other eIF4G interacting proteins were retained in the nucleus upon poliovirus-infection and if the results we observed with eIF4E were a consequence of altered nuclear permeability arising as a consequence of poliovirus replication (Gustin

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and Sarnow 2001). To track these possibilities, we monitored the behaviour of PABP upon poliovirus infection since it also interacts with the N-terminal domain of eIF4GI and eIF4GII (Imataka, Gradi et al. 1998). Our results indicate that PABP distribution is not altered up to 3 hrs following poliovirus infection (Fig. 3.3A-B). In order to rule out non-specific effects of poliovirus infection on nucleo-cytoplasmic trafficking, we compared the localization of eIF4E to the nuclear protein, hnRNPA1. HnRNPA1 protein has been previously documented to translocate to the cytoplasm upon poliovirus infection at 3 hrs post-infection (Gustin and Sarnow 2001). We confirmed these findings and found that the majority of hnRNPA1 is indeed re-located in the cytoplasm 3 hrs post-adsorption, whereas PABP localization remains unchanged and eIF4E becomes equally distributed between the nucleus and cytoplasm (Fig. 3.3C).

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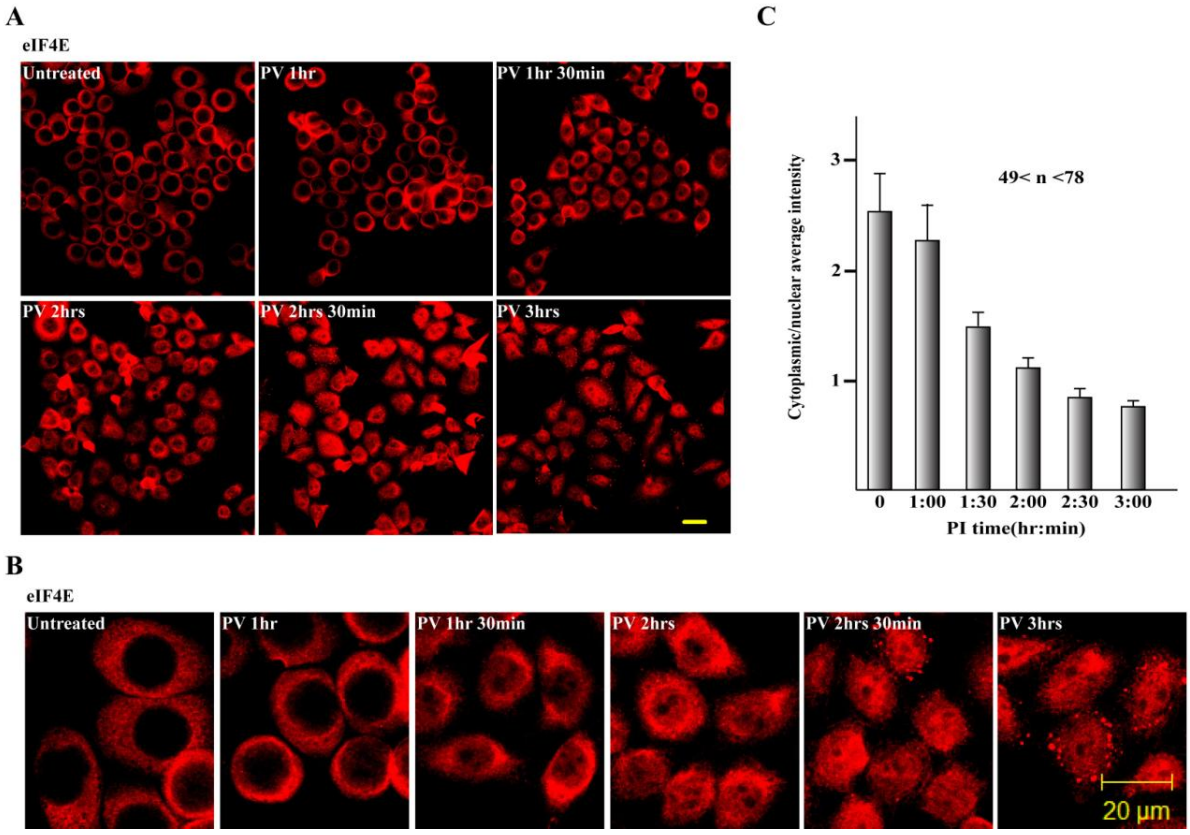

Figure 3.1: **Sub-cellular localization of eIF4E is altered upon poliovirus infection. (A)** HeLa cells at 80% confluency were infected with poliovirus, fixed, and stained for eIF4E at the indicated times post-adsorption. **(B)** Higher magnification of cells from **(A)**. **(C)** Quantitation of relative cytoplasmic to nuclear eIF4E abundance. Using MetaMorph (Molecular devices), we measured the average intensity of nuclear and cytoplasmic eIF4E and present the calculated ratio. The Y axis represents the ratio of the average intensity of the cytoplasm to the nucleus. 'n' represents the number of cells that were inspected in this experiment. The numbers ranged between 49 and 78. Scale bars: 20µm.

