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Eukaryotic initiation factor 4B (eIF4B): regulation by signaling pathways and its role in translation

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Այս աշխատանքը նվիրվում է հորս հիշատակին

Abstract

Due to the high energetic expenditure for the cell, the protein biosynthesis in eukaryotes is an extensively controlled process predominantly regulated at the ribosomal biogenesis and translation initiation steps. The ribosomal biogenesis defines the global translational aptitude of the cell. It is a mainly nucleolar process which is regulated at multiple steps (e.g. transcription, rRNA processing and modification, ribosomal protein translation etc). However, the most extensively regulated and the rate limiting step of translation is the initiation. Multiple eukaryotic translation initiation factors (eIFs) function to facilitate this priming step of translation. The initial recognition of the mRNA molecule happens through the 5' cap structure found in all mRNAs of nuclear origin. This event is mediated through the recruitment of heterotrimeric complex eIF4F consisting of cap-binding protein eIF4E, scaffolding protein eIF4G and the RNA helicase eIF4A unwinding secondary structures found in 5'UTR of mRNA and thus thought to facilitate the scanning process. The helicase activity of eIF4F complex or of eIF4A alone is further potentiated by eIF4B *in vitro*. The latter protein is at the focus of present thesis.

Signal transduction regulates multiple cellular processes including mitogenesis, differentiation, apoptosis, chemotaxis etc. Signaling pathways also regulate ribosomal biogenesis to coordinate mitogenic cues, nutrient and energy availability with the translational capacity of the cells. Mounting evidence links PI3K-Akt-mTOR and Ras-MAPK cascades to the translational control. In this thesis, I show that PI3K/mTOR and MAP kinase cascades converge to phosphorylate eIF4B on Ser422. This phosphorylation results in an increased interaction with eIF3, an essential factor bridging between eIF4F and the small ribosomal subunit. Physiological significance of eIF4B phosphorylation on Ser422 has been demonstrated by the stimulatory effect of eIF4B Ser422Asp phosphomimetic mutant on cap-dependent translation. Taken together, this represents a new paradigm of translational control mechanism regulated by signaling crosstalk. The function of eIF4B *in vitro* is well characterized but its *in vivo* effects are disputed in literature. To address this I established HeLa cell line stably expressing shRNA targeting eIF4B. eIF4B silencing inhibits proliferation rates and anchorage-independent growth. Expression of luciferase reporter gene containing 5' terminal oligopyrimidine tract (TOP) is selectively repressed in eIF4B-silenced cells and can be rescued by exogenous eIF4B

regardless of Ser422 phosphorylation status. Moreover, the *de novo* synthesis rates of endogenous ribosomal proteins in serum starved cultures recapitulate the luciferase reporter assay data. Utilizing polysomal analysis, I was able to show more significant inhibition of translation initiation in serum starved eIF4B-silenced cells. Our attempt to discover novel eIF4B-interacting proteins by Mass Spectrometry approach led to the identification of nucleolar RNA helicase DDX21. Confocal microscopy has shown partial co-localization of tagged eIF4B and DDX21 in nucleolar periphery. Pulse chase experiments metabolically labeling rRNA show an attenuated 28S rRNA production and concomitant accumulation of 36S intermediates in eIF4B-silenced cells. Since ribosomal biogenesis is highly coordinated process and requires strict stoichiometry maintenance of ribosomal components the observed inhibition of rRNA processing could be consequential to the decreased ribosomal protein expression. However, given the fact that eIF4B is associated with the nucleolar pre-ribosomal particle complexes its direct effect on rRNA processing cannot be ruled out. Regulation of ribosomal biogenesis by translation initiation factor may represent an important control mechanism allowing cells to co-ordinate these two processes.

Résumé

La synthèse protéique étant couteuse en dépenses d'énergie, c'est un processus extrêmement contrôlé, essentiellement au niveau de la biogenèse des ribosomes et les étapes d'initiation de la traduction. La biogenèse des ribosomes reflète la capacité traductionnelle de la cellule. C'est un processus majoritairement nucléolaire, régulé à des multiples niveaux (p.e. transcription, maturation et modifications post-transcriptionnelles des ARN, traduction des protéines ribosomales, etc). Cependant, la phase la plus régulée et l'étape limitante de la traduction est l'initiation. De multiples facteurs de traduction eucaryotes facilitent cette étape d'amorçage de la traduction. La reconnaissance initiale de la molécule d'ARN messager se fait au niveau de la structure appelée « coiffe », présente à l'extrémité 5' de tous les ARN messagers nucléaires. Ce processus est assuré par le complexe hétérotrimerique eIF4F, constitué de la protéine de liaison de la coiffe eIF4E, la protéine d'échafaudage eIF4G et l'ARN hélicase eIF4A, qui permet de dérouler les structures secondaires de la partie 5' de ARN messagers, ce qui est supposé faciliter la processus de scan. De plus, l'activité hélicase du complexe eIF4F est stimulée par eIF4B *in vitro*. L'étude de cette dernière protéine fait l'objet de ce travail de thèse.

Les voies de signalisation régulent différents processus cellulaires, tels que la mitogenèse, la différenciation ou l'apoptose. Ces voies de signalisation régulent aussi la biogenèse des ribosomes, permettant ainsi la coordination entre les signaux mitogéniques, la disponibilité des nutriments et de l'énergie avec la capacité traductionnelle de la cellule. De plus en plus d'évidences montrent des liens entre les cascades PI3K-Akt-mTOR et Ras-MAPK et le contrôle de la traduction. Dans ce travail de thèse je montre que les voies PI3K-Akt-mTOR et Ras-MAPK convergent pour phosphoryler le résidu Ser422 de eIF4B. Cette phosphorylation facilite l'interaction avec eIF3, un facteur essentiel faisant le pont avec la petite sous-unité ribosomale. L'importance physiologique de cette phosphorylation a été démontrée par l'effet stimulateur de la mutation Ser422Asp qui mime un état phosphorylé sur la traduction coiffe-dépendante. Ceci représente un niveau mécanisme de contrôle traductionnel par les voies de signalisation. Les fonctions de eIF4B sont bien caractérisées *in vitro*, mais ses fonctions *in vivo* font partie de controverses dans la littérature. Pour aborder les fonctions physiologiques de

eIF4B j'ai établi une lignée de cellules HeLa exprimant de façon stable un petit ARN (shRNA) dirigé contre eIF4B. La perte de eIF4B inhibe la prolifération et la croissance en milieu sémi-liquide. L'expression d'un rapporteur luciférase contenant une séquence 5' polypyrimidine (TOP) est sélectivement réprimée dans les cellules où eIF4B est perdu et cette expression peut-être restaurée par l'expression exogène de eIF4B, indépendamment de son état de phosphorylation sur Ser422. De plus, dans des conditions de culture en absence du sérum, le taux de synthèse des protéines ribosomales endogènes *de novo* récapitule les effets observés sur le rapporteur luciférase. Grâce à une analyse des polysomes j'ai été capable de démontrer que l'inhibition de la traduction en conditions de privation en sérum est plus forte dans les cellules où l'expression de eIF4B est réprimée. Notre tentative d'identification de nouvelles protéines interagissant avec eIF4B par la Spectrométrie de Masse a permis la découverte de DDX21, une nouvelle hélicase nucléolaire. La microscopie confocale a démontré une colocalisation partielle entre la protéine eIF4B contenant une étiquette (« tag ») et DDX21 dans la périphérie nucléolaire. Des expériences de « pulse-and-chase » permettant le marquage métabolique de ARN ribosomaux ont montré une diminution de la synthèse des ARN ribosomaux 28S et une accumulation concomitante des intermédiaires 36S dans les cellules appauvries en eIF4B. Etant donné que la biogenèse des ribosomes est un processus hautement coordonné, qui demande une stoichiométrie précise des différents composants ribosomaux, l'inhibition observée de la maturation des ARN ribosomaux peut être la conséquence d'une diminution de l'expression des protéines ribosomales. Cependant, sachant que eIF4B est associé avec les complexes des particules pre-ribosomales nucléolaires, son effet direct sut la maturation des ARN ribosomaux ne peut être exclu. La régulation de la biogenèse des ribosomes par des facteurs d'initiation de la traduction représente un mécanisme de contrôle important permettant à la cellule de coordonner ces deux processus.

Preface

This thesis is a compilation of one published article for which I am the first author and unpublished data.

Chapter 2

David Shahbazian, Philippe P Roux, Virginie Mieulet, Michael S Cohen, Brian Raught, Jack Taunton, John WB Hershey, John Blenis, Mario Pende and Nahum Sonenberg. (2006). The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO Journal 25, 2781–2791.

Chapter 3

David Shahbazian, Bernard F Gibbs and Nahum Sonenberg. Mammalian eIF4B silencing reveals its role in ribosomal biogenesis and is inhibitory for cellular proliferation and anchorage independent growth

I would like to acknowledge the work of my co-authors for each of the chapters, and thank them for their collaboration. In addition, colleagues who helped me with materials and cells are acknowledged at the end of each chapter.

Chapter 2

Philippe P Roux from John Blenis' laboratory performed the experiments shown in Figure 4.A and Figure 5.A and B, Virginie Mieulet from Mario Pende's laboratory performed the experiments shown in Figure 2.A, Michael S Cohen from Jack Taunton's laboratory designed and synthetized the RSK inhibitor fmk used in Figure 4.D and E, Brian Raught and John WB Hershey provided expert advice. I have performed all other experiments and wrote the entire manuscript under supervision of Nahum Sonenberg.

Chapter 3

Bernard F Gibbs performed the Mass Spec analysis of eIF4B interacting proteins. I have performed all other experiments.

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Finally, I thank my family members who encouraged and supported me over the years of graduate studies.

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Chapter 3 – Mammalian eIF4B silencing reveals its role in ribosomal biogenesis and is inhibitory for cellular proliferation and anchorage independent growth.

Chapter 1 – General Introduction

1.1 Translational control

Gene expression is controlled at two major checkpoint levels: transcription and translation. Translational control becomes the predominant form of regulation in systems with silent transcription (such as oocytes and early embryogenesis) and in enucleated cells (e.g. reticulocytes) (Mathews, 2007). Inactive mRNAs in somatic cells have been recently shown to accumulate in discrete cytoplasmic foci called P bodies. mRNAs associated with these granules can be either degraded or returned to translation (Buchan and Parker, 2007). Packaging of mRNAs into granules followed by transport to neurites and ensuring local synaptic translation has been also described in neurons (Kiebler and Bassell, 2006). The latter findings provide an example of translational regulation in somatic cells. The cytoplasmic accumulation of a specific mRNA to effective levels may be a time consuming process. It can involve pre-transcriptional events (promoter demethylation, chromatin remodeling through histone modifications and recruitment of trans-acting factors), pre-mRNA processing events (capping, polyadenylation, splicing), quality control (e.g. NMD) and eventually nucleocytoplasmic export (Behm-Ansmant et al., 2007; Calvo and Manley, 2003; D'Alessio and Szyf, 2006; Shatkin and Manley, 2000). Nonetheless, bypassing these processes and hence having pre-synthesized mRNA pools in the cytoplasm grants cells the ability to respond promptly to extracellular signals. The importance of translational control is also underscored by the fact that many human diseases are etiologically linked to aberrant translation (Holland et al., 2004; Pandolfi, 2004; Petroulakis et al., 2006; Scheper et al., 2007).

1.2 Eukaryotic translation: an outline

Eukaryotic translation can be subdivided into four major steps: initiation, elongation, termination and recycling. During the initiation step (see Appendix 1), the complex containing the 40S ribosomal subunit is recruited to mRNA with the assistance of translation initiation factors. This complex then scans the 5' untranslated region of the message until it detects the start codon in a favorable consensus (Mathews, 2007). At this point, the 60S large ribosomal subunit binds to the complex and initiation factors are released, giving way to the elongation step. Codon specific aminoacyl transfer RNAs (tRNAs) are recruited to the ribosome during elongation and sequential cycles of peptide bond formation are catalyzed. This process ends when the ribosome "reads" the entire

coding region of the mRNA and encounters the stop codon. At this point termination occurs as eukaryotic release factors (eRFs) result in the discharge of the nascent polypeptide from the ribosome. Additional factors accelerate dissociation of ribosomal subunits from each other and from the mRNA, making all of these components available for subsequent rounds of translation (Pisarev et al., 2007).

1.3 Composition of mRNA

Eukaryotic mRNA contains a 5' cap structure, a 5' untranslated region, the protein coding region or open reading frame (ORF), a 3' untranslated region and a poly-A tail. These elements of the messenger transcript regulate its stability and accessibility to the translational machinery. As opposed to the bacterial polycistronic messages, eukaryotic mRNAs are typically monocistronic (or code for a single protein). However, eukaryotic mRNAs might have short upstream open reading frames of regulatory significance.

1.3.1 The Cap structure

With the notable exception of mitochondria and chloroplast derived mRNAs, the 5' cap structure is the hallmark of all mRNAs of nuclear origin. It may be represented as m^7GpppN (where 'N' is any nucleotide and 'm' is a methyl group). The 'cap' is added post-transcriptionally in the nucleus by formation of an unusual 5' to 5' bond between the positively charged 7-methylguanosine and the foremost 5' nucleoside of the message. This structure interacts with eIF4F complex via its cap-binding subunit eIF4E (Sonenberg et al., 1978). The 'cap' dependent mode of translation is unique to eukaryotes since bacteria utilize a different ribosome-recruitment mechanism. The bacterial transcripts have initiatory sequences (stretches of approximately 6 nucleotides) complementary to the specific region in the ribosomal RNA (rRNA) referred to as Shine-Dalgarno sequences. Translation then takes place from the first adjacent start codon (usually 4-7 nucleotides downstream) (Alberts B, 1989). In eukaryotes, it has been demonstrated that introduction of such rRNA-complementary sequences strongly inhibits translation of the message carrying such elements upstream of the start codon (Verrier and Jean-Jean, 2000). Viruses have evolved different ways to bypass the cap-dependent translation mechanism. Many of them cleave translation initiation factors rendering them inactive and resulting in a host protein synthesis shut-off (Clemens, 2005). Hence, these viruses attain the advantage of preferential translation of viral RNA by "hijacking" the

translational machinery of the host cell (Bushell and Sarnow, 2002). This is achieved through recruitment of ribosomes to special secondary structures encoded by the viral RNA known as internal ribosome entry sites (IRESes). The dependence of these viruses on canonical translation initiation factors is decreased (if not completely absent). This strategy is employed by such viruses as poliovirus (Pelletier and Sonenberg, 1989), HCV (Lancaster et al., 2006), and Cricket paralysis virus (Pisarev et al., 2005).

1.3.2 5' Untranslated regions (5'UTRs)

Untranslated regions found between the cap-structure and the protein coding region (or ORF, for open reading frame) are referred to as 5'UTRs. These sequences vary in length and in secondary structure complexity. The current consensus is that long structured 5'UTRs are inhibitory to translation, possibly due to interference with the scanning process. Specific regulatory sequences (or cis-acting elements) have been reported for some of these transcripts. Translation of transcripts containing such regulatory elements is tightly controlled and is induced under specific conditions such as hypoxia, apoptosis, mitogenesis etc. They might also require trans-acting factors. In fact, transcripts of many mitogenic proteins and growth factors have long structured 5'UTRs (e.g. myc, VEGF, FGF etc). For instance, c-myc can be translated via conventional cap-dependent as well as IRES-dependent mechanisms (Stoneley et al., 2000). In the case of VEGF, there has been an even higher level of regulatory complexity reported: an uORF found within the VEGF IRES sequence has been shown to control the expression of particular VEGF isoforms (Bastide et al., 2008). Another example of messages regulated through the 5'UTR is 5' terminal oligopyrimidine tract (5'TOP) containing transcripts (Hamilton et al., 2006). All 5'TOP mRNAs known to date code for proteins involved in translation (e.g. ribosomal proteins, elongation factors, PABP). Short upstream ORFs are found in up to 25% of mammalian mRNAs (Crowe et al., 2006). The vast majority of experimentally tested eukaryotic uORFs are translational repressors. These cis-acting elements have been demonstrated to decrease translational efficiency through a variety of modes, including ribosome-blocking by the encoded peptide, ribosome stalling at the uORF termination codon, induction of the nonsense-mediated decay (NMD) pathway, and failure of the ribosome to re-initiate at the main translation start site after disengaging from the uORF (Gaba et al., 2001).

1.3.3 The coding region of mRNA or the open reading frame

As mentioned above, the coding region of mRNA is the only sequence (except some uORFs) that serves as a template for protein synthesis by the ribosome. Upon recognition of the start codon, the ribosome reads this sequence sliding in a 5' to 3' direction, catalyzing a series of peptide bonds in the process of elongation until it meets the stop codon.

1.3.4 3' Untranslated regions (3'UTRs)

The 3'UTRs of mRNAs are the sequences flanked by the stop codon of the ORF and the poly-A tail (with the exception of histone transcripts which are not polyadenylated). It may also contain secondary structures and other regulatory sequences affecting the efficiency of translation as well as the stability of the transcript (Kuersten and Goodwin, 2003). microRNA mediated gene silencing is a recently described mechanism of post-transcriptional control of expression. All microRNAs described to date inactivate translation by annealing to target sequences found in the 3'UTR of the mRNA (Filipowicz et al., 2008).

1.3.5 Poly-A tail

All capped mRNAs are subject to polyadenylation in the nucleus (with exception of histone transcripts) (Hall, 2002). The presence of a poly-A tail both stabilizes the mRNA and stimulates its translation through the activity of the poly-A binding protein PABP. Translational synergy between the 5' cap structure and the poly-A tail has been attributed to the eIF4G:PABP interaction-induced circularization of the transcript (Kahvejian et al., 2001; Kahvejian et al., 2005). The polyadenylation state of the transcript can regulate its translational activity and poly-A tail elongation may also take place in the cytoplasm. This phenomenon is observed in oocytes where relatively short poly-A tails of translationally silent mRNAs undergo rapid extension in a progesterone-dependent manner (Sarkissian et al., 2004).

1.4 Ribosomes: anatomy, mode of action and biogenesis

Ribosomes are the protein producing factories of the cell. The mammalian ribosome is a large ribonucleoprotein particle consisting of four rRNAs (28S, 5.8S, 18S and 5S) and 79 ribosomal proteins (Mayer and Grummt, 2006). Ribosomes of all living organisms consist of two subunits, however prokaryotic and eukaryotic ribosomes differ in size (70S

and 80S for an assembled ribosome rerespectively). In eukaryotic cells, ribosomal subunits are referred to as the small 40S ribosomal subunit and the large 60S ribosomal subunit (based on their sedimentation coefficients). Upon association, they assemble into a particle of 80S. The ribosome has three binding sites for tRNAs in the vicinity of the mRNA binding groove namely, A (aminoacyl), P (peptityl) and E (exit). There are three known modes of translation initiation by the ribosome: scanning, shunting and IRES-dependent initiation. The classical scanning mode of initiation involves ribosome recruitment and linear scanning of the 5'UTR of the mRNA. The shunting model was described in several viral and cellular systems and is suggested for the transcripts with unusually long 5'UTRs containing numerous short upstream ORFs (Futterer et al., 1993; Sherrill and Lloyd, 2008). In the shunting model, the ribosome scans the 5'UTR nonlinearly, or "jumps" over the uORFs to a region adjacent to the true start codon. This is believed to be achieved by looping out of the uORFs containing the 5'UTR segment. The last mode of ribosome recruitment and initiation is cap-independent and has diminished dependence on eIFs (Pelletier and Sonenberg, 1989). It involves internal binding of the ribosome to the secondary structures adjacent to the start codon of the transcript. These ribosome "landing pads" are referred to as IRESes (for internal ribosome entry sites) (Holcik and Sonenberg, 2005; Pelletier and Sonenberg, 1989). Elongation factor 1-bound aminoacyl-tRNA enters the A-site where it anneals through its anticodon to the mRNA. Upon codon recognition, peptide bond formation is catalyzed and the ribosome slides downstream, translocating the nascent peptide-bound tRNA to the P-site and positioning the downstream mRNA codon in the A-site. This translocation event requires the activity of elongation factor 2. Upon the transfer of the growing polypeptide chain to the next acceptor tRNA from the A-site, the now empty (deacylated) tRNA from the P-site will be translocated to the E-site, and will be released from the translating ribosome. The fidelity of protein synthesis is 10^4, meaning that only every ten thousandth amino acid incorporated by the ribosome in a protein is incorrect. The rather high fidelity of translation is controlled at two major levels. The first being the tRNA loading step by the cognate aminoacyl-tRNA synthetase that ensures the perfect match between the tRNA anticodon and the amino acid loaded. These enzymes have dual action: they can either catalyze bond formation between an amino acid and the cognate

tRNA or hydrolyze this bond if an aberrantly loaded aminoacyl-tRNA is detected. The second quality control checkpoint is the step of elongation factor 1:aminoacyl-tRNA complex binding to the A-site of the ribosome. The dissociation of elongation factor and subsequent peptide bond formation is conditional on codon-anticodon recognition (Alberts B, 1989).