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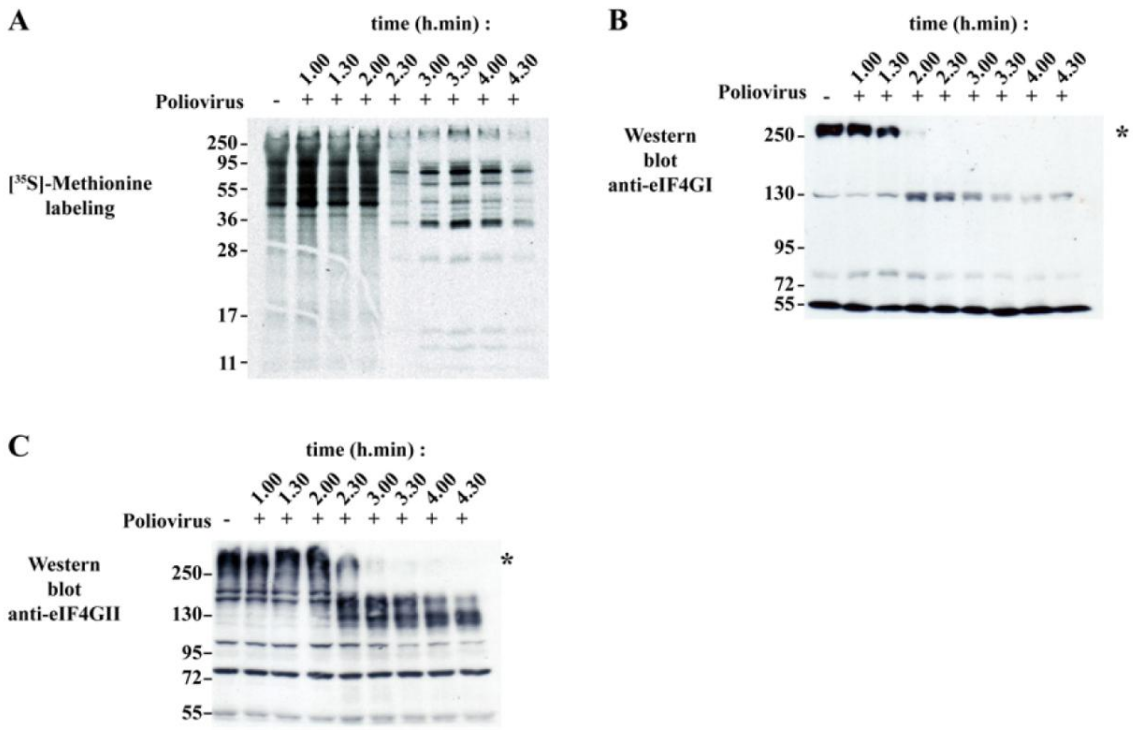

Figure 3.2: **Kinetics of host protein synthesis and eIF4G cleavage in poliovirus infection HeLa cells. (A)** HeLa cells at 80% confluency were adsorbed with virus at RT, followed by incubation at 37°C and labeled for 15 min with [^{35}S]methionine before harvesting at the indicated times. Protein extracts (5 µg) were analyzed on 15% polyacrylamide-SDS gels. (**B and C)** Western blot analysis of the samples prepared in **(A)** (20µg) following fractionation on a 6% SDS-PAGE. Both anti-eIF4GI (1:1000) and anti-eIF4GII (1:1000) are polyclonal rabbit antibodies that recognize the N-terminal domain of the proteins. The results shown are representatives of experiments repeated at least twice with similar results.

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Poliovirus replication is not required for nuclear re-localization of eIF4E.

To address if nuclear re-localization of eIF4E is dependent on poliovirus replication, we blocked replication using the inhibitor guanidine-HCl [GuHCl] (Bablanian 1972; Rose, Trachsel et al. 1978). In the presence of GuHCl, only a partial shut off of host protein synthesis is achieved in infected cells (Bonneau and Sonenberg 1987). GuHCl prevents the translation of viral proteins but does not prevent cleavage of eIF4GI or eIF4GII (Fig. 3.4A-C) (Bonneau and Sonenberg 1987). Exposure of uninfected or poliovirus-infected cells to GuHCl had no effect on the subcellular distribution of eIF4E (Fig. 3.4D). These results indicate that the translocation of eIF4E is not dependent on viral replication.

Pyrithione prevents eIF4G cleavage and eIF4E translocation.

Pyrithione, a zinc ionophore, is known to inhibit replication of picornaviruses by impairing viral polyprotein processing (Krenn, Gaudernak et al. 2009). Hence, pyrithione can be used to block eIF4G cleavage by the 2A protease. In order to assess if eIF4GI and eIF4GII cleavage during poliovirus infection is required for eIF4E nuclear re-localization, we exposed mock- or poliovirus-infected Hela cells to pyrithione. The translation of viral proteins and the cleavage of eIF4GI and eIF4GII are prevented by pyrithione (Fig. 3.5A-B). In mock-infected cells, pyrithione did not alter the cytoplasmic localization of eIF4E (Fig. 3.5C). However, the nuclear retention of eIF4E in poliovirus-infected cells was clearly reduced by the presence of pyrithione (Fig. 3.5C-D). These results are consistent with cleavage of eIF4G being required for eIF4E nuclear translocation.

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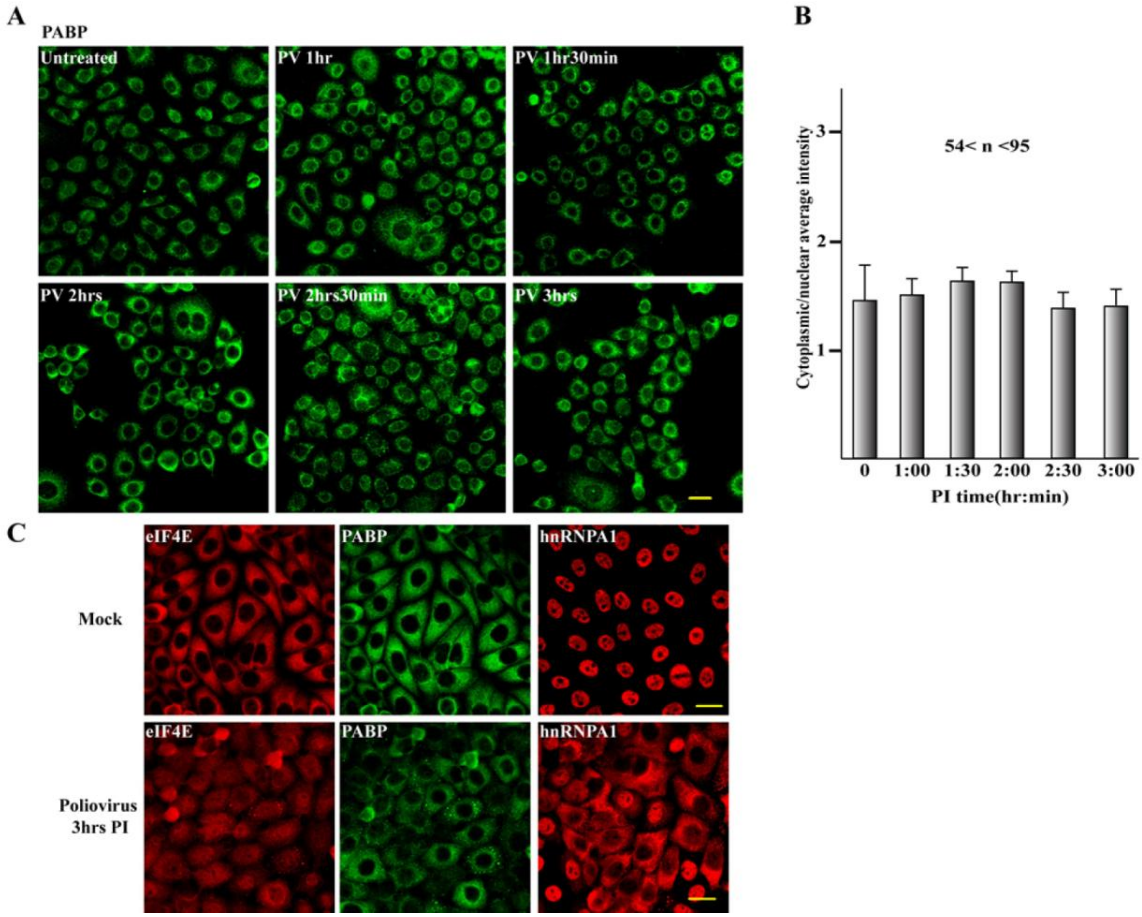

Figure 3.3: **Poliovirus infection stimulates nucleo-cytoplasmic export of hnRNPA1 while nuclear import of eIF4E. (A)** Subcellular distribution of PABP in poliovirus-infected HeLa cells. HeLa S3 cells were infected with poliovirus, fixed and probed for PABP at the indicated times after adsorption. Scale bars: 20µm. **(B)** Quantitation of cytoplasmic and nuclear PABP. Using MetaMorph, we measured the average intensity of the PABP signal in the nuclear and cytoplasmic area of the cells and present the calculated ratio. The Y axis represents the ratio of the average intensity of the cytoplasm to the nucleus. 'n' represents the number of cells that were inspected in this experiment. The number range between 54 and 95. Scale bars: 20µm. **(C)** Mock- or poliovirus-infected cells (3hrs) were fixed and immuno-stained for eIF4E, PABP, and hnRNP A1. Scale bars: 20µm.

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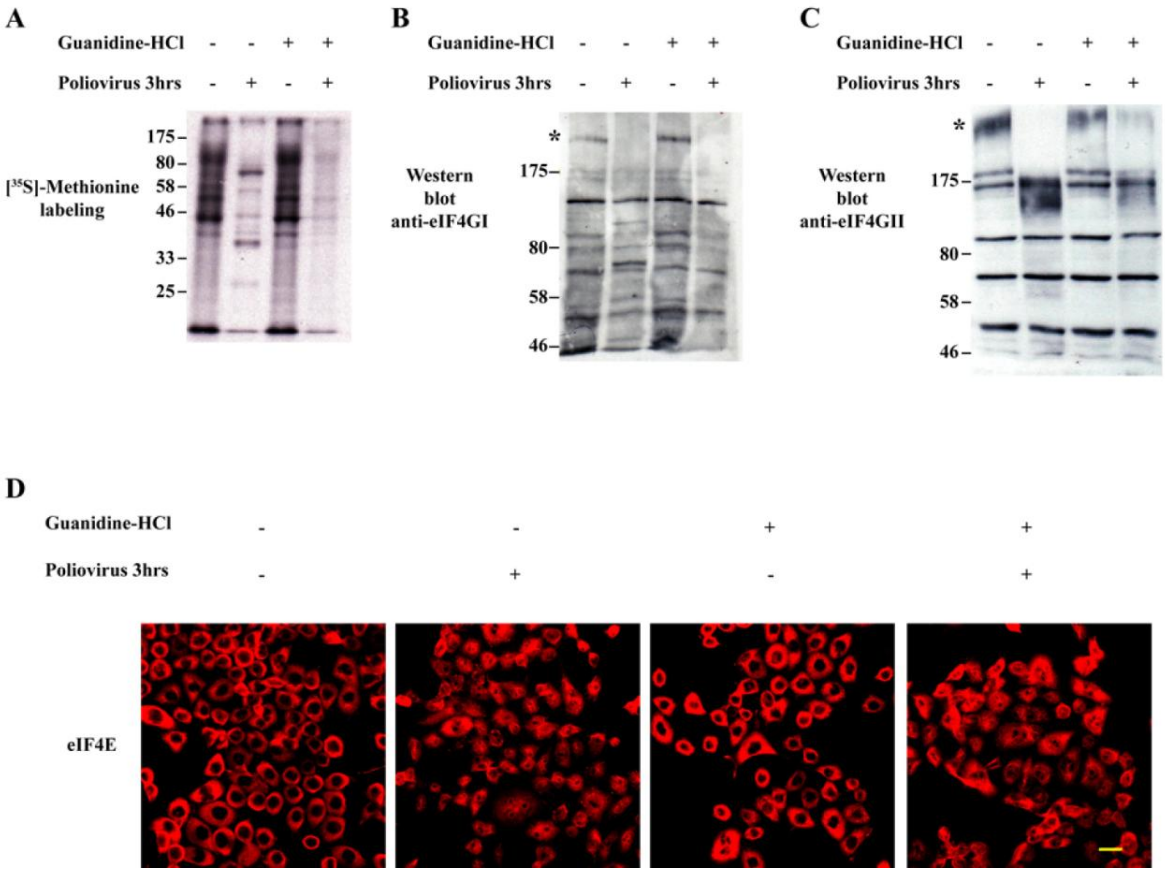

Figure 3.4: **Effect of GuHCl on the subcellular distribution of eIF4E in poliovirus-infected HeLa cells. (A)** HeLa cells were infected with poliovirus in the presence or absence of GuHCl (1.5 mM) and labelled for 15 min with [^{35}S] methionine three hours after infection. Equal amounts of cytoplasmic extract (5 µg) were fractionated on a 12% SDS-PAGE. **(B and C)** Equal amounts of cytoplasmic extract (20µg) were fractionated on a 6% SDS-PAGE and analyzed by Western blotting for eIF4GI (1:1000) and eIF4GII (1:1000). The position of migration of eIF4GI and eIF4GII are denoted by an asterisk. **(D)** Sub-cellular localization of eIF4E in poliovirus-infected cells treated with GuHCl. HeLa cells were fixed and probed for eIF4E three hours after infections. The absence or presence of 1.5 mM GuHCl during infection is indicated. Scale bars: 20µm.