Eukaryotic ribosomal biogenesis consumes a significant amount of cellular energy. In fact, 60% of yeast transcriptional activity accounts for rRNA synthesis. Similar figures are reported for the transcription of ribosomal protein mRNAs as a segment of all RNA Pol II initiation events (Warner, 1999). Energy expenditure is also required for rRNA processing and modifications, RNA helicase activities, ribosomal protein synthesis, pre-ribosomal particle folding and transport. The initial steps of ribosomal biogenesis take place in the nucleolus. Here, clusters of rRNA precursors are transcribed, modified, associated with ribosomal proteins and partially processed. Pre-ribosomal particles then transit through the nucleoplasm to the cytoplasm where additional processing steps take place (Rouquette et al., 2005). Multiple proteins participate in these processes: approximately 350 human nucleolus-associated proteins have been identified (Scherl et al., 2002). However, more recent study suggested that the human nucleolar proteome contains over 700 proteins (Leung et al., 2006). The ribosomal content of the cell is tightly regulated and adjusted to the nutritional conditions and hence to the translational needs of the cell. Signaling pathways stimulate ribosome biogenesis to coordinate the translational capacity of the cell with nutrient availability and mitogenic cues (Avruch et al., 2005; Holland et al., 2004). The protein kinase TOR (for target of rapamycin) in yeast and its mammalian homolog mTOR (mammalian TOR), is a key player integrating extracellular signals (mitogens and growth factors), with the nutritional and energetic status of the cell (availability of amino acids, ATP and glucose) to regulate gene expression and multiple aspects of cell growth, proliferation and metabolism. With regard to ribosomal biogenesis, mTOR inhibition by rapamycin results in rapid downregulation of rRNA precursor production and processing (reviewed in (Mayer and Grummt, 2006)). Moreover, both transcription and translation of ribosomal proteins is regulated by mTOR. Maintenance of stoichiometry of the ribosomal components in eukaryotic cells is of paramount importance and is controlled at several levels. One such control mechanism is

the synchronous mTOR-dependent activation of all three RNA polymerases and ribosomal protein synthesis: RNA Pol I transcribes the rRNA precursors cleaved into 28S, 18S and 5.8S rRNAs, RNA Pol II transcribes mRNAs (including those of ribosomal proteins), and RNA Pol III transcribes the 5S rRNA and tRNAs(Mayer and Grummt, 2006). Any deficit in a single ribosomal component is sensed by the cell and can arrest the ribosomal biogenesis process or result in ribosomal subunit depletion. For instance, conditional knockout of ribosomal protein S6 in murine liver has been shown to suppress 40S subunit production (Volarevic et al., 2000). Knockdown of another ribosomal protein, L11, has been reported to cause 60S ribosomal subunit depletion (Bhat et al., 2004). PI3K and MAPK cascades have also been reported to cooperate with mTOR in the regulation of rRNA synthesis downstream of insulin-like growth factor 1 (James and Zomerdijk, 2004).

1.5 eIF4 family

As mentioned above, the 5' cap structure is the hallmark of all eukaryotic mRNAs of nuclear origin. It is this structure that directly interacts with the eIF4F heterotrimeric complex, which facilitates 40S ribosomal subunit recruitment to the mRNA and melts secondary structures found in 5'UTR of the transcript, accelerating the scanning process. eIF4F consists of the cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A. The relatively low intrinsic helicase activity of eIF4A alone, or as a part of eIF4F is significantly stimulated by eIF4B and to lesser extent by eIF4H *in vitro*. Individual members of the eIF4 family, their regulation and interactions are described in the following sections.

1.5.1 eIF4E

The discovery of eIF4E (Sonenberg et al., 1978) has helped to elucidate the complex regulatory mechanisms involved in eukaryotic translation initiation. The interaction of eIF4E with the cap structure is strengthened by the interaction with its binding partner eIF4G (Gross et al., 2003). The family of eIF4E inhibitors or 4E-binding proteins consists of three members (4E-BP1, 2 and 3) (Gingras et al., 2001).The 4E-BPs compete with eIF4G for binding to the same site on eIF4E and hence their binding is mutually exclusive. The best studied member of this family is 4E-BP1. The interaction of 4E-BP1 with eIF4E is regulated by phosphorylation. The hypophosphorylated form of the protein

binds avidly to the eIF4E, whereas 4E-BP1 is expulsed form the eIF4E upon mTOR-mediated hierarchical phosphorylation on four sites. eIF4E itself can undergo phosphorylation on Ser209 (Pyronnet, 2000). This phosphorylation is carried out by Mnk1/2 proteins activated downstream of the MAPK cascade and has been reported to affect the affinity of eIF4E for the cap and is proposed to alter its activity. Interestingly, Mnk proteins and eIF4E have to bind simultaneously to eIF4G, which serves as a scaffold for this phosphorylation event. Hence, phosphorylation of eIF4E on Ser209 is dependent on simultaneous activation of mTOR (maintaining the eIF4E-eIF4G complex intact) and MAPK cascade (activating eIF4E Ser209 kinases Mnk1/2). The precise physiological significance of this phosphorylation is under current investigation in several laboratories, but preliminary data suggest that it alters susceptibility to viral infection (Barbara Herdy, personal communication) and carcinogenesis (Topisirovic et al., 2004).

1.5.2 eIF4G

The largest eIF4F subunit, eIF4G, possesses no reported catalytic activity. Instead, with its domains directly interacting with eIF4E, RNA, eIF4A, eIF3, Mnk1/2 and PABP, it can be confidently regarded as the scaffolding hub of the translation initiation complex (Mathews, 2007). Its interactions with eIF4E and eIF3 are dependent on mTOR activity, since rapamycin treatment results in dissociation of eIF4G from both proteins (Gingras et al., 2001; Harris et al., 2006). Interestingly, both of these interactions are necessary to tether the eIF3-bound small 40S ribosomal subunit to the cap structure of the mRNA. The interaction between eIF4E and eIF4G is believed to allosterically stabilize eIF4E's interaction with the 5'cap structure (Gross et al., 2003). The ATP-dependent RNA helicase eIF4A, the only catalytic subunit in the eIF4F complex, is brought to the initiation complex through its interaction with eIF4G. Its activity is believed to be necessary for scanning of 5'UTR (described in more detail in the following sections). As mentioned above, simultaneous interaction between eIF4E, eIF4G and Mnk1/2 proteins is required for eIF4E phosphorylation on Ser209. By virtue of eIF4G-PABP binding, the mRNA is believed to undergo circularization and this configuration is thought to contribute to the synergistic translation stimulatory effect of the cap and polyA tail (Kahvejian et al., 2005).

1.5.3 eIF4A

As many other members of the DEAD-box protein family, eIF4A is an ATP-dependent RNA helicase. Members of the DEAD-box protein family are thought to remodel RNA:RNA and RNA:protein interactions and are implicated in ribosome biogenesis, NMD (nonsense-mediated RNA decay) and translation. Since the central helicase core of these proteins lacks sequence specificity, most DEAD-box proteins have flanking domains interacting with accessory proteins which target helicases to specific RNAs and/or alter their activities (Silverman et al., 2003). The helicase activity of eIF4A is targeted to 5'UTRs of mRNAs by the eIF4F complex. There are three isoforms of eIF4A in mammals referred to as eIF4AI, eIF4AII and eIF4AIII. The first two are believed to be functionally redundant since they show a high degree of sequence similarity, catalytic activity and are cytoplasmic. The nuclear eIF4AIII protein is the least similar to the other isoforms by sequence. This isoform has been shown to participate in the priming round of translation in the nucleus and is involved in NMD (Ferraiuolo et al., 2004). The activity of all eIF4A isoforms is stimulated by their cognate co-factor eIF4B *in vitro* (Li et al., 1999). Another eIF4A co-factor homologous to eIF4B, eIF4H, has been shown to stimulate eIF4AI activity *in vitro* (Richter-Cook et al., 1998). The complexity of the capped mRNA 5'UTR has been shown to be directly proportional to its dependence on eIF4AI activity for efficient translation *in vitro* (Svitkin et al., 2001). However, Pateamine A-induced hyperactivation of eIF4A results in RNA-mediated sequestration of eIF4A, causing inhibition of translation initiation *in vivo* (Bordeleau et al., 2006).

1.5.4 eIF4B

The translation initiation factor 4B was purified and identified in the late 70's as a translation-stimulating activity *in vitro*. Later studies have shown that it functionally interacts with eIF4F and eIF4A potentiating their RNA helicase activity *in vitro* (Rozen et al., 1990). eIF4B has been reported to interact with the eIF3 subunit p170 and the 18S rRNA, which led to a model proposing that eIF4B forms auxiliary bridges between the message and the 40S ribosomal subunit (Methot et al., 1996a; Methot et al., 1996b). Toe printing studies using mammalian eIF4B have underscored its importance for 48S initiation complex assembly even on messages with relatively unstructured 5'UTRs (Dmitriev et al., 2003). Interestingly, the recombinant factor purified from bacteria poorly

substituted for the native protein, suggesting that eIF4B activity depends on posttranslational modifications characteristic to eukaryotic cells (e.g. phosphorylation). Indeed, eIF4B has been reported to undergo phosphorylation in rabbit reticulocytes (Benne et al., 1978) and its phosphorylation changed in a serum and mitogen-dependent manner (Duncan and Hershey, 1985; Morley and Traugh, 1989). Recent work by Raught et al identified eIF4B Ser422 as a rapamycin-sensitive S6K target site (Raught et al., 2004). We and others demonstrated the significance of Ser422 phosphorylation for eIF3 binding and stimulation of cap-dependent translation (Holz et al., 2005; Shahbazian et al., 2006). However, little is known regarding other eIF4B phosphosites and the physiological significance of these modifications is yet to be discovered. Ectopic overexpression of eIF4B in *Drosophila* cultured cells and in developing eye imaginal discs stimulated proliferation (Hernandez et al., 2004). In the same study, knockdown of eIF4B resulted in a minor inhibition of general translation and affected the survival of insect cells grown in reduced serum-containing media. The reports regarding the effects of eIF4B overexpression in mammalian systems are controversial. Some studies utilizing cells transiently overexpressing eIF4B have shown translational stimulation by this factor (Holz et al., 2005) whereas others demonstrated translational inhibition (Naranda et al., 1994; Raught et al., 2004). This discrepancy, however, could be attributed to the difference in the overexpression levels reached in these studies. In yeast, the eIF4B gene (named Tif3 and STM1 in two independent studies) was found to be non-essential, since its disruption resulted in a cold- and temperature-sensitive slow growth phenotype (Altmann et al., 1993; Coppolecchia et al., 1993). The polysomal profiles of STM1 mutants were interpreted as having a defect in translation initiation. In extracts lacking Tif3, the translation of β-galactosidase reporters with increasing 5'UTR complexity was preferentially suppressed under suboptimal temperatures. General translation under restrictive conditions (20^0 C) could be restored with the addition of wt extract or mammalian eIF4B in a dose dependent manner (Altmann et al., 1993). Interestingly, however, the yeast Tif3 protein could not stimulate the helicase activity of eIF4A *in vitro* but was efficient for strand exchange and annealing activities (Altmann et al., 1995; Niederberger et al., 1998). Moreover, C-terminally truncated Tif3 could complement the Tif3 null strain for translational activity, suggesting that the translational defect was most

likely eIF4A independent (eIF4A stimulating activity resides in the C-terminal portion of mammalian eIF4B). ATP-independent strand exchange and annealing activities are the hallmarks of RNA-chaperone proteins (Rajkowitsch et al., 2007). Hence, eIF4B might be acting in this experimental setting by modifying RNA architecture and improving the accessibility of the messages for translational machinery. Another study in yeast has identified a temperature-sensitive mutation, *dob1-1 (dependent on eIF4B 1)*, in a screen for dependence on overexpression of the yeast translation initiation factor eIF4B (Tif3). Dob1p is an essential ATP-dependent RNA helicase participating in rRNA processing as a component of 3'-> 5' nuclear exosome complex (de la Cruz et al., 1998). This suppression was *TIF3* specific, since multicopy plasmids encoding other translation initiation factors (eIF4A, eIF4E and eIF4G1) failed to confer suppression. Interestingly, despite the fact that eIF4B is predominantly cytoplasmic, it has been shown to increase the helicase activity of a nuclear isoform of eIFA (eIF4AIII) *in vitro* (Li et al., 1999). Also, eIF4B has been identified among nuclear phosphoproteins, and in association with nuclear pre-ribosomal complexes, pointing at the existence of nuclear eIF4B (Beausoleil et al., 2004; Sekiguchi et al., 2006).

1.6 Signal transduction and translational regulation

There is a growing body of evidence that links signaling cascades to translational regulation (reviewed in (Sonenberg and Hinnebusch, 2007)). The two major pathways that signal to the translation machinery are the PI3K/Akt/mTOR and the Ras-MAPK signaling cascades. One of the best studied examples for translational regulation via signaling is the inactivation of the translational repressor 4E-BP1 through hierarchical phosphorylation by mTOR (Gingras et al., 2001). Interestingly, the signaling by MAP kinases Erk and p38 has been shown to repress 4E-BP1 expression (Rolli-Derkinderen et al., 2003). These signaling modules have also been reported to regulate global translation through inactivation of eukaryotic elongation factor 2 (eEF2) kinase, resulting in derepression of eEF2 (Everett et al., 2001; Wang et al., 2001). Co-operation of these two signaling pathways at the level of translational regulation has been reported in human cancers and mouse model systems. For instance, targeting ribosomal biogenesis and translation initiation in human non-small cell lung cancer was shown to be more effective when cells were co-treated with inhibitors of MAPK and mTOR (Legrier et al., 2007). In

a glioblastoma model system, preferential recruitment of a large number of mRNAs encoding oncogenic proteins to polysomes has been attributed to aberrant signaling by upstream regulators of MAPK and mTOR modules (Ras and Akt, respectively) (Rajasekhar et al., 2003). In a later study, activated MAPK and Akt/mTOR/S6K proteins were found to be markers of poor prognosis in glioblastoma patients (Pelloski et al., 2006). The MAPK and PI3K/mTOR cascades' signals converging on the same downstream effectors are not completely independent from each other. In fact, the complex interactions between these modules form crosstalk networks. For instance, Ras binds PI3K directly (Rodriguez-Viciana et al., 1996) and the biological relevance of this interaction has been confirmed in *Drosophila* and human neutrophils (Orme et al., 2006; Suire et al., 2006). The hyperactivation of mTOR signaling has been reported in neurofibromin (Ras-GAP) deficient tumor cells of human and murine origins and their aberrant proliferation was shown to be sensitive to rapamycin treatment (Dasgupta et al., 2005). A negative upstream effector of mTOR, TSC2, can undergo inhibitory phosphorylation by Akt (Inoki et al., 2002), ERK (Ma et al., 2005) and RSK (Roux et al., 2004). Prolonged activation of Erk 1/2 was shown to be partially dependent on PI3K signaling (Grammer and Blenis, 1997).

Translational control in long-term synaptic plasticity and memory has also been shown to depend on MAPK and mTOR signaling (Gelinas et al., 2007; Kelleher et al., 2004). Brain-derived neurotrophic factor-stimulated neuronal translation was reported to be sensitive to pharmacological inhibitors of PI3K, mTOR and MEK (Takei et al., 2001). To conclude, the paradigms describing translational control by PI3K-mTOR and MAPK cascades are abundant. In some cases phosphorylation of translation factors by these signaling cascades have been demonstrated, but the data clarifying the biological relevance of these modifications are lacking (e.g. eIF4G (Raught et al., 2000a), several subunits of eIF3 (Damoc et al., 2007) etc.).

1.7 Translation and pathogenesis

The etiology of many diseases stems from aberrant translation. In a recent review by Sceper et al, the authors summarized our knowledge regarding the inherited diseases caused by mutations affecting mRNA translation (Scheper et al., 2007). Among those are mutations in the cis-acting elements of particular mRNAs resulting in either

overproduction of the protein (e.g. ferritin, trombopoietin, myc) or its insufficiency (e.g. connexin 32, dyskerin and p16). Another heterogeneous category of "translational diseases" is caused by mutations in ribosomal components, tRNAs and their charging enzymes, and translation factors and their upstream regulators. Mutations in genes that encode components of the translational machinery can give rise to a wide spectrum of diseases. For instance, mutations in eIF2B (in any of its 5 subunits) and PERK genes affect the guanine nucleotide exchange factor (GEF) activity of eIF2B and yet result in different disorders. Overexpression of certain eukaryotic translation initiation factors results in cellular transformation (e.g. eIF4E, eIF4G, eIF2α Ser51Ala non-phosphorylatable mutant, several eIF3 subunits etc). Also, initiation factors are overexpressed in many types of cancer. For instance, eIF4E overexpression is observed in a large number of malignansies: e.g. colon, breast, bladder, lung, prostate, cervix, gastrointestinal tract, head and neck, Hodgkin's lymphoma and neuroblastoma (reviewed in (Mamane et al., 2006). eIF4G is most frequently overexpressed in squamous cell carcinomas of the lung. The eukaryotic translation initiation factors 4A1, 2B and 4B as well as the poly(A)-binding protein PABPC1 were also found to be overexpressed in lung cancer (Comtesse et al., 2007). Upregulation in the level of eIF4A mRNA has been reported in human melanoma and hepatocellular carcinoma cells (Eberle et al., 1997; Shuda et al., 2000). Overexpression of eIF4 family members in human malignancies is bound to lead to increased eIF4F formation and consequently, enhanced translation initiation and cell growth. Thus, inhibition of translation initiation through targeting the mTOR-signalling pathway is emerging as a promising therapeutic option (Petroulakis et al., 2006).

1.8 Rationale

The mammalian eIF4B protein was purified in the 1970's as a protein that stimulates translation in a reconstituted rabbit reticulocyte translation system *in vitro*. Further biochemical studies revealed its ability to stimulate the helicase activity of the eIF4F complex and identified eIF4B as a co-factor for eIF4A (Rozen et al., 1990). Toe printing analysis revealed that 48S complex formation on mRNAs possessing even moderately structured 5'UTRs is greatly dependent on eIF4B (Dmitriev et al., 2003). Importantly, in

the same report bacterially expressed recombinant eIF4B poorly substituted for the native counterpart. The latter observation points at the crucial posttranslational modifications lacking in bacteria (e.g. phosphorylation). The mammalian eIF4B is a phosphoprotein (Beausoleil et al., 2004; Benne et al., 1978) and in a recent study one of its rapamycin- and serum-sensitive phosphorylation sites, Ser422, was identified (Raught et al., 2004). S6K has been shown to be responsible for this phosphorylation. However, rapamycin resistant Ser422 phosphorylation was consistently observed in cells under different stimulation conditions. Hence, we set out to identify the signaling pathways leading to activation of the alternative Ser422 kinase using pharmacological inhibitors, knock out cells and molecular biology approaches.