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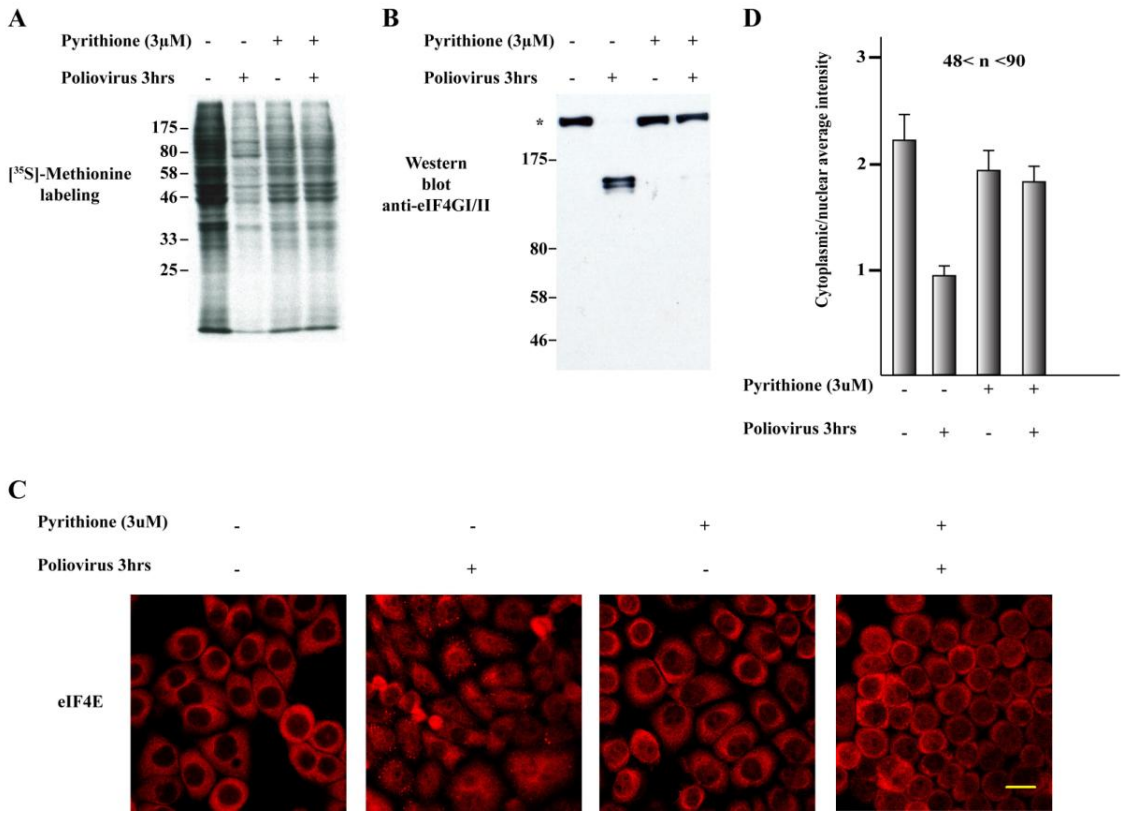

Figure 3.5: **Effects of Pyrithione on the subcellular distribution of eIF4E in poliovirus-infected HeLa cells. (A)** HeLa cells were infected with poliovirus in the presence or absence of pyrithione (3μM) and labelled for 15 min with [^{35}S] methionine three hours after infection. Equal amounts of cytoplasmic extract (5 μg) were fractionated on a 10% SDS-PAGE. **(B)** Equal amounts of cytoplasmic extract (20μg) were fractionated on a 6% SDS-PAGE and analyzed by Western blotting for eIF4G (1:1000) (this antibody recognizes both eIF4GI and eIF4GII). The position of migration of eIF4GI/II is denoted by an asterisk. **(C)** Sub-cellular localization of eIF4E in poliovirus-infected cells treated with pyrithione. HeLa cells were fixed and probed for eIF4E three hours after infections in the absence or presence of pyrithione. **(D)** Quantitation of cytoplasmic and nuclear eIF4E. Using MetaMorph (Molecular devices), we measured the average intensity of the eIF4E signal in the nuclear and cytoplasmic area of the cells and present the calculated ratio. The Y axis represents the ratio of the average intensity of the cytoplasm to the nucleus. 'n' represents the number of cells that were analyzed in this experiment. The numbers range between 48 and 90. Scale bars: 20μm.