Relatively little is known regarding mammalian eIF4B function *in vivo*. Few reports utilizing an eIF4B overexpression system in mammalian cells disagree on its role: there are articles in favor of the inhibitory effect of eIF4B overexpression on translation (Naranda et al., 1994; Raught et al., 2004) and one article showing the opposite (Holz et al., 2005). To address this controversy, I established a cell line stably expressing eIF4B targeting shRNA and studied the effect of eIF4B silencing on HeLa cell proliferation and translation. Additionally, in a search for novel eIF4B-interacting proteins we identified a nucleolar protein DDX21 using Mass Spec analysis. The latter protein, as well as many other components of pre-ribosomal particle complexes, has been independently found associated with eIF4B by another group (Sekiguchi et al., 2006). Hence, this prompted us to assess the effect of eIF4B on ribosomal biogenesis. In summary, we studied eIF4B regulation by the MAPK and PI3K/mTOR signaling pathways. To understand the function of eIF4B *in vivo*, the effect of eIF4B silencing on proliferation, translation initiation and ribosomal biogenesis was also examined.

CHAPTER 2 – The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity

2.1 Abstract

The eukaryotic translation initiation factor 4B (eIF4B) plays a critical role in recruiting the 40S ribosomal subunit to the mRNA. In response to insulin, eIF4B is phosphorylated on Ser422 by S6K in a rapamycin-sensitive manner. Here we demonstrate that the p90 ribosomal protein S6 kinase (RSK) phosphorylates eIF4B on the same residue. The relative contribution of the RSK and S6K modules to the phosphorylation of eIF4B is growth factor-dependent, and the two phosphorylation events exhibit very different kinetics. The S6K and RSK proteins are members of the AGC protein kinase family, and require PDK1 phosphorylation for activation. Consistent with this requirement, phosphorylation of eIF4B Ser422 is abrogated in PDK1 null embryonic stem cells. Phosphorylation of eIF4B on Ser422 by RSK and S6K is physiologically significant, as it increases the interaction of eIF4B with the eukaryotic translation initiation factor 3.

2.2 Introduction

Translation initiation is the step at which the ribosome is recruited to the mRNA (Gingras et al., 1999; Hershey and Merrick, 2000). Multiple eukaryotic initiation factors (eIFs) are involved in this process. The heterotrimeric eIF4F consists of the cap-binding protein, eIF4E, the scaffolding protein eIF4G, and the helicase eIF4A. eIF4F, through eIF4E, recognizes the mRNA 5' cap structure. The eIF4A subunit is thought to unwind secondary structure in the mRNA 5'UTR to facilitate ribosome binding. eIF4B stimulates eIF4F activity by potentiating the eIF4A RNA helicase activity (e.g. (Rozen et al., 1990), for reviews see (Gingras et al., 1999; Hershey and Merrick, 2000)). eIF4G bridges the mRNA with the ribosome through its interaction with eIF3 (Etchison et al., 1982), which was demonstrated to interact directly with eIF4B (Methot et al., 1996b; Vornlocher et al., 1999).

Initiation is a critical step and a checkpoint of translation. Translational control is exerted by many different types of extracellular stimuli, which activate various signaling pathways and nutrient-sensing modules (Raught et al., 2000b). Signaling pathways regulate the activities of components of the translational machinery and stimulate ribosome biogenesis to coordinate the translational capacity of the cell with nutrient availability and mitogenic cues (Avruch et al., 2005; Holland et al., 2004). Two well-studied pathways that exhibit a paramount effect on translational regulation are the Ras-MAPK and PI3K/Akt/mTOR signaling modules.

Ras, through Raf, activates the dual threonine/tyrosine kinase MAPKKs, MEK1/2 , which in turn phosphorylate and activate the ERK1/2 protein kinases, resulting in phosphorylation of multiple cytoplasmic (e.g. RSK, Mnk1/2) and nuclear (e.g. transcription factors) substrates (Roux and Blenis, 2004). PI3K phosphorylates the membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) at position 3 to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 serves as a membrane docking signal for PH-domain containing proteins such as the serine/threonine kinases Akt/PKB and PDK1 (phosphatidylinositol-dependent kinase 1). PDK1 activates Akt/PKB by phosphorylating Thr 308 (in Akt1) within the T loop of the catalytic domain (Alessi et al., 1996). PDK1 also phosphorylates the homologous site in multiple AGC family kinases (Williams et al., 2000). Among these are the different isoforms of S6K and RSK.

The highly homologous S6K1 and S6K2 proteins (>80% identity) are encoded by distinct genes. Both S6K1 and S6K2 are phosphorylated and activated in a rapamycin-sensitive manner by mTOR, which phosphorylates a threonine residue in the linker domain (Burnett et al., 1998; Park et al., 2002; Volarevic and Thomas, 2001), allowing phosphorylation by PDK1 in the catalytic domain (Alessi et al., 1998; Balendran et al., 1999).

The RSK family consists of four members (RSK1 to 4) (Blenis, 1993; Roux and Blenis, 2004). Activation of the RSKs requires coordinated input from the Ras/MAPK cascade (Blenis, 1993) and PDK1 (Jensen et al., 1999). The RSKs are involved in multiple processes in the cell, including transcriptional regulation, cell cycle control, protein synthesis and feed-back inhibition of the Ras/MAPK cascade via Sos phosphorylation (reviewed in (Roux and Blenis, 2004)). Here we identify RSK as an *in vivo* and *in vitro* eIF4B Ser422 kinase.

2.3 Results

2.3.1 Rapamycin-resistant eIF4B Ser422 phosphorylation is mediated by ERK1/2 MAPK signaling

Insulin-stimulated eIF4B phosphorylation at Ser422 was previously demonstrated to be rapamycin-sensitive, and the kinase responsible for Ser422 phosphorylation was identified as S6K, (Raught et al., 2004) see also Fig.1A, compare lanes 9 and 10, upper panel). Interestingly, however, when HeLa cells are stimulated with serum, a significant fraction of Ser422 phosphorylation becomes resistant to inhibition by rapamycin (Fig.1A, compare lane 6 to 5, upper panel).

In addition to the mTOR/PI3K pathway, the MAPK signaling module appears to play an important role in translational control (Naegele and Morley, 2004; Rajasekhar et al., 2003). It was thus pertinent to examine the contribution of this pathway to eIF4B phosphorylation. To determine whether the MAPK cascade is responsible for rapamycin-resistant eIF4B Ser422 phosphorylation, cells were treated with the MEK 1/2/5 inhibitor U0126 (Duncia et al., 1998) prior to serum or insulin stimulation. To monitor for the efficiency of rapamycin and U0126 treatments, immunoblotting assays using phosphospecific antibodies raised against phosphorylated Thr389 of S6K, or active ERK1/2 (dually phosphorylated on Thr202 and Tyr204) were also carried out (Fig. 1A). A rapamycin-resistant component of eIF4B Ser422 phosphorylation is observed in serum-stimulated but not in insulin-stimulated cells (compare lanes 6 and 10). This residual phosphorylation is abrogated by U0126 treatment (compare lanes 6 and 8). U0126 by itself has a minor effect on eIF4B phosphorylation in serum-stimulated cells, Thus, rapamycin-resistant phosphorylation of eIF4B Ser422 phosphorylation is mediated by ERK1/2 MAPK signaling. Experiments using specific inhibitors of p38 (SB203580) and JNK1/2 (JNK inhibitor II) ruled out an involvement of these MAP kinases in Ser422 phosphorylation, since these inhibitors failed to reduce serum-stimulated phosphorylation of Ser422 in rapamycin pretreated cells (data not shown).

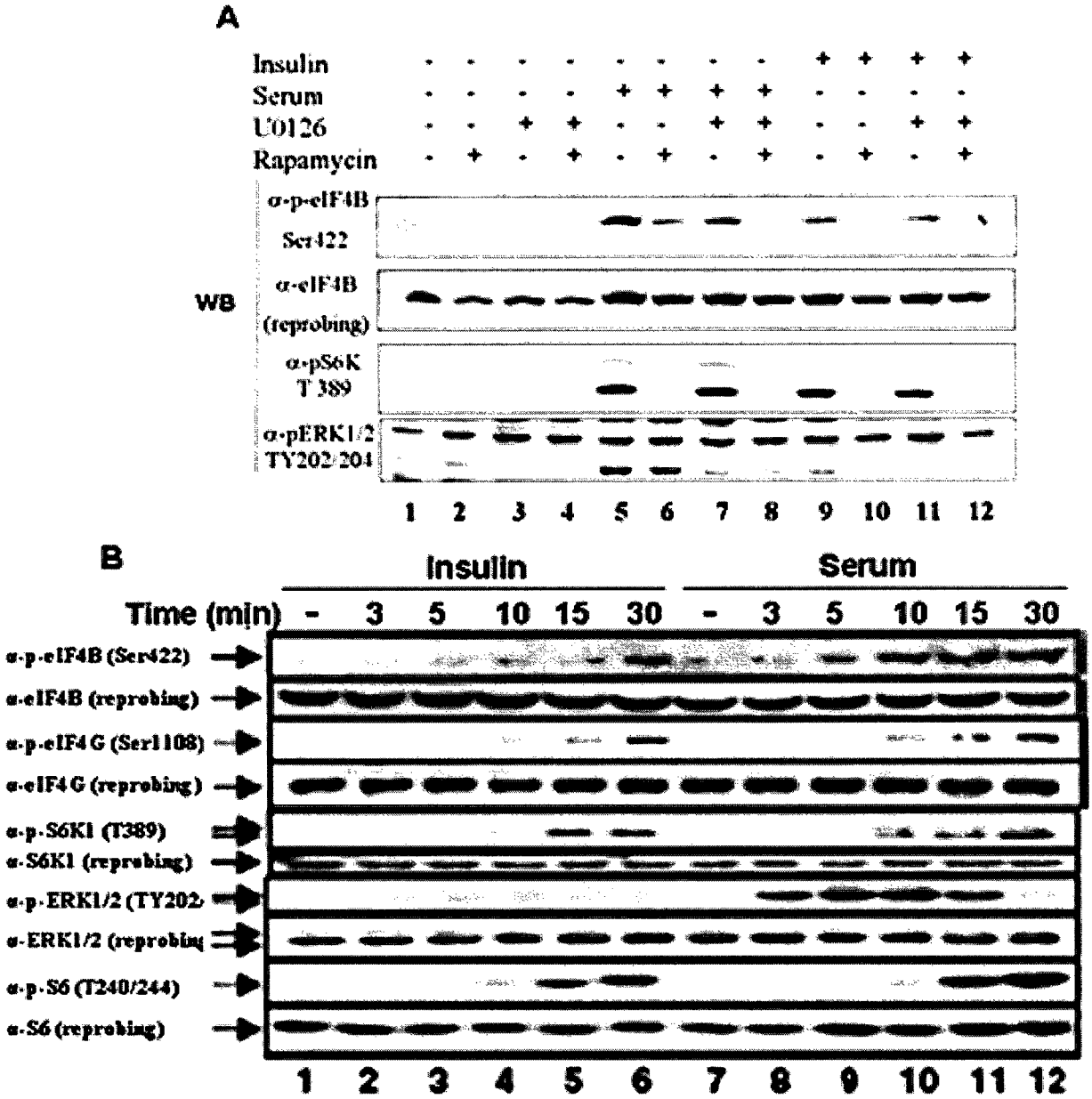
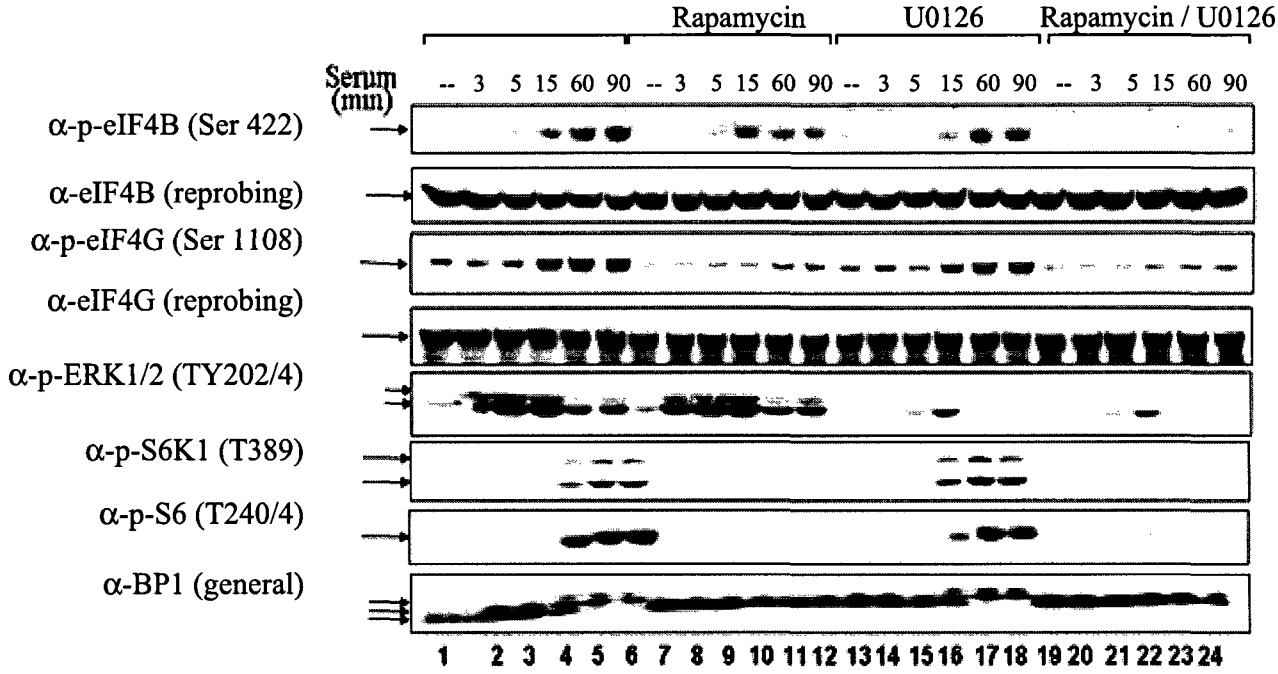


Figure 1 Rapamycin-resistant eIF4B Ser422 phosphorylation is mediated by ERK1/2 MAPK signaling. (A) HeLa cells were deprived of serum in the presence or absence of 20nM rapamycin for 16–18 h. Cells were pretreated with 10 mM of U0126 for 2 h, and then stimulated with either 20% serum or insulin (100 nM) for 30 min. Total cell extracts were subjected to SDS–PAGE followed by immunoblotting with phospho-eIF4B S422, phospho-S6K1 T389, and phospho-ERK1/2 T202/Y204 antibodies and the membrane was reprobed with anti-eIF4B antiserum. (B) HeLa cells were starved for serum as in (A) and stimulated for the indicated times with either 20% serum or insulin (100 nM). Cell extracts were resolved by SDS–PAGE and immunoblotted with phospho-eIF4G S1108, phospho-eIF4B S422, phospho-S6K1 T389, phospho-ERK1/2 T202/Y204, phospho-S6 S240/244 antibodies and the indicated total proteins.

C



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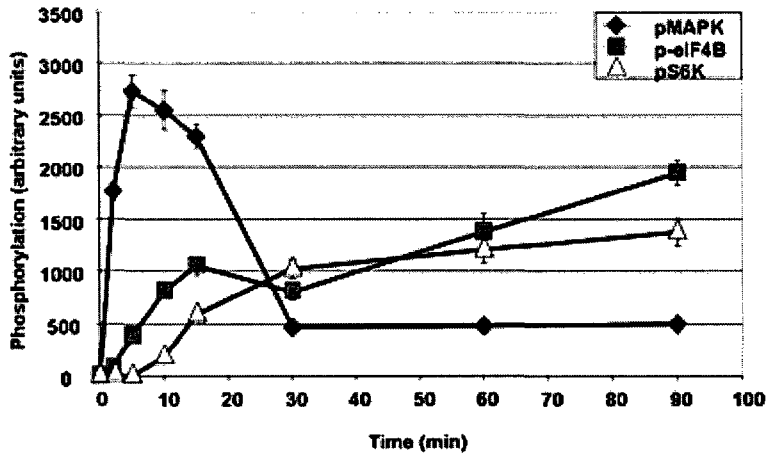


Figure 1 (continued) (C) HeLa cells were deprived of serum in the presence or absence of 20 nM rapamycin for 16–18 h. Cells were pretreated with 10 mM of U0126 for 2 h, and then stimulated with 20% serum for the indicated times. Total cell extracts were resolved by SDS–PAGE, immunoblotted with phospho-eIF4G S1108, phospho-eIF4B S422, phospho-S6K1 T389, phospho-ERK1/2 T202/Y204, and phospho-S6 S240/244 antibodies and reprobed for the indicated proteins with pan-specific antibodies. (D) Sequential activation of signaling pathways involved in eIF4B Ser422 phosphorylation. HeLa cells were deprived of serum for 16–18 h. Cells were then stimulated with 20% serum for the indicated amounts of time. Protein extracts were resolved by SDS–PAGE and probed for phospho-eIF4B S422, phospho-ERK1/2 T202/Y204, and phospho-S6K T389.

and no effect in insulin-stimulated cells, consistent with the lack of ERK activation by insulin (lanes 7 and 11, respectively). Total eIF4B protein levels were not affected by any of the treatments, as determined by reprobing the membrane with pan-eIF4B antibody.

To study the differential sensitivity of eIF4B phosphorylation to rapamycin and U0126, a time course experiment was carried out (Fig.1B). Both serum and insulin stimulated the phosphorylation of the PI3K/Akt/mTOR pathway substrates, eIF4G (Ser1108) and S6K1 (Thr389), with similar kinetics, although the insulin-induced S6K phosphorylation is somewhat delayed and less intense (compare lanes 4 and 10). A phosphorylation time-course of the S6K substrates rpS6 (Ser240/244) and eIF4B (Ser422) is similar in insulin-stimulated cells. However, in serum-induced cells eIF4B Ser422 phosphorylation appears faster than S6 phosphorylation (Fig.1B, lanes 9 to 12), and is detectable before S6K activation (compare lanes 3 and 9). Importantly, in contrast to serum, insulin is incapable of activating the MAPK ERK1/2 cascade in these cells (lanes 1-6). The inability of insulin to effect signaling through the MAPK module is the most likely explanation for the complete rapamycin-sensitivity of eIF4B phosphorylation in insulin-stimulated cells.