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3.4 DISCUSSION

Cleavage of both eIF4GI and eIF4GII is associated with shut-off of host cell protein synthesis upon poliovirus infection (Gradi, Svitkin et al. 1998). Herein, we show that eIF4E translocation to the nucleus increases concomitantly with cleavage of eIF4G and onset of host protein synthesis shut-off (Figs. 3.1-3.2). Although PABP also binds to the N-terminus of eIF4G near the eIF4E binding site, poliovirus-induced cleavage of eIF4G does not induce PABP translocation to the nucleus (Fig. 3.3). Thus, accumulation of eIF4E into the nucleus does not appear to be dependent on the N-terminal truncated eIF4G fragment or due to breakdown of the nuclear envelope. In addition, hnRNPA1 is actually transported out of the nucleus suggesting that bi-directional transport is still active in poliovirus-infected cells. We suggest that depletion of eIF4E from the cytoplasm may play a role in poliovirus mediated shut off of host translation by stimulating linearization of mRNA templates. Cooperative binding of the eIF4F complex and PABP to the mRNA engenders a more stable eIF4E association with the cap structure than eIF4G/4E binding alone and is thought to maintain the mRNA in a circular state during translation (Kahvejian, Svitkin et al. 2005). mRNA circularization is thought to increase translation initiation possibly by allowing for more efficient re-initiation of terminating ribosomes (Sachs 2000). Cleavage of eIF4G between the eIF4E and eIF4A binding sites [as occurs during poliovirus infection [reviewed in (Lloyd 2006)] is not predicted to perturb the circular state of the mRNA template and such transcripts may continue to translate. Active re-assignment of eIF4E from the cytoplasm to the nucleus upon viral infection as described herein would favor linearization of cellular transcripts and lead to a further reduction in translation efficiency. Although we have not defined the mechanism by which eIF4E is relocalized to the nucleus, this could be mediated by the N-terminal fragment of eIF4G which harbors a motif that is sufficient for nuclear localization (Coldwell, Hashemzadeh-Bonehi et al. 2004). The 4E-BPs may also be involved since these have been previously identified as modifiers of eIF4E localization (Sukarieh, Sonenberg et al. 2009). Our results indicate that eIF4E re-assortment to the nucleus during poliovirus infection is reinforcement, in conjunction with eIF4GI/II

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cleavage, for the host cell translation shut-off in favour of the viral IRES-dependent translation.

In order to confirm our finding and hypothesis further, we will test the effect of poliovirus on eIF4E localization in the presence of an eIF4G mutant known to be resistant to the viral protease cleavage. We expect to see a rescue of the nuclear eIF4E translocation. In addition, we will test the effect of encephalomyocarditis virus, EMCV, on the localization of eIF4E. This virus has a similar mode of action to the poliovirus in term of shutting down host cell protein synthesis, but it does not cleave eIF4G.

3.5 MATERIALS AND METHODS

General methods and cell maintenance

HeLa S3 cells were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin. For poliovirus infections, adsorptions were performed at room temperature for 30 min, after which time the cells were incubated at 37°C. Infections were performed with the Mahoney strain of poliovirus type 1 at a multiplicity of infection of 10 pfu/cell. For labelling experiments, cells were incubated with [^{35}S] methionine (50 µCi ml^{-1}) for 15 min before the end of the experiment. After radiolabeling, cells were lysed in RIPA lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 µg/ml leupeptin, 1 µg/ml pepstatin). Guanidine-HCl (Sigma-Aldrich, G3272) and pyrithione (Sigma-Aldrich, H6377) were added to cells after viral adsorption and were used at final concentrations of 1.5 mM and 3 µM, respectively.

Antibodies

The anti-eIF4E antibody (mouse monoclonal) was a gift from S. Kimball (The Pennsylvania State University College of Medicine, Hershey, PA, USA) (Kimball, Horetsky et al. 2003). Anti-eIF4GI and anti-eIF4GII polyclonal antibodies have been previously described (Gradi, Imataka et al. 1998). The anti-PABP antibody was a kind gift of Dr. NK Gray (MRC, Edinburg, UK). Anti-eIF4G (Cat#2498) was obtained from Cell Signaling Technology (Danvers, MA, USA) and anti-hnRNPA1 antibody (9H10) was from Abcam (Cambridge, MA, USA).

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Fluorescence Microscopy

For immunofluorescence, cells were seeded in 4 well slide chambers at 50,000 cells per well the day before the experiment. After infection and treatments, cells were fixed using 4% formaldehyde in PBS, permeabilized with 4% formaldehyde/0.1%Triton X-100 in PBS, and blocked with 50%FBS/6%milk/3%BSA/0.1%Triton X-100/0.05%NaN_3 in PBS [modified from ref (Dostie, Ferraiuolo et al. 2000)]. Cells were then interrogated with anti-eIF4E (1:100), anti-PABP (1:200), or anti-hnRNPA1 antibodies (1:1000) for 16 hrs at 4^{o}C, washed with PBS, and incubated with secondary antibodies conjugated to Alexa fluor 488 or 594. Nuclei were stained with DAPI (5mg/ml) (Molecular Probes, D-1306) diluted at 1:2000. The immunofluorescence acquisition was performed more than three times, and a representative result is shown in each case. Images were taken from a 63X objective of a Zeiss LSM 5 PASCAL confocal microscope.

The cell scoring module from MetaMorph (Molecular devices) was used to quantify the average intensity of eIF4E or PABP in the cytoplasm and the nucleus of the cells. We used the DAPI stained image to mask for the nuclear area. The software then created an overlay with the image obtained with the anti-eIF4E or anti-PABP antibodies. This overlay image delimits the nuclear and the cytoplasm area of eIF4E or PABP and quantifies the average intensities in the nucleus and the cytoplasm. Using these values, the ratio of the average intensities was calculated.

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CHAPTER4:
GENERAL DISCUSSION

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Since its discovery in the late ‘70s (Sonenberg, Morgan et al. 1978), eIF4E has been linked to the binding of the mRNA cap structure and therefore its role in translation initiation in the cytoplasm (Sonenberg 2008). eIF4E has gained interest for its potential nuclear role in mRNA metabolism, such as exporting a subset of mRNA from the nucleus (Rousseau, Kaspar et al. 1996; Lai and Borden 2000).

The nuclear import of eIF4E was reported to occur via the importin αβ pathway which requires a nucleocytoplasmic protein 4E-T (Dostie, Ferraiuolo et al. 2000). Since eIF4E does not have a Nuclear Localization Sequence (NLS), it has to rely on the NLS of 4E-T for import into the nucleus (Dostie, Ferraiuolo et al. 2000). In the cytoplasm, eIF4E binds the cap structure of the mRNA in order to assure proper cap-dependent translation initiation. In addition, eIF4G and 4E-BPs bind eIF4E and control its activity. All those cytoplasmic interactions would favour eIF4E localization and retention in the cytoplasm under normal conditions. Sonenberg and colleagues (Dostie, Ferraiuolo et al. 2000) showed eIF4E is retained in the cytoplasm in the absence of 4E-T or upon mutation of the binding site for eIF4E. This work suggests that eIF4E requires 4E-T as a carrier for nuclear import under normal conditions. As a result, controlling 4E-T import or export might be a key player to control the import of eIF4E. One way of controlling 4E-T would be through phosphorylation since 4E-T is a phosphoprotein (Pyronnet, Dostie et al. 2001). Phosphorylation affects the nuclear import and/or export of other proteins such as nucleoplasmin (Vancurova, Paine et al. 1995) and Jak family members (Leonard and O'Shea 1998).

The cap is involved in several aspects of RNA metabolism in the nucleus such as efficient cleavage of the poly (A) site (Hart, McDevitt et al. 1985), pre-mRNA splicing (Konarska, Padgett et al. 1984), and nuclear export of RNA polymerase II transcripts (Jarmolowski, Boelens et al. 1994). It was shown that several nuclear cap-binding proteins assist the cap in its function such as the nuclear cap binding complex (CBC) (Rozen and Sonenberg 1987; Izaurralde, Lewis et al. 1994). Based on this, it was suggested that the nuclear function of eIF4E is related to RNA metabolism (Dostie, Ferraiuolo et al. 2000).

The role of eIF4E in promoting nuclear export of mRNAs was investigated (Rousseau, Kaspar et al. 1996; Culjkovic, Topisirovic et al. 2005). Sonenberg and

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colleagues showed that overexpression of eIF4E enhances translation of certain mRNAs, such as ODC, due to an increase in the cytoplamic to nuclear ODC mRNA ratio. On the other hand, the same phenotype was not seen for Actin and GAPDH. The hypothesis to explain this discrepancy is based on differences among 5'UTR structures. Actin and GAPDH possess 5'UTR of average length (50-100 nucleotides), and a low G+C content. Whereas the 5'UTR of ODC is longer and has a high G+C content (~70%). Sonenberg and colleagues (Rousseau, Kaspar et al. 1996) suggested that eIF4E had a variable sensitivity to these structures which would influence the nucleocytoplasmic transport of the mRNAs. As a result, higher concentrations of these mRNAs in the cytoplasm would increase availability of these mRNAs to the translational machinery (Rousseau, Kaspar et al. 1996).

Similarly, Cyclin D1 mRNAs was shown to be more sensitive to eIF4E binding than Actin or GAPDH (Rousseau, Kaspar et al. 1996; Lai and Borden 2000; Topisirovic, Culjkovic et al. 2003). To follow up on this, Borden and colleagues (Culjkovic, Topisirovic et al. 2005) showed the presence of a nuclear interaction between eIF4E and the 3'UTR structure of Cyclin D1 mRNA. This interaction was limited to 100 nucleotides referred to as 4E-sensitive element (4E-SE). Importantly, this element is highly conserved between human, mouse, rat, and chicken sequences (Culjkovic, Topisirovic et al. 2005). What needs to be clarified further is the significance of the eIF4E/4E-SE interaction and whether other factors are involved.

As a conclusion, eIF4E was shown by multiple groups to be a nucleocytoplasmic dynamic protein having essential roles in the control of cellular translation. The binding capacity of eIF4E to the cap structure of the mRNAs, as well its potential interaction with the 5' and 3' UTR structures, would make it an excellent target for upstream control mechanism. Therefore, modifying the localization of eIF4E as influenced by its binding partners in the nucleus or the cytoplasm should have a direct effect on its function. In order to further understand the role of eIF4E binding partner in the control of eIF4E activity under various conditions, we investigated several scenarios in which 4E-BPs and eIF4G levels were altered.

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4.1 eIF4E nuclear retention

4E-BPs, a family of translational suppressors, control the activity of eIF4E. Binding of the 4E-BPs to eIF4E is controlled by the phosphorylation status of 4E-BPs. The hypophosphorylated forms of 4E-BP bind to eIF4E and prevent interaction of eIF4E with eIF4G, thus impairing cap-dependent translation (Haghighat, Mader et al. 1995).

Biochemical fractionation experiments have previously led to the conclusion that 4E-BP1 localization is restricted to the cytoplasm (Kim and Chen 2000; Zhang, Shu et al. 2002). These findings would limit the interaction of 4E-BPs/eIF4E to the cytoplasm. Unfortunately, these techniques are subject to limitations due to leakage of small proteins from the nucleus. We showed that ~30% of 4E-BPs localize to the nucleus using direct immunofluorescence techniques and live imaging (Rong, Livingstone et al. 2008). This observation prompted us to investigate the potential eIF4E/4E-BP nuclear interaction. In addition, it is of interest that mTOR (the upstream kinase activator of 4E-BPs) was reported to be a nucleocytoplasmic shuttling protein whose nuclear localization is required for 4E-BP1 phosphorylation (Kim and Chen 2000; Zhang, Shu et al. 2002).

We demonstrated that 4E-BP1 induce the nuclear retention of eIF4E under serum starvation and rapamycin treatment. Those conditions are known to inhibit the activity of mTOR and therefore lead to dephosphorylation of 4E-BPs (Gingras, Raught et al. 2004). The 4E-BP–dependent regulation of eIF4E localization correlates well with the dephosphorylation of 4E-BP1 and its increased binding to eIF4E. This conclusion was further supported by data demonstrating that HA-4E-BP1(4Ala) mutant, which is constitutively bound to eIF4E, causes nuclear accumulation of eIF4E even under standard conditions. Thus, 4E-BP phosphorylation status could control the amount of nuclear eIF4E by limiting its release from the nucleus. This retention could be an effective means to inhibit cap-dependent translation. Furthermore, we hypothesized that sequestering eIF4E in the nucleus is more efficient than in the cytoplasm, because 4E-BPs do not need to compete with the mainly cytoplasmic eIF4G and 4E-T (Rong, Livingstone et al. 2008).In parallel, we wanted to study the effect of 4E-BPs on the localization of eIF4E under other stressful conditions. To do so, we investigated the effect of heat stress on eIF4E localization in the absence of 4E-BPs.