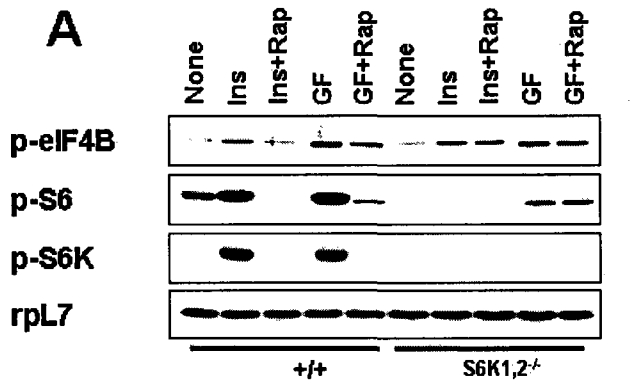
To further characterize the biphasic pattern of eIF4B phosphorylation, a time-course experiment using serum in the presence or absence of U0126 or rapamycin was performed (Fig.1C). Activation of the MAPK cascade was monitored by immunoblotting with phosphospecific antibodies directed against activated ERK1/2. Cell extracts were also examined for phosphorylation of the rapamycin-sensitive substrates eIF4G and S6K1, using phosphospecific antibodies, and 4E-BP1 using a pan-specific antibody. Serum-induced MAPK phosphorylation is very rapid, detected as early as 3 minutes after serum stimulation, and reaches a peak at 5 minutes post-induction (Fig.1C). In contrast, S6K phosphorylation and activity (as determined by rpS6 Ser240/244 phosphorylation) are undetectable at these early time points, but are sustained for much longer (compare 60 and 90 minutes). These data demonstrate that eIF4B phosphorylation in response to serum is mediated by both the MAPK and PI3K/mTOR pathways. Importantly, eIF4B phosphorylation can be temporally divided into two phases: an early phase which is sensitive to U0126, but not to rapamycin (compare the 15 minutes time points with the two inhibitors, lanes 10 and 16), and a late phase, which is inhibited by rapamycin

(compare 60 and 90 minutes, lanes 11 and 12 versus 17 and 18). Simultaneous treatment of cells with both inhibitors abrogates eIF4B phosphorylation at all times (lanes 20-24). A detailed time course experiment (Fig. 1D) clearly demonstrates that serum-induced eIF4B phosphorylation is detectable prior to the activation of S6K (as judged by S6K1 T389 and rpS6 S240/244 phosphorylation).

2.3.2 eIF4B Ser422 phosphorylation persists in cells lacking S6K1 and S6K2

To further corroborate the existence of an eIF4B kinase that is distinct from S6K in cells other than HeLa, primary hepatocyte cultures from S6K1/2$^{-/-}$ double knockout (DKO) mice were used. The extent of eIF4B phosphorylation was similar in wild type and S6K-deficient hepatocytes under serum-deprived conditions, and upon insulin or serum stimulation (Fig. 2A).

However, the wild type and mutant cells differed in their sensitivity to rapamycin. Whereas rapamycin abrogated eIF4B phosphorylation following insulin stimulation, and partially after serum stimulation in wild type cells, Ser422 phosphorylation was completely resistant to rapamycin treatment in S6K DKO hepatocytes. Thus, S6K phosphorylates eIF4B in insulin-stimulated hepatocytes, but an mTOR-independent kinase efficiently compensates for the S6K deletion. Consistent with the data in HeLa cells, serum activates an mTOR-independent mechanism that leads to phosphorylation of eIF4B in wild type and S6K deficient hepatocytes. The amino-terminal kinase domain of the RSKs shares a high degree of homology with the S6K proteins, and the consensus phosphorylation sequence recognized by RSK1 (and presumably the highly homologous RSK2-4 isoforms) is almost identical to that of the S6K proteins (Leighton et al., 1995). Unlike S6K, which is activated by PDK1 (Alessi et al., 1998) and mTOR (Burnett et al., 1998), RSK activity is regulated by the ERK 1/2 MAPKs (Frodin and Gammeltoft, 1999; Frodin et al., 2000) and PDK1 (Jensen et al., 1999). Since the amino acid sequence surrounding eIF4B Ser422 conforms to both the RSK and S6K consensus sequences (Fig.2B), and the RSKs are regulated by the ERK MAPKs, the RSKs appear to be the most likely candidates to effect the MAPK-dependent rapamycin-resistant phosphorylation of Ser422.



B

S6K consensus	RXRXXS*XX
RSK consensus	K/RXRXXS*XX
eIF4B Ser 422	RERSRTGS*ESS

Figure 2 (A) eIF4B Ser422 phosphorylation persists in cells lacking S6K1 and S6K2.
Hepatocytes derived from wt and S6K1/2 DKO animals were starved for nutrients and serum and stimulated with 1 mM insulin or 10% serum in the presence or absence of 20nM rapamycin. Total cell lysates were immunoblotted with phosphoeIF4B S422, phospho-S6 S235/236, phospho-S6K1 T389, and rpL7 antibodies. (B) Substrate consensus sequences of S6K and RSK as compared to the eIF4B fragment encompassing the Ser422 phosphorylation site.

2.3.3 Ser422 is dephosphorylated in PDK1 null and PIF pocket mutant ES cells

Members of the AGC family of kinases are phosphorylated by PDK1 on the T-loop; this phosphorylation event is required for their full activation. PDK1 null cells are defective for both RSK and S6K activation (Mora et al., 2004). To determine whether Ser422 phosphorylation is affected in these cells, wild type and PDK1 KO embryonic stem (ES) cells were serum-starved for 16 hours in the presence or absence of rapamycin, and then stimulated with serum for 15 minutes. Consistent with previously published data (Williams et al., 2000) phosphorylation of S6K T389 and rpS6 S240/244 is abrogated in PDK1 null cells (Fig.3A, lanes 5-8). Unlike their wt counterparts, PDK1 null ES cells exhibit no detectable eIF4B kinase activity upon serum stimulation (lanes 5-8). Similar to the data shown for HeLa cells (Fig.1C, lanes 4 and 10), rapamycin did not abrogate serum-induced eIF4B phosphorylation in wt ES cells (compare lane 4 to 3).

Another member of the AGC family that phosphorylates a consensus sequence similar to that of the RSKs and S6Ks is Akt (Obata et al., 2000). Mutation of Leu155 to glutamate in the PDK1 substrate docking site, also known as the "PIF-pocket" (for PDK1 interacting fragment), prevents PDK1 from interacting with and phosphorylating S6K and RSK, but does not affect its ability to activate Akt (Biondi et al., 2001; Collins et al., 2003). To determine whether Akt is able to phosphorylate eIF4B, we studied Ser422 phosphorylation in PDK1 PIF-pocket mutant knock-in ES cells. Similar to PDK1 null cells, PDK1 PIF-pocket mutant cells are devoid of Ser422 kinase activity (Fig.3B, compare lanes 5-8 to 1-4). These data, although consistent with the idea that both S6K and RSK are *bona fide* eIF4B kinases, do not completely rule out the participation of other AGC kinases in eIF4B phosphorylation. However, given that eIF4B phosphorylation is sensitive to pharmacological inhibitors that fail to inhibit other AGC kinases, it is very likely that the major kinases responsible for eIF4B phosphorylation are S6K and RSK.

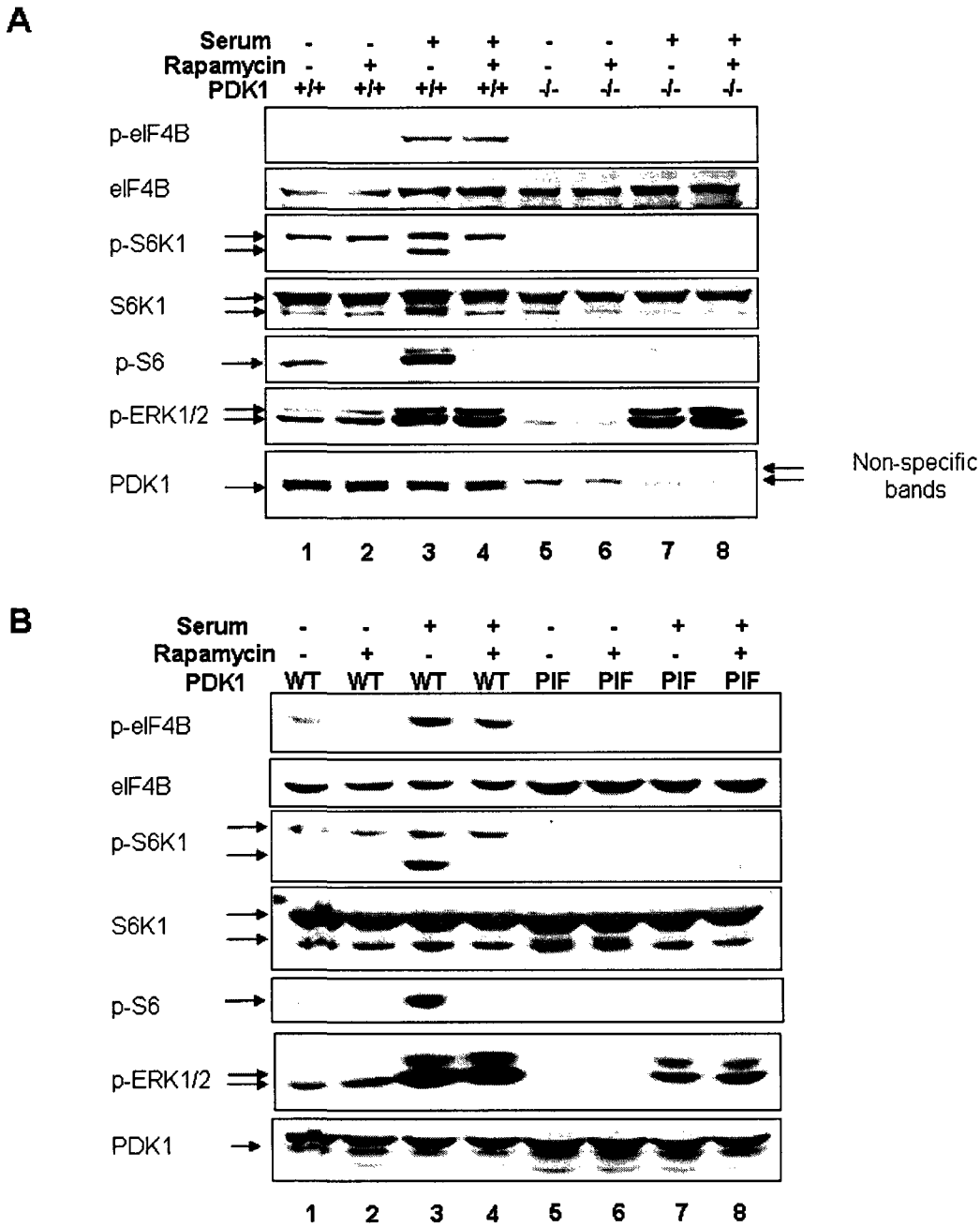


Figure 3 eIF4B Ser422 is dephosphorylated in PDK1 null and PDK1
PIF pocket mutant ES cells. Wt and PDK1-/- knockout (A) or PDK1 PIF pocket mutant (B) ES cells were starved for 16–18 h in the presence or absence of 20nM rapamycin and then stimulated with 20% serum for 15 min. Total cell extracts were resolved by SDS–PAGE and proteins were detected by immunoblotting using phospho-eIF4B S422, phospho-S6K1 T389, phospho-S6 S240/244, and phospho-ERK1/2 T202/Y204 antibodies. Membranes were reprobed with antibodies against the indicated total proteins and against PDK1 (arrows on the right indicate nonspecific bands).

2.3.4 Catalytically active RSK variants phosphorylate eIF4B in vitro and in vivo

To demonstrate that RSK can directly phosphorylate eIF4B, we examined eIF4B phosphorylation in an *in vitro* kinase assay. HeLa cells were transfected with plasmids encoding HA-tagged wild type RSK1 and S6K1, as well as a kinase-inactive RSK1 mutant. Cells were serum starved for 16-18 hours, and pretreated with U0126 (U0) or rapamycin (RAP) prior to serum or insulin stimulation (Fig.4A). Immune-complex kinase assays were then carried out with recombinant eIF4B as a substrate *in vitro*. Wild type (but not kinase-dead) RSK elicited a 3.5-fold increase in eIF4B phosphorylation (Fig.4A), indicating that RSK, but not a co-purifying kinase activity is responsible for the phosphorylation. Pretreatment of cells with U0126 abrogated RSK-mediated eIF4B phosphorylation *in vitro*. S6K immunoprecipitated from insulin-treated cells exhibited a 5-fold increase in eIF4B phosphorylation relative to basal phosphorylation levels (an unstimulated sample expressing HA-S6K1). This phosphorylation was abrogated by rapamycin pretreatment (Fig.4A).

To further demonstrate that RSK can phosphorylate eIF4B *in vivo,* HeLa cells were co-transfected with various RSK1 mutants, and Flag-tagged eIF4B. Cells were stimulated with serum for 15 (Fig.4B) or 90 minutes (Fig.4C), following pretreatment with U0126 or rapamycin, respectively. While catalytically active wild type RSK and MyrRSK (a constitutively active, membrane-targeted form) potently phosphorylated Ser422, the kinase-dead RSK variant not only failed to do so, but actually suppressed serum-induced eIF4B phosphorylation (compare Fig 4B lane 8 to lanes 2 and 5). Ser422 phosphorylation was readily detectable in unstimulated cells transfected with MyrRSK (Fig.4B and C). This basal phosphorylation was increased after 15 minutes of serum stimulation, but not when cells were treated with U0126 (Fig.4B). A fraction of the Ser422 phosphorylation induced by MyrRSK is not inhibited by U0126. Consistent with the earlier report MyrRSK retains some activity even in the presence of MEK inhibitors (Roux and Blenis, 2004). Flag-eIF4B phosphorylation in cells stimulated with serum for 90 minutes exhibited rapamycin sensitivity, unless co-expressed with MyrRSK (Fig. 4C).

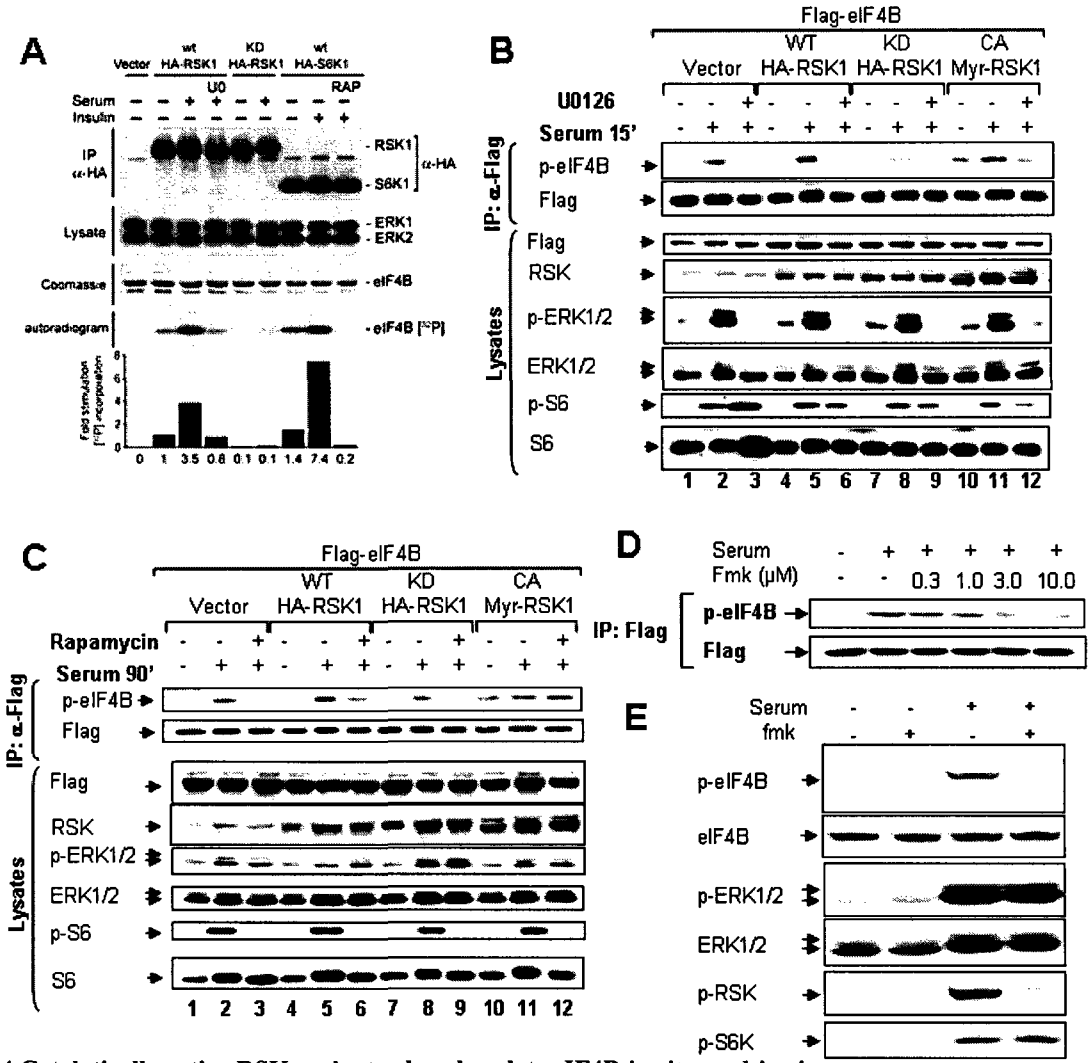


Figure 4 Catalytically active RSK variants phosphorylate eIF4B *in vitro* and *in vivo*.
(A) Wt and kinase-dead HA-RSK- and wt HA-S6K1-transfected HeLa cells were serum starved for 16–18 h, pretreated with either U0126 (10 mM; U0) or rapamycin (20 nM; RAP) as indicated, and stimulated with either serum or insulin for 15 min. An aliquot of the total cell lysate was immunoblotted for ERK1/2. Another aliquot was used to immunoprecipitate RSK variants and S6K1 using anti-HA antibody. Immunoprecipitates were split. Half was subjected to SDS–PAGE and probed for HA and the remaining half was assayed for in vitro kinase activity by using recombinant eIF4B as substrate. Samples were resolved by SDS–PAGE, stained with Coomassie brilliant blue, and exposed to an X-ray film. 32P incorporation was quantified using a phosphorimager. A representative autoradiogram is shown. (B, C) HeLa cells cotransfected with Flag-tagged eIF4B together with wt, kinase-dead, and constitutively active RSK variants were serum starved for 16–18 h in the presence or absence of 10 mM U0126 (B) or 20nM rapamycin (C) before serum stimulation for 15 min (B) or 90 min (C). Cell lysates were used to immunoprecipitate exogenous Flag-tagged eIF4B using anti-Flag (M2) antibody. Immune complexes were subjected to SDS–PAGE and probed with antibodies directed against phosphorylated eIF4B Ser422. Membranes were reprobed with anti-Flag antibody. Aliquots of total cell lysates were run on gel and probed with indicated antibodies. (D) HeLa cells were transfected with Flag-eIF4B. After 24 h, cells were deprived of serum in the presence or absence of increasing concentrations of RSK1/2 inhibitor fmk for 16–18 h. Cells were stimulated with 20% serum for 15 min. eIF4B was immunoprecipitated using anti-Flag antibody. Immune complexes were subjected to SDS–PAGE and Western blotting with phospho-eIF4B S422 antibody. The membrane was stripped and reprobed with Flag antibody. (E) HeLa cells were deprived of serum in the presence or absence of 10 mM RSK1/2 inhibitor fmk for 16–18 h. Cells were stimulated with 20% serum for 15 min. Total cell extracts were subjected to SDS–PAGE followed by immunoblotting with phospho-eIF4B S422, phospho-ERK1/2 T202/Y204, phospho-RSK S380, and phospho-S6K1 T389 antibodies and then reprobed for total eIF4B and ERK1/2.