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4.2 eIF4E localization to stress granules (SG)

Stress granule formation upon environmental stress is considered a posttranscriptional regulatory mechanism utilized by the cell to overcome stressful conditions. Since eIF4E binds to the mRNAs and it is controlled by 4E-BPs, it is of interest to study the localization of eIF4E to SGs in the absence of 4E-BPs. To do so, wild type (wt) and DKO MEFs were used. Under standard growth conditions, endogenous eIF4E is localized primarily in the cytoplasm of both wt and DKO MEFs, with a minor amount of eIF4E (10-15%) present in the nucleus of wt MEFs (Sukarieh, Sonenberg et al. 2009). Upon exposure to heat stress, eIF4E was differently redistributed in both cell types. In wt MEFs, eIF4E was concentrated into SGs and relocated to the nucleus, whereas eIF4E was only located to the SGs in DKO MEFs. These results suggested that 4E-BP is reducing the localization of eIF4E to SGs and sequestering eIF4E into the nucleus. Whereas in DKO MEFs, eIF4E was not retained into the nucleus and it was further located to SGs. These data were further confirmed by a rescue experiment in the DKO MEFs where 4E-BP (5Ala) mutant, where 4E-BP phosphorylation sites are mutated to alanine, was capable of retaining eIF4E into the nucleus and away from SGs. We hypothesized that the absence of 4E-BP renders eIF4E more available to bind to other binding partners - mainly eIF4G. This interaction might enhance the localization of eIF4E to SGs especially that eIF4G localization to SGs was not affected by the state of 4E-BP. On the other hand, we reported that 4E-BP was not retained in SGs although it binds eIF4E. The hypothesis to explain this phenotype could be due to the competition between 4E-BP and eIF4G on eIF4E binding site. Upon localization into SGs, eIF4G is present at a high concentration compared to 4E-BP and therefore eIF4G is favoured to bind eIF4E. That’s why 4E-BP is not retained into SGs. This balance is tilted upon the addition of a 4E-BP (5Ala) mutant or pre-treatment of the cells with rapamycin which will enhance the eIF4E/4E-BP interaction and eIF4E is retained in the nucleus.

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Since the induction of SG formation upon heat stress is dependent on the activation of the HRI-kinase activity (Chen 2000), and since oxidative stress (arsenite treatment) activate the same kinase, we monitored the localization of eIF4E to SGs in the absence of 4E-BP upon arsenite treatment. In this case, both wt and DKO MEFs showed similar phenotype i.e. eIF4E was not retained in the nucleus but rather was localized to the SGs. This experiment confirmed our previous observation in DKO MEFs upon HS where eIF4E was not retained in the nucleus but rather localized to SGs.

We should keep in mind that the downstream effect of HRI-kinase is to induce the phosphorylation of the α-subunit of eIF2, leading to the reduction in the formation of the ternary complex. If the eIF2α subunit was mutated for the phosphorylation site Ser51, the cells loose the capacity to form SGs upon heat stress and oxidative stress (Kedersha, Stoecklin et al. 2005). Therefore, the localization of eIF4E to SGs, that is controlled by the presence of 4E-BPs, is dependent on the general formation of SGs as induced by the phosphorylation of eIF2α. This was a general belief until we showed that it is possible to localize eIF4E to SGs without the need for eIF2α phosphorylation (Mazroui, Sukarieh et al. 2006). We demonstrated that perturbing eIF4A and/or inhibiting eIF4F activity induces SG assembly without the need to phosphorylate eIF2α. We used multiple stimuli that affect ribosome recruitment to mRNAs during translation initiation. These stimuli include compounds treatment such as hippuristanol and pateamine, poliovirus infection and eIF4A siRNA. In all these cases, the cap dependent translation was affected. eIF4E was also localized to SGs upon hippuristanol (Pelletier, Mazroui et al. 2008) and pateamine treatment of cells (R.S. unpublished data). Consequently, we hypothesized that reducing the availability of ternary complex (and its subsequent effects on global protein synthesis), and not phosphorylation of eIF2α, is responsible for SG formation.

4.3 eIF4E re-assortment to the nucleus upon eIF4G cleavage.

Poliovirus infection induces host-cell protein synthesis shut-off and is associated with cleavage of both eIF4GI and eIF4GII (Gradi, Svitkin et al. 1998). The cleavage of eIF4Gs produce an N-terminus of eIF4G containing the binding site for eIF4E and PABP, whereas the C-terminal fragment contains the binding sites for eIF4A and eIF3.

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Therefore, the eIF4F complex is dissociated and the cap-dependent translation of the host cell is inhibited favouring the IRES-dependent translation of the viral RNA. We showed (chapter 3) that eIF4E translocates to the nucleus upon poliovirus infection concomitantly with cleavage of eIF4G. Although PABP also binds to the N-terminus of eIF4G near the eIF4E binding site, poliovirus-induced cleavage of eIF4G does not induce PABP translocation to the nucleus. Based on this observation, we hypothesized that the different localization behaviour of eIF4E and PABP could be related to the difference in their requirement for viral RNA translation. Binding of eIF4E to the cap structure, and being part of the eIF4F/PABP interaction favour mRNA circularization during translation (Kahvejian, Svitkin et al. 2005). Therefore, nuclear translocation of eIF4E would favour linearization of cellular transcripts and lead to a further reduction in translation efficiency. On the other hand, PABP was shown to play an important role in poliovirus replication, as its removal dramatically reduces the infectivity of poliovirus RNA (Sarnow 1989). eIF4E translocation could be mediated by the N-terminal fragment of eIF4G which harbours a motif that is sufficient for nuclear localization (Coldwell, Hashemzadeh-Bonehi et al. 2004). Further experiments are required to support this hypothesis.