Additional evidence for an *in vivo* contribution of RSK to eIF4B phosphorylation was obtained through the use of a recently designed and characterized fluoromethylketone (fmk), which potently and selectively inactivates RSK1 and RSK2 in mammalian cells (Cohen et al., 2005). The inhibitor targets the C-terminal kinase domain of RSK1 and RSK2, preventing autophosphorylation on S380 and S386 of human RSK1 and RSK2, respectively. This phosphorylation enables PDK1 docking which then phosphorylates and activates the RSK N-terminal kinase domain (Frodin et al., 2000; Jensen et al., 1999). To determine the optimal inhibitory concentration of fmk in HeLa cells, a dose response experiment was carried out. HeLa cells were transfected with Flag-tagged eIF4B, then deprived of serum in the presence of increasing concentrations of fmk for 16-18 hours. Cells were then stimulated with serum for 15 minutes, and eIF4B was immunoprecipitated using anti-Flag antibody and subjected to SDS-PAGE and Western blotting with the phosphospecific eIF4B Ser422 antibody. Fmk addition resulted in a dose-dependent inhibition of serum-induced eIF4B phosphorylation, reaching a plateau at 3 μM (Fig. 4D). To determine whether endogenous eIF4B phosphorylation is also sensitive to fmk treatment, HeLa cells were starved of serum in the presence or absence of 10 μM fmk for 16 hours, prior to serum stimulation for 15 minutes. Fmk strongly reduced serum-stimulated phosphorylation of eIF4B and RSK1, whereas S6K and MAPK activation remained unaffected (Fig.4E). In conclusion, RSK phosphorylates Ser422 both *in vitro* and *in vivo*. The early phase of eIF4B phosphorylation is more dependent on RSK activity than at later times.

2.3.5 RNA interference of the RSK1 and RSK2 isoforms leads to reduced eIF4B Ser422 phosphorylation and inhibits cap-dependent translation

To further substantiate the involvement of RSK in eIF4B Ser422 phosphorylation, HeLa S3 cells were co-transfected with siRNAs targeting RSK1 and RSK2, or with mock siRNA. The siRNA treatment resulted in a >90% knockdown of both RSK1 and RSK2 expression. Twenty-four hours post-transfection cells were starved of serum, pretreated with inhibitors, and then stimulated with either serum or insulin for 15 min. Both serum

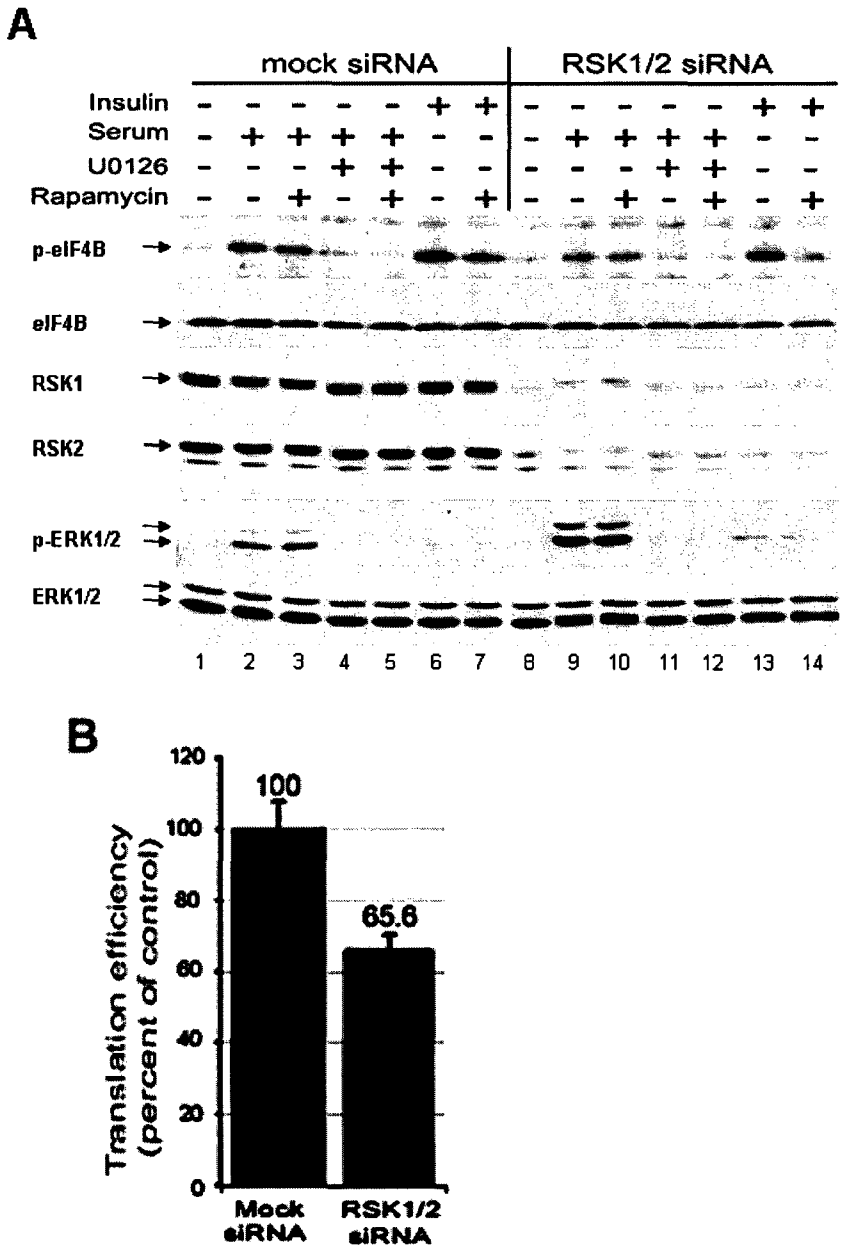


Figure 5 RNAi-mediated silencing of RSK1 and RSK2 isoforms expression leads to reduced eIF4B Ser422 phosphorylation and inhibition of cap-dependent translation.

(A) HeLa cells were subjected to RNAi using synthetic oligos nonspecific (Mock) or specific to RSK1 and RSK2 isoforms. At 24 h post-transfection, cells wereserum starved for 16–18 h in the presence or absence of rapamycin, then indicated samples were treated with U0126 and stimulated with serum or insulin as shown. Total cell extracts were immunoblotted with phospho-eIF4B S422 and phospho-ERK1/2 T202/Y204 antibodies followed by reprobing for the corresponding total proteins. RSK1 and RSK2 Western blots were also carried out to demonstrate the efficiency of the knockdown. (B) HEK293 cells were transfected with the bicistronic luciferase construct and indicated siRNAs. After 48 h, cells were harvested and assayed for Renilla (RL) and firefly (FL) luminescence. Results are presented as average of RL/FL ratio±standard error from three independent experiments carried out in triplicate

and insulin elicited phosphorylation of eIF4B on Ser422 in mock siRNA-transfected cells (Fig.5A, lanes 2 and 6). As expected, serum-induced phosphorylation of eIF4B was sensitive to U0126 (lanes 2 and 4), whereas insulin-stimulated eIF4B phosphorylation was sensitive to rapamycin (lanes 6 and 7). Serum-induced (compare lanes 2 and 3 to lanes 9 and 10), but not insulin-induced (compare lanes 6 and 13), eIF4B Ser422 phosphorylation was prevented by RNAi directed against RSK1/2.

To assess the effect of RSK1/2 RNAi on cap-dependent translation, HEK293 cells were co-transfected with RSK1 and RSK2 targeting siRNAs and bicistronic Renilla-HCV IRES-Firefly luciferase reporter (see Fig.5B). Forty-eight hours later cells were harvested and analyzed for luciferase activity. The data suggest that RSK1/2 RNAi leads to ~34% inhibition in cap-dependent translation. These results provide further evidence that RSK proteins are playing an important role in regulating cap-dependent translation in part through eIF4B Ser422 phosphorylation.

2.3.6 Phosphorylation of eIF4B on Ser422 enhances its affinity for the eIF3 complex

eIF4B co-purifies with eIF3 through several purification steps [e.g. (Brown-Luedi et al., 1982)], eIF3 can be immunoprecipitated with eIF4B (Methot et al., 1996b), and eIF4B directly interacts with eIF3 in yeast and mammalian cells (Methot et al., 1996b; Vornlocher et al., 1999). To examine whether the association of eIF3 with eIF4B is affected by the phosphorylation state of eIF4B a co-immunoprecipitation experiment was carried out. Cells co-expressing Flag-eIF4B and various mutants of RSK were starved of serum overnight, pretreated with U0126 for 2 hours, and then stimulated with serum for 15 minutes. Immunoprecipitates were subjected to SDS-PAGE and probed with phosphospecific-Ser422 eIF4B and anti-eIF3a (p170 subunit) antibodies. To monitor for the amount of Flag-tagged eIF4B loaded on the gel, the membrane was stripped and reprobed with anti-Flag antibody (Fig.6A). Serum stimulation strongly enhanced the interaction between eIF4B and eIF3 in cells expressing wt and constitutively active RSK variants (lanes 2 and 8, 3.5 and 7.5 fold, respectively), but not in cells expressing the kinase-dead RSK (lane 5). Thus, phosphorylated eIF4B is enriched in a complex containing eIF3 as compared to its hypophosphorylated counterpart.

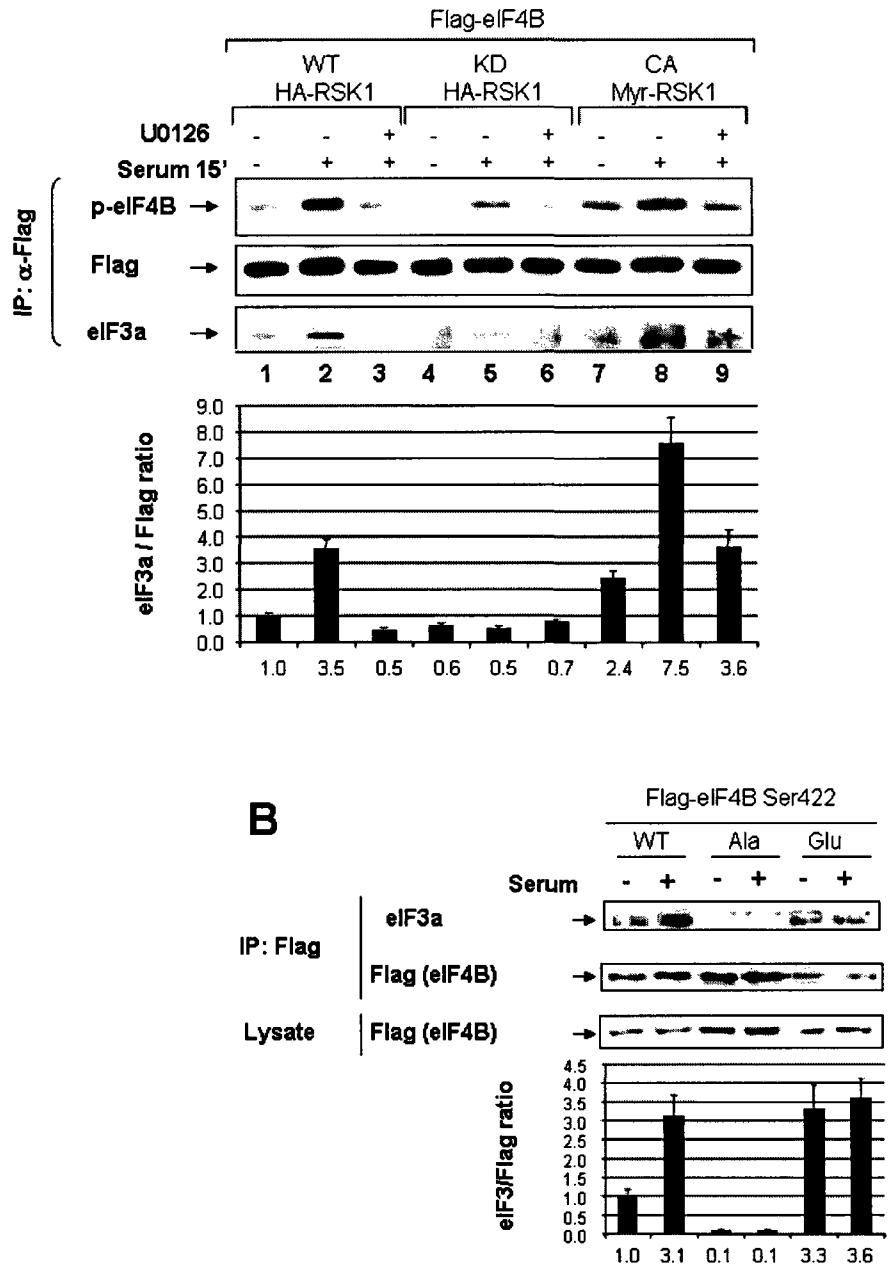


Figure 6 eIF4B Ser422 phosphorylation results in enhanced interaction between eIF4B and a complex containing eIF3.

(A) HeLa cells cotransfected with Flag-tagged eIF4B and wt, kinase-dead, and constitutively active RSK variants were starved for 16–18 h in the presence or absence of 10 mM U0126 before serum stimulation for 15 min. Immunoprecipitation of Flag-tagged eIF4B was carried out using anti-Flag (M2) antibody. Immune complexes were subjected to SDS–PAGE and probed with a phosphospecific eIF4B S422 antibody and an eIF3a (p170) antibody. Membranes were reprobed with anti-Flag antibody. (B) HeLa cells were transfected with Flag-tagged wt eIF4B and Ser422 point mutants: Ser422Ala and Ser422Glu. After 16–18 h of serum starvation, cells were stimulated with serum for 15 min, and samples were processed as in (A).

Finally, to demonstrate that eIF4B Ser422 phosphorylation is important for its interaction with eIF3, we examined the ability of eIF4B point mutants to bind eIF3. HeLa cells were transfected with plasmids encoding Flag-tagged wild type, Ser422Ala (non-phosphorylatable) and Ser422Glu (phosphomimetic) point mutants of eIF4B. Cells were serum starved for 16-18 hours prior to serum stimulation for 15 minutes (Fig.6B). Cells were lysed and immune complexes precipitated with anti-Flag antibody were subjected to SDS-PAGE and Western blot analysis using anti-Flag and anti-eIF3a antibodies. As shown above, wild type eIF4B exhibited enhanced interaction with eIF3a upon serum stimulation (~3.1 fold increase; Fig.6B). Strikingly, the non-phosphorylatable Ser422Ala mutant showed a decreased interaction with eIF3 under both serum starved and serum stimulated conditions (~10% of unstimulated wt control). A phosphomimetic mutant of eIF4B (Ser422Glu) exhibited a constitutive high level of interaction between eIF4B and eIF3 (~3.3-3.6 fold increase as compared to wt control). These data thus indicate that the interaction between eIF4B and eIF3 is regulated through the phosphorylation of eIF4B on Ser422. Similar results were recently published by Holz et al (Holz et al., 2005).

2.4 Discussion

Here we demonstrate that two major signaling pathways involved in translational control converge to phosphorylate eIF4B on Ser422 (Fig.7). This conclusion is based on the following results: a) Ser422 phosphorylation is sensitive to both a pharmacological inhibitor of MEK, U0126, and the mTOR inhibitor rapamycin, b) Ser422 phosphorylation is observed in S6K1/2 double knock out cells, c) eIF4B phosphorylation is dependent upon functional PDK1, and serum-induced Ser422 phosphorylation requires active RSK protein. d) RSK directly phosphorylates eIF4B *in vitro*. In addition, we show that eIF4B phosphorylation results in an enhanced interaction between eIF4B and eIF3. Importantly, the expression of a phosphomimetic Ser422Asp mutant of eIF4B in cells resulted in increased translation (Holz et al, 2005). Moreover, RNAi against RSK1/2 resulted in reduced eIF4B phosphorylation and inhibited cap-dependent translation. Thus, the temporal serum-induced biphasic phosphorylation of Ser422, first by the MAPK signaling module, and subsequently by the PI3K/Akt/mTOR cascade (Fig.1D) is likely to be of biological significance.

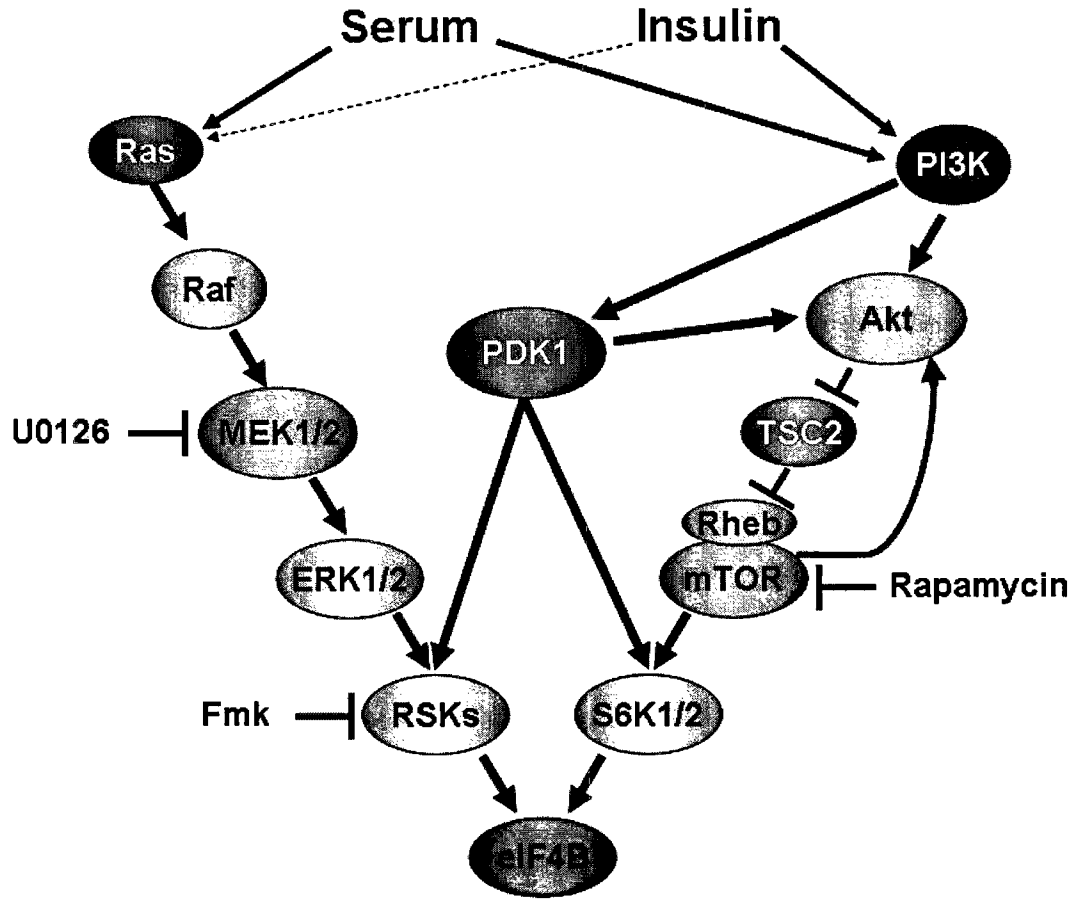


Figure 7 Signaling pathways involved in eIF4B Ser422 phosphorylation.
Growth factor-activated MAPK and PI3K cascades activate RSK and S6K proteins correspondingly and converge at the level of eIF4B phosphorylation. In systems where insulin is a marginal activator (dashed arrow) of MAPK cascade, insulin-induced eIF4B phosphorylation is absolutely sensitive to rapamycin. PDK1 protein plays a central role in activation of both RSK and S6K proteins and is indispensable for eIF4B phosphorylation.

Consistent with our data, the recovery of translation in human kidney cells after hypertonic stress-induced translational shut off requires phosphorylation of downstream substrates of both the ERK1/2 MAPK and PI3K signaling modules (Naegele and Morley, 2004). Also, activation of the MAPK and PI3K signaling pathways results in the recruitment of a large number of mRNAs (~200) to polysomes (Rajasekhar et al., 2003). It is noteworthy that cytokine-driven mitogenesis is also dependent on two temporally distinct phases of signaling; the first is the ERK1/2 MAPK cascade activity, and the second is the PI3K pathway (Jones and Kazlauskas, 2001; Mirza et al., 2004). IL-2 induced hematopoetic cell proliferation is dependent on MAPK effectors (c-myc, c-fos and c-jun) and rapamycin-sensitive bcl-2 expression (Miyazaki et al., 1995). It is, however, unlikely that the two converging signaling cascades have a redundant role in eIF4B phosphorylation and compensate for each other's function, because of the transient nature of the ERK1/2 MAPK cascade-mediated eIF4B phosphorylation as opposed to a later, more sustained phosphorylation mediated by the PI3K-mTOR-dependent pathway. Kinetics of mitogen-stimulated ERK1/2 MAPK cascade activation in cells is typically faster than PI3K-mTOR module activation, and thus allows for a more precise regulation and an immediate response (e.g. transcription, translation, etc.). Thus, it is plausible that ERK1/2 MAPK-mediated transient phosphorylation of eIF4B fills the temporal gap that exists between mitogenic stimuli and PI3K-mTOR pathway activation to more closely orchestrate mitogenic cues and rates of translation.

In addition to eIF4B, the S6K and RSK family members phosphorylate upstream components of the signaling pathways that lead to eIF4B phosphorylation. These include TSC2 and Sos by RSK, and mTOR and IRS1 by S6K. Inactivation of Ras-GAP results in robust phosphorylation of S6 through Ras mediated PI3K/mTOR pathway activation (Dasgupta et al., 2005). Also, ERK1/2 phosphorylate TSC2, leading to the dissociation of TSC2 from TSC1 and subsequent inactivation of the complex (Ma et al., 2005) and derepression of mTOR activity. This complex pattern of phosphorylation is a hallmark of all signaling pathways, as it engenders essential signal amplification and establishes checkpoints and feed-back regulation loops. The RSKs have previously been implicated in translational control: activated RSK translocates to polysomes, where it stimulates the phosphorylation of several ribosome-associated proteins (Angenstein et al., 1998). The

RSKs also phosphorylate and inactivate GSK3 to stimulate translation (Eldar-Finkelman et al., 1995; Torres et al., 1999). Both S6K and RSK phosphorylate and inhibit elongation factor 2 kinase (Wang et al., 2001).

eIF4B stimulates the helicase activity of eIF4A (Lawson et al., 1989; Rozen et al., 1990), and interacts with eIF3 (Methot et al., 1996b). This interaction is presumably required for stabilization of the bridge between the mRNA and eIF3 through eIF4G. Here, we present evidence that eIF4B phosphorylation on Ser422 stimulates the interaction between eIF4B and eIF3. Although we have not demonstrated this, it is likely to stimulate the direct interaction between eIF4B and eIF3. The eIF4B-eIF3 interaction correlates with increased translation rates in cells upon eIF4B phosphorylation. It is also possible that Ser422 phosphorylation increases the stimulatory effect of eIF4B exerted on the eIF4A-mediated helicase activity. Recently, Dmitriev *et al* showed that eIF4B is obligatory for 48S ribosome initiation complex formation on mRNAs which possess even a relatively low complexity in their 5'UTRs (Dmitriev et al., 2003). They reported that recombinant eIF4B protein poorly substituted for the native factor suggesting that a post-translational modification which is absent in bacteria (e.g. phosphorylation) is important for eIF4B function. Importantly, as aforementioned, Holz et al 2005, demonstrated recently that phosphorylated eIF4B stimulates cap-dependent translation *in vivo* (Holz et al., 2005). Although these results are in contrast to earlier reports which showed an inhibition of translation by eIF4B overexpression (Naranda et al., 1994; Raught et al., 2004), it is possible that the discrepancies are due to different expression levels of the exogenous eIF4B. Highly overexpressed eIF4B (25-50 fold) can be inhibitory to translation due to its potential interference with endogenous complexes by creating inactive pools of physiological eIF4B interacting partners (eIF4A, eIF3, PABP etc).

MAPK and PI3K signaling pathways stimulate translation by increasing the rates of translation initiation, and elongation, and by stimulating ribosome biogenesis (Holland et al., 2004). Cooperation between these two major signaling pathways results in preferential increase in ribosome recruitment of mRNAs which encode oncogenic proteins in glial cells (Rajasekhar et al., 2003). In light of the importance of eIF4B phosphorylation for its function, this report presents a new paradigm for the interaction between PI3K/Akt/mTOR and Ras/MAPK cascades in controlling translation.

2.5 Materials and methods

2.5.1 Constructs

Flag-tagged eIF4B in a pcDNA3 vector was previously described (Raught et al., 2004). Plasmids encoding the HA-tagged wt S6K, HA-tagged wt and kinase-dead avian RSK1 and constitutively active myristoylated avian RSK1 were described elsewhere (Roux et al., 2004). The bicistronic Renilla-HCV IRES-Firefly luciferase plasmid was published (Kruger et al., 2001).

2.5.2 Cell culture/transfections

Human cervical carcinoma derived HeLa R19 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Gibco). Transfections were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were grown to 80-90% confluence prior to overnight serum withdrawal. Cells were treated with 20 nM rapamycin (Sigma), or 10 μM fluoromethylketone (fmk) (Cohen et al., 2005) overnight, or 10 μM U0126 (Promega) for 2 hours prior to stimulation with 20% serum or 100 nM insulin (Sigma) as indicated in the figure legends. Murine $PDK1^{+/+}$, $PDK1^{-/-}$ and PDK PIF pocket mutant cells were a kind gift of Dr. Alessi. ES cells were grown on gelatinized plasticware in KnockOut DMEM containing 15% KnockOut serum (Gibco) supplemented with 0.1 mM non-essential amino acids, antibiotics (100 U penicillin G, 100 μg/ml streptomycin), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1000 U/ml ESGRO [Leukemia inhibitory factor, used to prevent differentiation of ES cells] (Gibco). Cells were grown to 80% confluence, serum starved for 16 hours in presence or absence of 20 nM rapamycin and stimulated with 20% serum.

2.5.3 S6K mutant mice and primary cell cultures

The generation of S6K1 and S6K2 deficient mice was previously described (Pende et al., 2004; Shima et al., 1998). Adult male mice in a mixed C57Bl/6-129Ola genetic background were used. Primary hepatocytes from 12- to 14-weeks old male mice were isolated by liver perfusion as described previously (Pende et al., 2004). After 3 hours of adhesion, cells were incubated for 2 days in serum-free M-199 medium containing 1 mg of BSA/ml. Cells were incubated overnight in an amino acids- and glucose-free media.

On the next day, the hepatocytes were pretreated for 30 minutes with 20 nM rapamycin and stimulated for 1 hour with growth factors (10% FBS or 1μM insulin).

2.5.4 Antibodies/immunoprecipitation/Western blotting

Anti-Flag (M2) and anti-HA mouse monoclonal antibodies were from Sigma. Anti-4E-BP1, anti-RSK1 and anti-RSK2 rabbit polyclonal antibodies were from Zymed. Anti-avian RSK1 antibody was previously characterized (Roux et al., 2004). Anti-eIF4G and anti-eIF4B rabbit antisera were described before (Ferraiuolo et al., 2005; Methot et al., 1996b). A monoclonal antibody against eIF3a p170 was a kind gift of Dr. Altmann (Mengod and Trachsel, 1985). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). Flag-tagged eIF4B was immunoprecipitated from 1 mg cell lysate protein extracted from transiently transfected HeLa cells. The samples were incubated at 4^0C overnight with 4 μg of anti-Flag (M2) antibody and immune complexes were collected for two additional hours by 20 μl of protein G sepharose beads. Resultant pellets were washed 3 times with 1 ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 microgram/ml each of aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF). Proteins were denatured by addition of 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 5% SDS, 10 mM EDTA, 0.5 M DTT, 0.25 % bromophenol blue, 50% glycerol) and subjected to SDS-PAGE followed by blotting onto nitrocellulose membrane. Membranes were blocked with 5% BSA solution and probed with phosphospecific eIF4B Ser422 antibodies (Raught et al., 2004). For loading control membranes were stripped in acidic buffer (0.2 M glycine, 0.5 M NaCl, pH 2.8) and reprobed using anti-Flag (M2) antibody. Experiments were repeated at least three times. Data were quantified using NIH Image software (unless stated otherwise) and standard deviations ranged between 4-21%. Representative results are shown.

2.5.5 In vitro kinase assay

HeLa cells were transfected with HA-tagged wt RSK1 and S6K1 or kinase-dead RSK1 using Fugene 6 according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Twenty-four hours following transfection, cells were serum starved for 16-18 hours, then stimulated with serum or insulin in the presence or absence of 10 μM U0126 or 20 nM rapamycin and lysed in cell lysis buffer (CLB: 10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM ß-glycerophosphate, 0.5% NP-40, 0.1%

Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate [Na_3VO_4], 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, 10 μg/ml of pepstatin). Lysates were incubated with anti-HA antibodies for 2 hours and then with protein A-Sepharose for an additional 1 hour at 4°C. Beads were washed three times in CLB and once in kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM ß-glycerophosphate). Kinase assays were performed with recombinant eIF4B (purified as in (Pause and Sonenberg, 1992)) as a substrate (2 μg per assay) and were completed in the linear range of substrate phosphorylation. Reaction products were subjected to SDS-PAGE, and ^{32}P incorporation was quantified using a Bio-Rad PhosphorImager.

2.5.6 RNAi against RSK1 and RSK2

For the small interfering RNA (siRNA) studies, 21 nucleotide complementary RNA with symmetrical two nucleotide overhangs were obtained from Qiagen (Valencia, CA). The DNA sequences against which double-stranded RNAs for RSK1 and RSK2 were created were CCCAACATCATCACTCTGAAA and AGCGCTGAGAATGGACAGCAA, respectively, and mock sequence was TATTCTCCGAACGTGTCACGT. HeLa S3 cells were transfected using Oligofectamine and 0.25-0.5 μg siRNA per 35 mm dishes according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). Transfection efficiency was determined to be greater than 95% using a fluorescently-labeled mock siRNA. Twenty-four hours following transfection, cells were serum starved for 16-18 hours, then stimulated with either serum or insulin, and then harvested in CLB. The lysates were centrifuged for 5 minutes at 4°C, adjusted for protein concentration using Bradford assay, and processed for immunoblotting.

2.5.7 Bicistronic Luciferase Assay

For luciferase reporter experiments, HEK293E cells were transfected with pRL-HCV-FL reporter plasmid and the indicated siRNAs. Forty-eight hours post-transfection, cells were harvested, and the luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) and Turner Designs TD-20/20 luminometer according to the manufacturers' instructions.

2.6 Acknowledgements

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point mutant constructs, and C. Lister for invaluable technical assistance. This work was supported by grants from the Canadian Institute of Health Research (CIHR) and the Howard Hughes Medical Institute (HHMI) to N.S; NIH grants #R37 CA46595 and #RO1 GM5140 to J.B; INSERM Avenir program (R01131KS) and Association Française contre les Myopathies (9971) grants to M.P; NIH grant GM22135 from the U.S.P.H.S. to J.H. JT is supported by NIGMS. P.R. is supported by International Human Frontier Science Program Organization (HFSPO). N.S. is a CIHR Distinguished Scientist and an HHMI International scholar.

Connecting text

Little is known regarding eIF4B function in mammalian cells *in vivo*. The attempts to overexpress eIF4B have been reported in literature and gave rise to controversial data (Holz et al., 2005; Milburn et al., 1990; Naranda et al., 1994; Raught et al., 2004). Hence, I set out to generate an alternative experimental *in vivo* system to study the effects of eIF4B and its Ser422 phosphorylation in particular. To this end, I established a HeLa cell line stably expressing a short hairpin RNA (shRNA) targeting eIF4B sequence encompassing the Ser422. The latter feature was necessary to allow expressing Ser422 mutants (carrying non-phosphorylatable Ala or phosphomimetic Glu substitutions) since their transcripts are not perfectly complementary to the shRNA sequence and hence should evade the silencing. Our assumption was that the eIF4B-dependent effects observed in eIF4B-silenced cells should be reversed by either non-phosphorylatable or phosphomimetic variants of eIF4B Ser422 mutants or both. The results of these studies are presented in Chapter 3.

CHAPTER 3 – Mammalian eIF4B silencing reveals its role in ribosomal biogenesis and is inhibitory for cellular proliferation and anchorage independent growth.

3.1 Introduction/Results

Translation initiation plays a critical role in protein expression and is the major checkpoint in translation. As discussed in the general introduction (Chapter 1), eIF4B is an important component of translational machinery stimulating recruitment of the 40S ribosomal subunit to the 5' cap structure of the mRNA. Messenger RNAs (mRNAs) containing even moderate level of complexity in 5' untranslated regions (UTRs) demonstrate increased dependence on eIF4B for 48S complex assembly *in vitro* (Dmitriev et al., 2003). Genetic manipulations have revealed the *in vivo* functions of eIF4B in yeast (Altmann et al., 1993; Coppolecchia et al., 1993) and in Drosophila (Hernandez et al., 2004). However, this factor is the least conserved among the eIF4 family members (Metz et al., 1999) and, hence, species-specific variations in function and/or activity of eIF4B are very likely. This notion is supported by several facts. First, the yeast homolog of eIF4B (Tif3) is incapable of stimulating eIF4A activity *in vitro* (Altmann et al., 1995). Second, the interaction between eIF4B and eIF3 (a factor consisting of 13 subunits) is attributed to eIF3a subunit binding in mammalian cells (Methot et al., 1996b), whereas binding through eIF3g subunit has been demonstrated in yeast (Vornlocher et al., 1999) and in plants (Park et al., 2004). Third, mammalian eIF4B is able to substitute for the yeast homolog (Tif3) in the eIF4B-deficient yeast extract (Altmann et al., 1993), whereas Drosophila eIF4B isoforms are unable to replace the function of its yeast counterpart (Hernandez et al., 2004). Previously, we and others have shown that eIF4B undergoes S6K and RSK dependent phosphorylation on Ser422, and this phosphorylation is important for eIF4B:eIF3 interaction (Holz et al., 2005; Shahbazian et al., 2006). Studies of eIF4B overexpression in a mammalian system and assessing its impact on translation have been performed in several independent reports. The conclusions from these data, however, are ambiguous since three reports have shown translational inhibition (Milburn et al., 1990; Naranda et al., 1994; Raught et al., 2004) and one report has shown stimulation by eIF4B (Holz and Blenis, 2005). Given its functional and/or physical interactions with mRNA and components of translational machinery (e.g. eIF4A, eIF4F, eIF3, PABP, 18S rRNA) (Bushell et al., 2001; Methot et al., 1996a; Methot et al., 1996b) eIF4B may also play a role of a scaffolding protein. Hence, high levels of its overexpression may be detrimental for the cells due to the

disruption of endogenous complexes and rendering them inactive by sequestration of their components.

3.1.1 Establishment of stable eIF4B-silenced HeLa cells.

In this study, we decided to assess the role of eIF4B and its Ser422 phosphorylation in mammalian cells utilizing RNAi approach. To this end, we established cells stably expressing shRNA targeting eIF4B sequence surrounding Ser422 (Fig.3.1A) and hence allowing the expression of eIF4B having Ala and Glu substitutions at this site (Fig.3.1B). As seen in panel (A) the resultant cells express significantly lower amounts of eIF4B. Furthermore, our assumption regarding the possibility of eIF4B Ser422 mutants' expression in eIF4B-silenced cells proved true (Fig.3.1B). The transcripts encoding eIF4B Ser422 mutants are likely to escape and not to "overwhelm" the RNAi since when transfected with the same amounts of wild type Flag-eIF4B expressing vector the eIF4B is efficiently and specifically knocked down in eIF4B-silenced cells (Fig.3.1C).

3.1.2 eIF4B silencing inhibits cell proliferation and anchorage independent growth.

To characterize eIF4B-silenced cells we first examined their proliferation rates. Mock and eIF4B-silenced cells were counted and equal numbers of cells were seeded in 6-well plates. Cells were harvested by trypsinization and counted in triplicates using hemocytometer for 5 consecutive days (Fig.3.2.A). The apparent doubling time of eIF4B-silenced cells was slightly increased: from 14.9 ± 0.77 hours in mock expressing cells to 17.1 ± 0.2 hours in eIF4B-silenced cells. The ability of transformed cells to overcome contact inhibition when grown in soft agar is a powerful tool, used to examine effects of oncogenes and tumor suppressors on anchorage independent growth. Silencing of growth promoting genes has been shown to revert the oncogenic potential of different types of cancer cells (Nakamura et al., 2000; Schmidt et al., 2007; Tomlinson et al., 2007). Thus, to examine the growth inhibitory effect of eIF4B silencing on anchorage independent proliferation of HeLa cells the soft agar assay has been performed. When eIF4B-silenced and mock cells were plated in soft agar and grown for 14 days mock cells formed much larger colonies as compared to eIF4B-silenced cells (Fig.3.2B). Hence, eIF4B silencing results in an inhibition of proliferation and anchorage independent growth of HeLa cells.

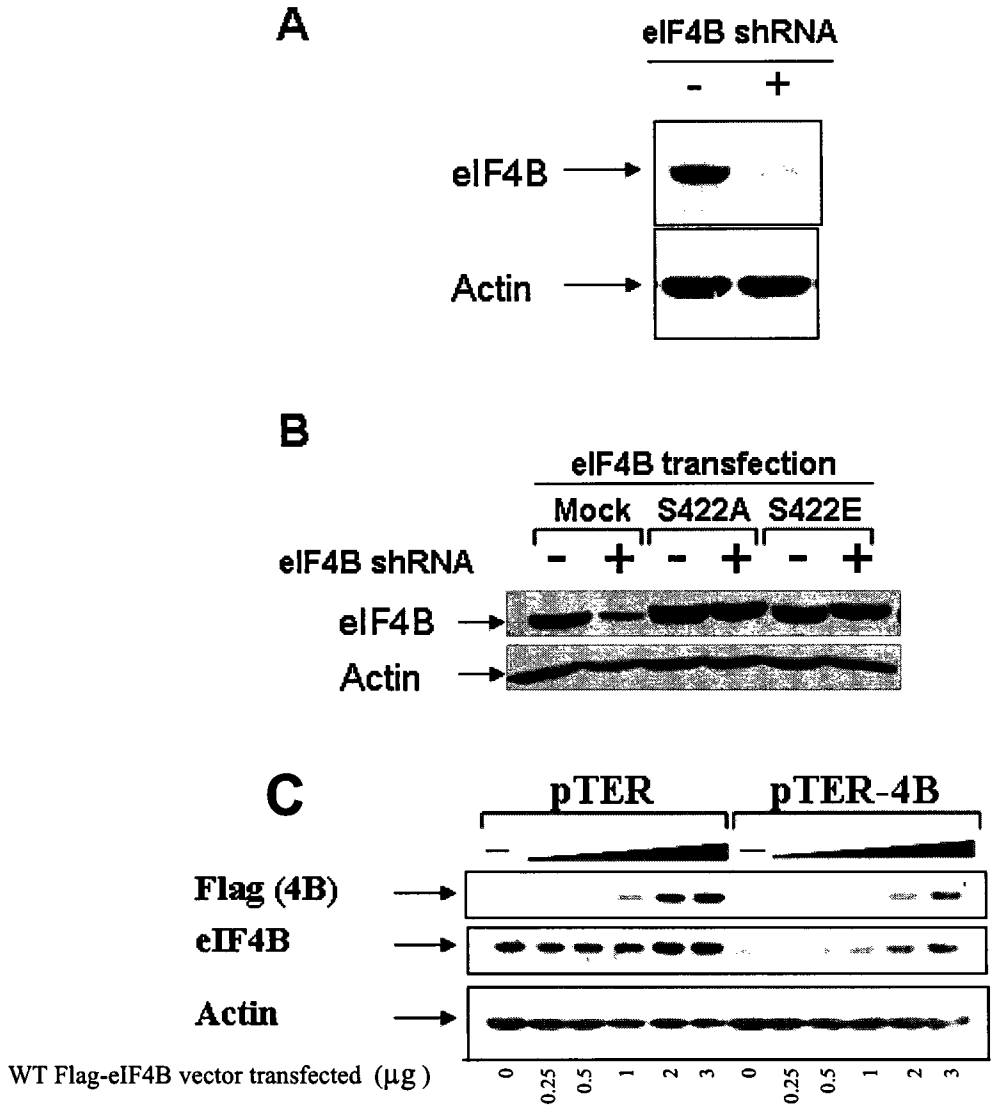
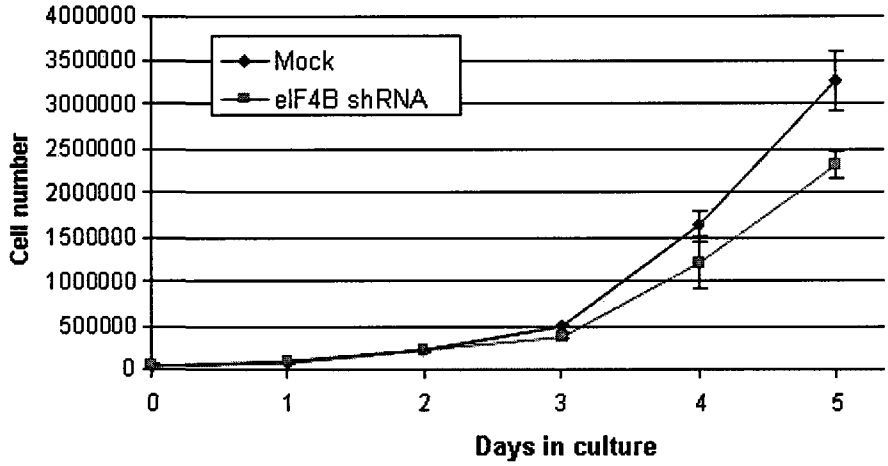


Fig.3.1 Establishment of eIF4B silenced cells. (A) To establish zeocin-resistant stable cell lines HeLa cells were transfected with pTER and pTER-4B (expressing short hairpin RNA targeting eF4B sequence surrounding Ser422 coding region). Cell extracts were resolved by SDS–PAGE and immunoblotted with eIF4B and actin antibodies. **Exogenous eIF4B Ser422 mutants escape RNAi in eIF4B silenced cells.** (B) eIF4B silenced and mock HeLa cells were transfected with 1 μg of empty pcDNA3-Flag plasmid (mock), or vectors coding for Flag-eIF4B Ser422A or Flag-eIF4B Ser422Glu. Cell extracts were resolved by SDS–PAGE and immunoblotted with eIF4B and actin antibodies. **Exogenous wild type eIF4B is targeted in eIF4B silenced cells.** (C) eIF4B silenced and mock HeLa cells were transfected with increasing amounts of wild type pcDNA3-Flag-eIF4B vector (0.25 to 3 μg/sample). Cell extracts were resolved by SDS–PAGE and immunoblotted with Flag, eIF4B and actin antibodies.