As a conclusion, translocating eIF4E into the nucleus results in inhibition of cellular translation whereas cytoplasmic eIF4E favours translation and so cellular survival. In the latter case, an increase in the availability of eIF4E for the eIF4F complex will favour cap-dependent translation. If eIF4E overexpression becomes chronic, translational control could be usurped and favour conditions favourable to transformation.

4.4 eIF4E function in cancer cells

Free eIF4E levels are due to the release of 4E-BPs from eIF4E, diminished expression of 4E-BPs or overexpression of eIF4E. Overexpression of eIF-4E has been reported in a wide variety of human cancers and cell lines, including non-Hodgkin's lymphomas, neuroblastomas, head and neck cancers, and cancers of the colon, breast, bladder, prostate and gastrointestinal tract (reviewed in (Graff and Zimmer 2003)).

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The overexpression of eIF4E increases the activity of the eIF4F complex. As a result, the expression of key genes involved in cell cycle progression and growth factor autonomy (ODC, c-myc, and cyclin D1), angiogenesis (VEGF, FGF-2), survival (BCL-2), and invasion (MMP-9, CD44v6) is enhanced. Therefore the development of reagents that selectively block eIF4E function, and as result availability, represents a promising anti-cancer treatment. For instance, the treatment of tumour cells with rapamycin prevents the liberation of eIF4E from 4E-BP and thereby blocks the formation of the eIF4F complex. Treatment of mouse tumour models with rapamycin suppressed the formation of experimental metastases (Guba, von Breitenbuch et al. 2002).

Another compound we were involved in studying its mechanism of action is called Silibinin (Lin, Sukarieh et al. 2009). Silibinin exerts both preventive and anticarcinogenic effects in different skin tumor models and inhibits the proliferation of human breast, lung, colon, pancreas, and prostate cancer cells *in vitro* and *in vivo* (Gazak, Walterova et al. 2007). We have investigated the ability of silibinin to inhibit cancer cell growth in MCF-7 human breast cancer cells by targeting the Akt/mTOR/eIF4E signaling pathway and showed that silibinin likely targets this pathway upstream of TSC2 but downstream of PTEN. Silibinin induces G_1 cell arrest and inhibits translation of cyclin D1 mRNA in MCF-7 cells. Since this compound has an inhibitory effect on mTOR and on the phosphorylation state of 4E-BP1, it was not a surprise to see eIF4E being translocated to the nucleus of the MCF-7 cells.

Another mechanism of targeting the function of eIF4E is to directly target the expression of the protein. For instance, Graff and colleagues developed eIF4E-specific antisense oligonucleotides (ASOs) (Graff, Konicek et al. 2007). Four ASO sequences targeting eIF4E are published and ASO4 is the best characterized. ASO4 binds specifically the 3'UTR of the *eIF4E* mRNA and target eIF4E mRNA for destruction. As a result, the expression of eIF4E-regulated proteins (e.g., VEGF, cyclin D1, survivin, c-myc, Bcl-2) is reduced and apoptosis is induced. The effects of ASO4 were seen in many systems such as cultured tumour cells, xenograft tumour tissues from mice, and human breast and prostate xenograft tumors. Proteins implicated in angiogenesis such as VEGF were reduced in ASO4 treated xenograph tumours. On the other hand, ASO4 has minor effects on general translation (Graff, Konicek et al. 2007).

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4.5 Conclusion and future directions

We identified new avenues for the control of eIF4E localization based on its interaction with its binding partners. Compartmentalization of eIF4E would define the novelty of cellular proliferation regulation. The diversity of eIF4E binding partners reflects the importance of eIF4E's roles in the cell. The interaction of 4E-T with eIF4E in cells has two consequences: import of eIF4E to the nucleus (Dostie, Ferraiuolo et al. 2000) and the targeting of eIF4E to sites of mRNA decay (Ferraiuolo, Basak et al. 2005). The dual role of 4E-T also might serve to sequester the limiting factor in translation initiation, eIF4E, as a means of maintaining translational homeostasis. It will be important to examine how 4E-T is regulated. Since 4E-T is a phosphoprotein (Pyronnet, Dostie et al. 2001), it would be interesting to know what pathways regulate its phosphorylation and activity.

In addition to the phosphorylation control of 4E-T, eIF4E has a phosphorylation site that could influence its function. Several lines of evidence suggest that inactivation of eIF4E by dephosphorylation is responsible for the shut-off of host protein synthesis in adenovirus (Huang and Schneider 1991) and possibly in part in influenza virus (Feigenblum and Schneider 1993). It would be of interest to determine whether eIF4E inactivation plays any role in the inhibition of mRNA transport in virus-infected cells. Consequently, upon poliovirus infection, eIF4E phosphorylation might induce its nuclear translocation or play a role in the host cell protein synthesis shut-off.

Even though eIF4E has emerged as a limiting factor for translation initiation in the cytoplasm, it has gained interest in the nucleus for its role in mRNA export (Rousseau, Kaspar et al. 1996; Topisirovic, Culjkovic et al. 2003; Culjkovic, Topisirovic et al. 2005). The export of mRNAs is subject to selectivity, that is favouring mRNA of proteins involved in cell cycle progression, anti-apoptosis, and angiogenesis. In addition, the overexpression of eIF4E leads to transformation and the development of malignancies. Therefore, it is an excellent target for anti-cancer therapies. Increasing the interaction between eIF4E and its inhibitor 4E-BPs upon serum starvation, rapamycin (Rong, Livingstone et al. 2008) or silibinin (Lin, Sukarieh et al. 2009) induced the translocation

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and the retention of eIF4E in the nucleus. Doing so would reduce the availability of eIF4E to be incorporated into the eIF4F complex. In parallel, localization of eIF4E into SGs and into the nucleus upon heat stress in a 4E-BP depend manner would put the cell in a stationary phase in order to prevent continuous translation under stress (Sukarieh, Sonenberg et al. 2009). Finally, translocation of eIF4E to the nucleus of poliovirus infected cells could contribute to the inhibition of host-cell protein synthesis (Chapter 3). Therefore, identifying reagents that induce the relocalization of eIF4E into the nucleus or into SGs could help in the development of anti-proliferative drugs.

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