3.1.3 5'TOP-Luciferase reporter is specifically repressed in eIF4B-silenced cells.

To examine the effect of eIF4B silencing on translation we used bicistronic Renilla-HCV IRES-Firefly luciferase reporter system that allows monitoring the ratio between cap-dependent and –independent translation initiation efficiency (Fig.3.3A). The translation of the second cistron of this reporter is driven by HCV IRES which is known to be eIF4B independent (Pestova et al., 1998). The data suggest that eIF4B silencing did not result in translational inhibition of cap-dependent cistron (Fig.3.3A). Similar results were obtained with bicistronic Renilla-Polio IRES-Firefly luciferase reporter (Fig.3.3B). Keeping in mind that eIF4B was previously shown to be non-essential for mRNAs lacking base pairing in their 5'UTRs (Dmitriev et al., 2003), we attributed the lack of translational difference to the unstructured nature of the reporters tested and decided to utilize a set of monocistronic luciferase reporters with or without a stable stem-and-loop (SL) structure in 5'UTR (Yang et al., 2004). Mock and eIF4B-silenced cells were transfected with either structured (Fig.3.3.C right panel) or unstructured (Fig.3.3.C left panel) reporters, grown for 48 hours and then analyzed for RL and FL luciferase luminescence. Surprisingly, the expression level of both structured and unstructured reporters was similar in mock and eIF4B-silenced cells (Fig.3.3C). These findings suggested that both 5' structured and unstructured reporters are efficiently expressed independently of eIF4B status in this experimental system (Fig.3.3A-C). As discussed in general introduction (Chapter 1), the major signaling pathways regulating translation initiation are PI3K/mTOR and MAPK modules. Translation of transcripts having 5'UTR TOP sequences has been shown to be most sensitive to pharmacological inhibitors of PI3K/mTOR pathway (Jefferies et al., 1994; Tang et al., 2001). Mitogenic stimulation of quiescent cells induces a rapid recruitment of TOP mRNAs from translationally inactive light messenger ribonucleoprotein particles to polysomes(Hamilton et al., 2006). Since eIF4B is regulated downstream of both PI3K/mTOR and MAPK signaling cascades (see Chapter 2) we decided to assess the effect of eIF4B silencing on TOP mRNA expression (Fig.3.3.D). To this end, mock and eIF4B-silenced cells were co-transfected with firefly luciferase reporter having 5'TOP sequence of eukaryotic elongation factor 2 (eEF2) (Kim and Chen, 2000) and non-TOP

A



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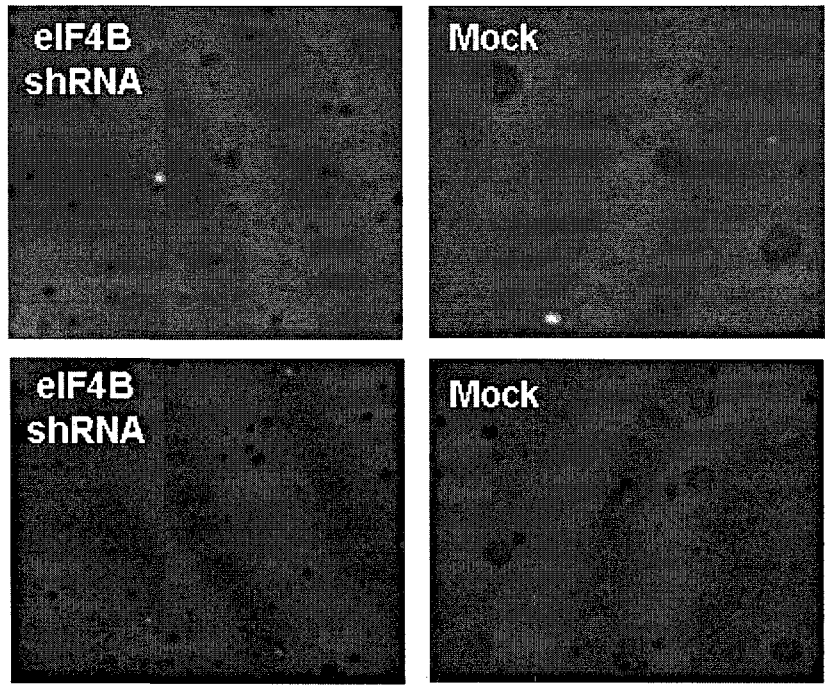

Fig3.2 eIF4B silencing results in attenuated proliferation. (A) 5 x 10^4 of mock and eIF4B silenced cells were plated per well in 6-well plates. Cells were trypsinized and counted in triplicates every day for 5 days. The results represent the data from 3 independent experiments ± standard deviation; **(B) eIF4B silencing affects anchorage independent growth.** 3 x 10^3 cells of indicated type were plated per well in soft agar and grown for 10 days. Colonies were visualized with crystal violet staining and photographed. Representative fields from two independent experiments are shown.

renilla luciferase reporter. The data shown in Fig3.3.D unambiguously indicate that TOP reporter is specifically repressed in eIF4B-silenced cells (2~2.5 fold). To determine if eIF4B Ser422 phosphorylation plays a role in TOP reporter expression, mock and eIF4B-silenced cells were co-transfected with eIF4B Ser422 mutants in addition to TOP-FL and NonTOP-RL. The data suggest that both non-phosphorylatable (Ser422Ala) and phosphomimetic (Ser422Glu) eIF4B variants, which are not targeted in eIF4B-silenced cells (see Fig3.1B), markedly restore the expression of 5'TOP luciferase reporter in cells silenced for the endogenous eIF4B (Fig.3.3E). The data from luciferase reporter assays identify eIF4B as a regulator of 5'TOP mRNA in Ser422 phosphorylation independent manner.

3.1.4 Synthesis of endogenous 5'TOP mRNA encoded proteins is inhibited in eIF4B-silenced cells.

At this point, we decided to examine the effect of eIF4B knockdown on expression of endogenous proteins encoded by TOP mRNAs. A large number of TOP mRNAs are coding for ribosomal proteins (Hamilton et al., 2006). For this reason, mock and eIF4B-silenced cells were analyzed for expression of ribosomal proteins S6, L7a and L18 by western blotting. The Western analysis demonstrates no significant difference in steady-state level of these ribosomal proteins under normal growth conditions (Fig.3.4.A). Serum and nutrient starvation suppresses the global translation and turns on catabolic programs in cells to reduce the energy expenditure. Under these conditions cells minimize anabolic processes by several mechanisms, and among others, by reducing ribosomal biogenesis. Thus, to monitor the *de novo* synthesis of ribosomal proteins we decided to subject cells to prolonged serum starvation followed by serum re-feeding. In eIF4B-silenced cells, serum starvation for 72 hours resulted in a faster decay of two TOP mRNA encoded proteins, ribosomal protein L18 and eukaryotic elongation factor 1α. Furthermore, re-accumulation of L18 upon 2 hours re-feeding with serum was faster in mock cells (Fig.3.4B). Similar kinetics could be demonstrated for the *de novo* synthesis of ribosomal proteins L7a, L11, S6 and L18 in cells recovering from 80 hours serum starvation in a time course experiment (Fig.3.4C). In both mock and eIF4B-silenced cells the level of these ribosomal proteins is upregulated within 3 hours of serum refeeding and

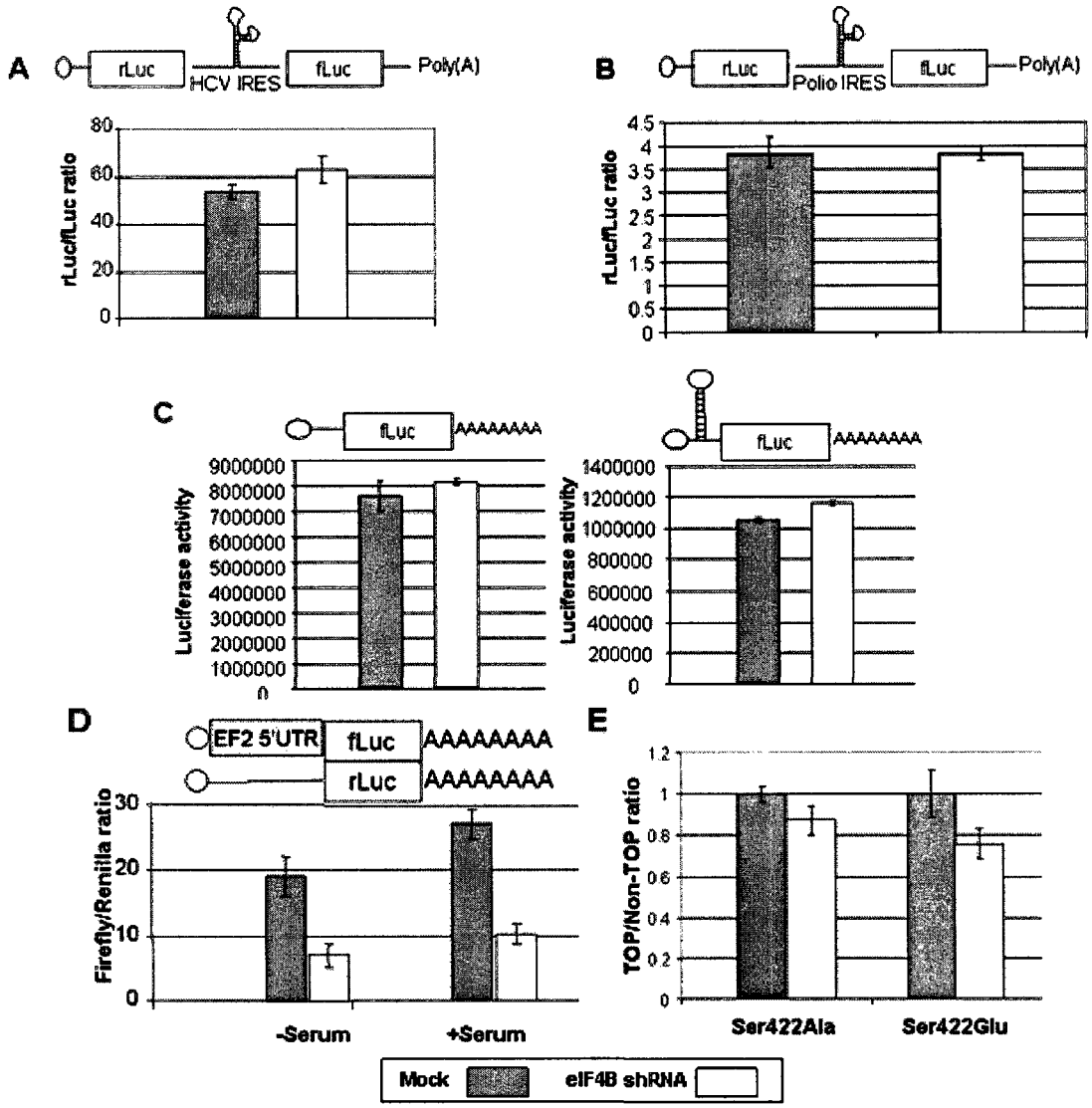

Fig3.3 eIF4B silencing affects translation of 5'TOP reporter in Ser422 phosphorylation independent manner. Mock (grey bars) and eIF4B silenced (white bars) cells were transfected with indicated luciferase constructs and grown for 48 hours, upon which cells were harvested and assayed for firefly (FL), or renilla (RL) and firefly (FL) luminescence. Transfections were performed with (A) RL-HCV IRES-FL, (B) RL-Polio IRES-FL. Results are presented as average of RL/FL ± standard deviation in A and B. (C) Monocistronic FL (left chart) and Stem-and-Loop-FL (right chart) reporters were transfected. Results are presented as average of luciferase activity counts ± standard deviation. (D) TOP-FL and NonTOP-RL monocistronic constructs were co-transfected, cells were grown for 24 hours, upon which cells were serum-starved for additional 24 hours or left in serum-rich media as indicated. (E) Transfections were carried out as in (D) except that eIF4B Ser422Ala and Ser422Glu encoding vectors were co-transfected. Cells were grown in serum-rich media for 48 hours before harvesting. The FL/RL ratio ± standard deviation in mock cells co-expressing either eIF4B Ser422 mutant was normalized to 1 and compared to eIF4B silenced cells expressing the same proteins.

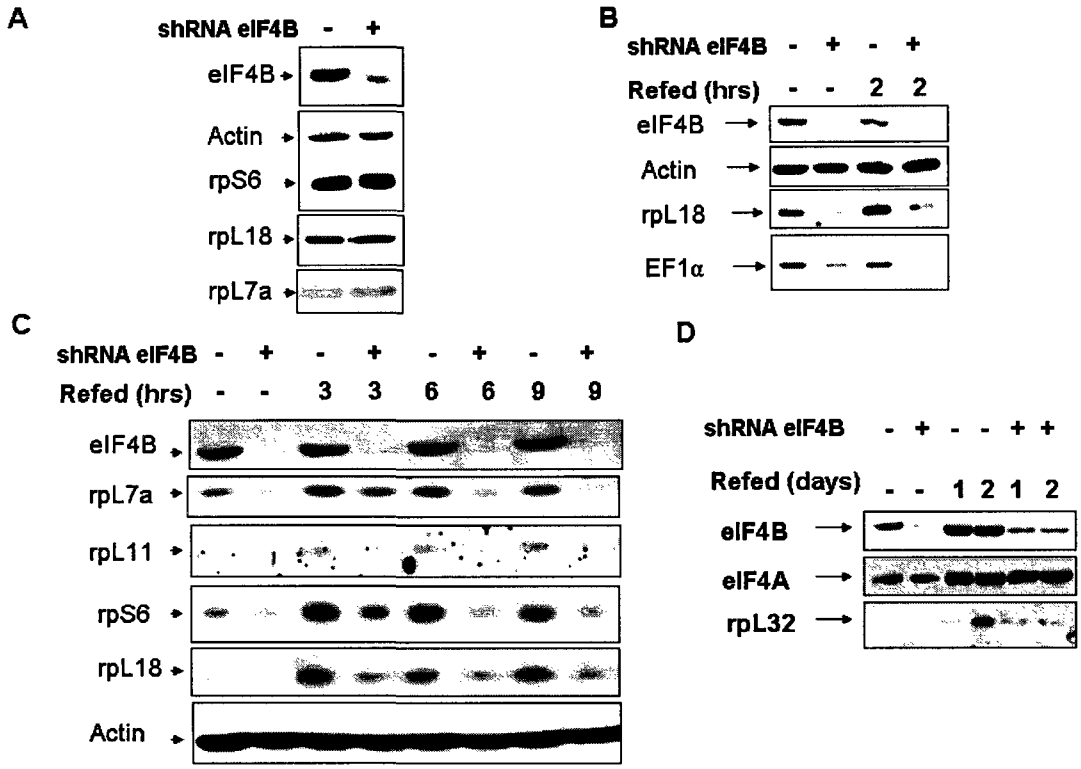

Fig3.4 eIF4B silencing affects *de novo* synthesis rate of 5'TOP mRNA encoded proteins. (A) Total cell extracts of normally growing mock and eIF4B silenced cells were resolved by SDS-PAGE and immunoblotted with eIF4B, actin, rpS6, rpL18, and rpL7a antibodies. (B) Cells were starved of serum for 72 hours and then refed for 2 hours with serum. Protein extracts were subjected to SDS-PAGE and immunoblotted with eIF4B, actin, rpL18 and eEF1α antibodies. (C) Mock and eIF4B silenced cells were serum starved for 80 hours and refed for indicated times. Proteins were detected by immunobloting with eIF4B, rpL7a, rpL11, rpS6, rpL18 and actin antibodies. (D) Cells were serum-starved for total duration of this experiment (96 hours, lanes 1 and 2), or were re-fed for last day or 2 days. Cell extracts were resolved by SDS-PAGE and immunoblotted with eIF4B, eIF4A (as a Non-TOP control) and rpL32 antibodies.

then stays relatively constant over the timecourse period. The plateau levels of ribosomal proteins, however, are different being considerably lower in eIF4B-silenced cells. When mock and eIF4B-silenced cells were incubated in serum-free media for 96 hours the ribosomal protein L32 decreased to undetectable levels (Fig.3.4D lanes 1 and 2). L32 level increased similarly in both cell types when cells were refed with serum for last 24 hours of the incubation (i.e. 72 hours of starvation followed by 24 hours of refeeding; compare lanes 3 and 5 in Fig.3.4D). When cells were refed with serum for the last 48 hours of the experiment (i.e. 48 hours of starvation followed by 48 hours of refeeding), L32 reached significantly higher levels of expression in mock cells (Fig.3.4D compare lanes 4 and 6). The level of eIF4A which is encoded by non-TOP mRNA is not affected by eIF4B silencing but is sensitive to the prolonged serum starvation (Fig.3.4D, middle panel). The effect of eIF4B silencing on synthesis of endogenous ribosomal proteins L7a, L11, L18, S6 and eEF1α is in concert with the results observed with 5'TOP luciferase reporter.

3.1.5 eIF4B silencing inhibits translation initiation and ribosomal biosynthesis.

We next assessed whether achieved eIF4B silencing level was sufficient to cause an inhibition of translation initiation *in vivo*. Polysomal analysis of normally growing cells demonstrated a slight decrease in lighter polysomes of eIF4B-silenced cells (Fig.3.5A). No significant difference was observed in the level of monosomes or free 40S and 60S subunits under this condition. This decrease might be suggestive of a specific suppression of a "translationally unfavored" subclass(es) of mRNAs having low ribosomal occupancy (e.g. highly structured, containing uORFs etc) or mRNAs with short ORFs (having low ribosomal density due to the length limitations) since these fractions contain transcripts with few bound ribosomes. However, we also cannot rule out the possibility that the overall number of ribosomes is slightly lower in eIF4B-silenced cells. In this case, ribosome availability would become a limiting factor for the translationally disadvantaged messages, whereas translation of efficient transcripts would not be significantly affected. When cells were serum starved for 48 hours and then re-fed for 24 hours the polysomal profiles of eIF4B-silenced cells were markedly different from those of mock cells (Fig.3.5B).

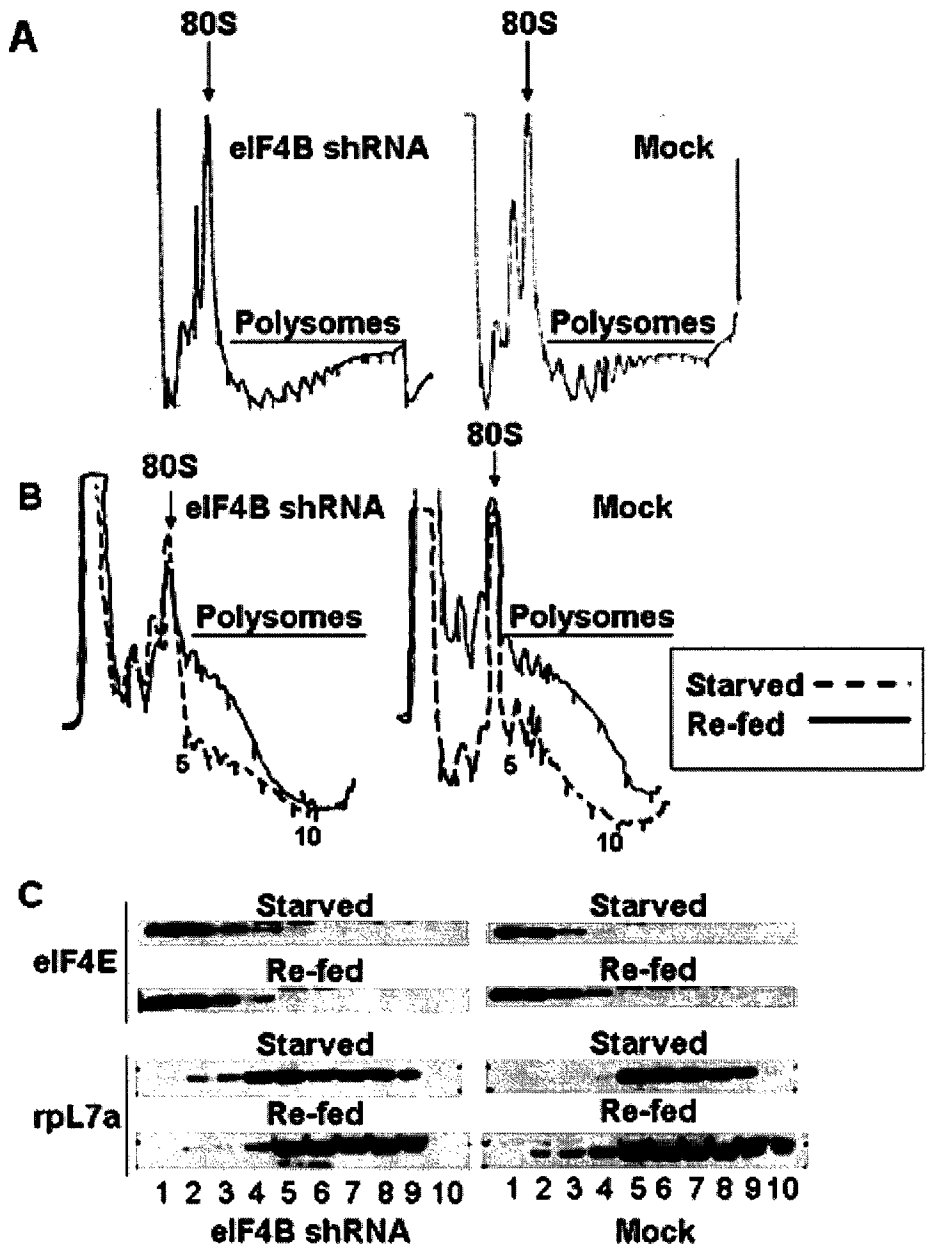

Fig.3.5 Effect of eIF4B silencing on HeLa cell polysomes. (A) Polysomes from normally growing mock and eIF4B silenced cells were analyzed on 10%–50% sucrose gradients. (B) Mock and eIF4B silenced cells were serum starved for 48 hours or serum starved for 48 hours (- - -) and then re-fed for 24 hours (—). Polysomes were analyzed on 10%–50% sucrose gradients, and fractions were collected from the gradients. (C) Western blots demonstrating the position of migration of L7a and eIF4E in fractions collected in (B).

Serum starvation led to the depletion of polysomes in both cell types but lighter polysomal peaks persisted in mock cells. The striking difference, however, was observed in the levels of free ribosomal subunits which were much higher in eIF4B-silenced cells, suggestive of a stronger inhibition of translation initiation. Serum replenishment increased the amount of material in both subpolysomal and polysomal fractions in mock cells, whereas only polysomal fractions increased in eIF4B-silenced cells. We then immunoblotted fractions from the polysome gradients to determine the extent of sedimentation of eIF4E and L7a (Fig.3.5C). eIF4B silencing caused L7a (as a marker of 60S subunits) to sediment into lighter complexes under starved condition (Fig.3.5C, compare fractions 2 to 4 in eIF4B-silenced and mock cells), which is consistent with the inhibition of translation initiation observed in polysomal profiles (Fig.3.5.B). In serum re-fed cells, the amount of L7a was higher in both lighter and heavier fractions of mock cells again paralleling the polysomal profiles (Fig.3.5.B and C). These differences could represent a combined effect of decreased translation initiation and an attenuated ribosomal biogenesis (due to the reduced ribosomal protein production as shown in Fig.3.4) in eIF4B-silenced cells.

3.1.6 eIF4B pull down complex contains DDX21 and the two proteins are partially co-localized in nucleolar periphery.

In parallel, we tried to identify novel eIF4B interacting partners. To this end, eIF4B interacting complexes were affinity purified from HeLa cell lysates using recombinant doubly tagged 6His-eIF4B-Flag wild type and Ser422Asp mutant baits. The 6His-eIF4B-Flag-containing complex was isolated under native conditions using Ni^{2+} column and Flag resin. Proteins associated with the purified 6His-eIF4B-Flag complexes were separated by SDS/PAGE and visualized by Coomassie staining (Fig.3.6A). Specific bands were excised from the gel and analyzed by mass spectrometry. The results were compared to the negative controls (i.e. samples with no bait, but subjected to all the purification steps in parallel with the experimental samples). Mass spectrometry analysis of individual bands has led to identification of known and unknown binding partners of eIF4B; however, no difference could be seen in this experimental setting between complexes precipitated by wt eIF4B and Ser422Asp phosphomimetic mutant. One of the

proteins consistently and specifically co-purified with eIF4B was identified as DDX21. Interestingly, DDX21 is a nucleolar RNA helicase and its depletion abrogates ribosomal RNA processing in mammalian cells (Henning et al., 2003) and in Xenopus oocytes (Yang et al., 2003). In mammalian cells, it has been previously described that under normal physiological conditions DDX21 localizes exclusively to nucleoli (Westermarck et al., 2002) and that eIF4B shows predominantly cytoplasmic staining (Low et al., 2005). Surprisingly, we (Fig.3.6A) and others (Sekiguchi et al., 2006) identified these two proteins as components of same complex(es). To address this apparent controversy and to verify our affinity precipitation data regarding the interaction between DDX21 and eIF4B, we decided to carry out a co-localization experiment. To this end, HeLa cells were co-transfected with GFP-DDX21 fusion protein and Flag tagged eIF4B. Twenty four hours later cells were trypsinized and 20% of transfectants were seeded on microscope slide-chambers. Extracts prepared from remaining transfected cells were immunoblotted with GFP and Flag antibodies and single bands of correct sizes were detected (Fig.3.6B). The subcellular localization of Flag-eIF4B (red) and GFP-DDX21 (green) in HeLa cells was then analyzed using confocal microscopy (Fig.3.6.C). As previously published, DDX21-GFP showed predominantly nucleolar staining, whereas majority of Flag-eIF4B was localized to the cytoplasm. However, small fraction of eIF4B could be seen in the nuclei in a speckled pattern and some of these speckles co-localized with DDX21-GFP in nucleolar periphery (yellow speckled signal in Fig.3.6C, "overlay" panel). The presence of eIF4B in the nuclear preparations has been reported in another study, which identified both DDX21 and eIF4B as nuclear phosphoproteins (Beausoleil et al., 2004). The incidence of eIF4B fraction associated with the nucleoli might be suggestive of a direct role in ribosomal biogenesis. And this notion is supported by the identification of eIF4B as a component of pre-ribosomal particles (Sekiguchi et al., 2006).

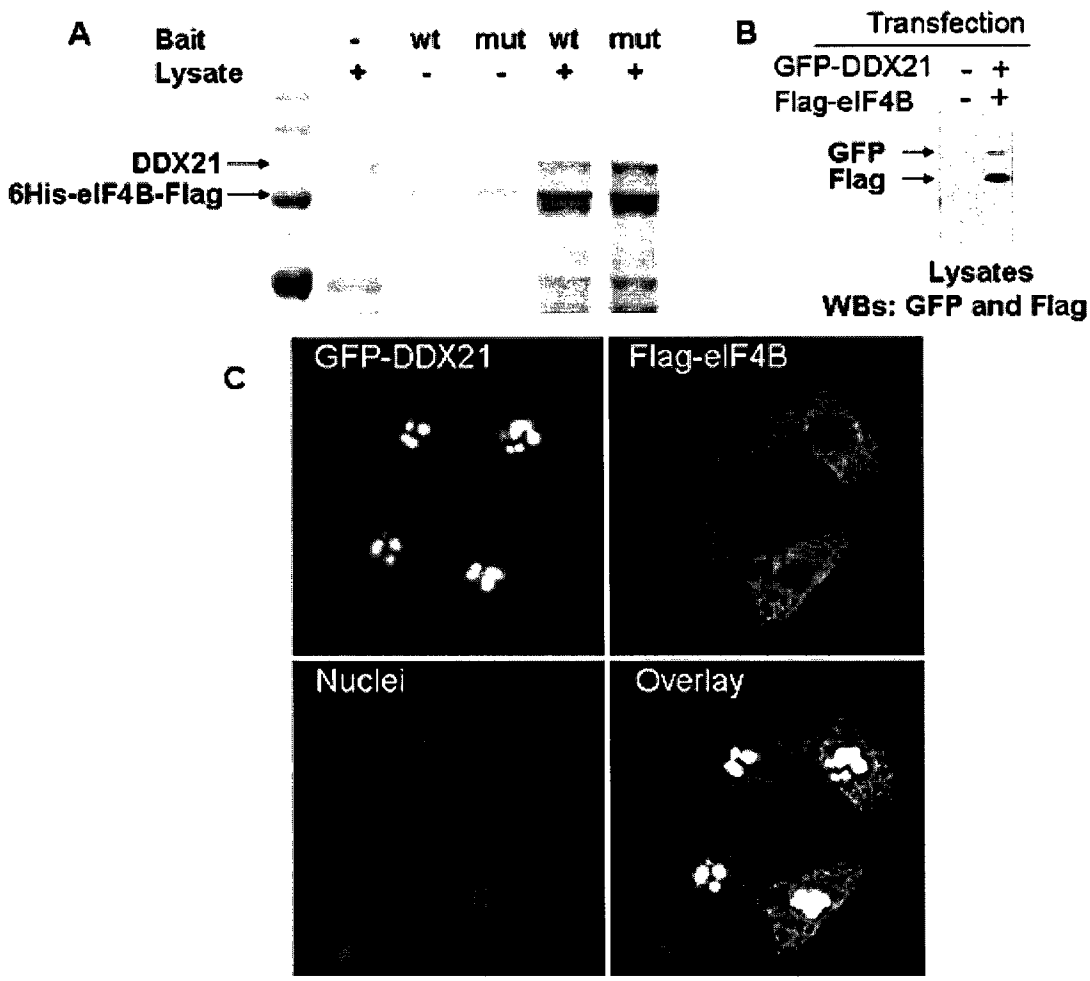

Fig3.6 Identification of DDX21 in an eIF4B-associated complex. (A) 6His-eIF4B-Flag-associated complexes from HeLa cell extract were doubly purified through 6His and Flag tags. Representative Coomassie-stained SDS–PAGE gel fragment is shown. The band of approximately 100 kDa was identified as DDX21 by mass spectrometry. Positions of eIF4B bait and DDX21 are indicated. Abbreviations: wt – wild type tagged eIF4B, mut – tagged eIF4B Ser422Asp mutant. **Co-localization of Flag-eIF4B and DDX21-GFP in HeLa cells.** Cells were co-transfected with Flag-eIF4B and DDX21-GFP. Twenty four hours later cells were split. (B) Protein extracts have been prepared from a portion of cells and probed for GFP and Flag expression. (C) Remaining cells were seeded on microscope chamber-slides, fixed and reacted with antibodies against Flag followed by staining with red fluorophore-labeled secondary antibody. The nuclei were stained with Hoescht dye. The images were visualized with a confocal microscope. Merged images are shown in yellow when green and red signals are co-localized [the "overlay" quarter of section (C)].

3.1.7 eIF4B silencing inhibits rRNA processing.

As mentioned above, DDX21 has an important role in rRNA processing and its silencing has been reported to inhibit production of 28S and 18S rRNAs (Henning et al., 2003; Yang et al., 2003). Since eIF4B and DDX21 were co-purified (Fig.3.6A and (Sekiguchi et al., 2006)) and co-localized in nucleoli (Fig.3.6C), we decided to assess the effect of eIF4B silencing on rRNA processing. Mock and eIF4B-silenced cells were pulse-labeled with [^{32}P] orthophosphate and then chased with unlabeled phosphate-enriched medium (Fig.3.7). The low ^{32}P activity at 0 h of chase indicates that little labeled phosphate had been incorporated into rRNA. Within 2 h, the labeling of RNA increased, and the positions of 32S, 28S, and 21S/18S rRNA were visualized. Compared with mock, eIF4B silencing resulted in a 35% and 20% decrease in the level of 28 and 18S rRNA after a 2-h chase, correspondingly (Fig.3.7). The level of 47/45S and 32S rRNA intermediate decreased to a lesser extent in eIF4B-silenced cells. After a 4 h chase, the level of 28S was lower by 45% in eIF4B-silenced cells, whereas 18S rRNA actually increased by 15%. It has been reported that multiple pathways of rRNA maturation may co-exist in the same cells. Therefore, the maturation of a given 18S molecule is not necessarily associated with a concomitant maturation of the 28S molecule present in the common 47/45S precursor rRNA. The alternative pathways of pre-rRNA cleavage and processing are marked with distinct intermediates (Diagram3.1). Interestingly, at 4 h time point 32S rRNA level decreased, but 36S rRNA species have accumulated to detectible level in eIF4B-silenced cells (Fig.3B middle panel). This also can explain why 18S species are produced at similar rate in mock and eIF4B-silenced cells, since 36S appearance is associated with direct excision of 18S from 41S precursor which might accelerate 18S production (see Diagram.3.1). This may also indicate that inhibition of a certain step in a preferential rRNA processing pathway possibly will induce a negative feedback and a switch to an alternative compensatory pathway. Hence, in eIF4B-silenced cells, 28S production is steadily attenuated from both 32S and 36S precursors and the difference in 18S production fluctuates (+/- 15-20% from the control).

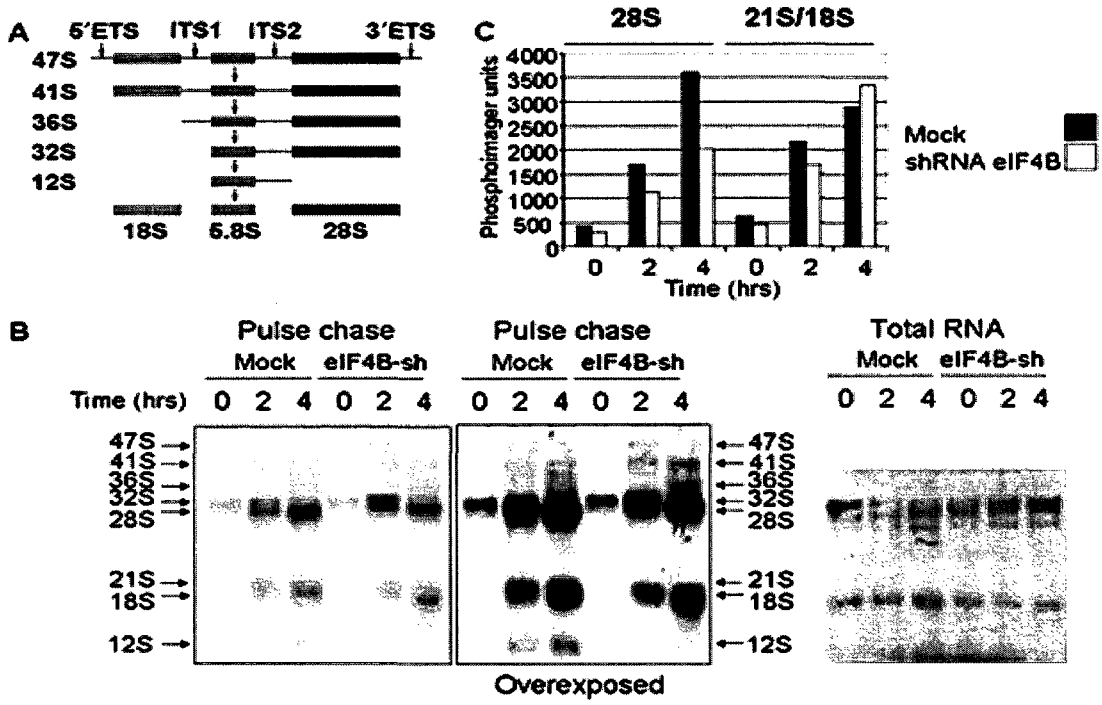

Fig.3.7 The effect of eIF4B silencing on rRNA processing. (A) rRNA species (the diagram is adopted from (Itahana et al., 2003)) (B) Mock and eIF4B silenced (indicated eIF4B-sh, for eIF4B shRNA) cells were starved of phosphate for 3.5 hours and then labeled with 40 μCi/ml [^{32}P]orthophosphate for 1.5 hours and chased in regular medium containing cold phosphate for 0, 2 and 4 hours. Total RNA was extracted and quantified. RNA was resolved on a denaturing 1% agarose-formaldehyde gel and blotted onto a Hybond-N nylon membrane. The membrane was dried and exposed to a PhosphoImager screen for 2 hours (left panel) or to a film overnight (middle panel). The sizes of the processing intermediates and the mature RNA species are indicated. After analysis, the RNA blot was stained with Blot Stain Blue reagent to visually check loading (right panel). (C) Signal intensity of 28S and 21/18S rRNA species was quantified by phosphoimager and plotted.

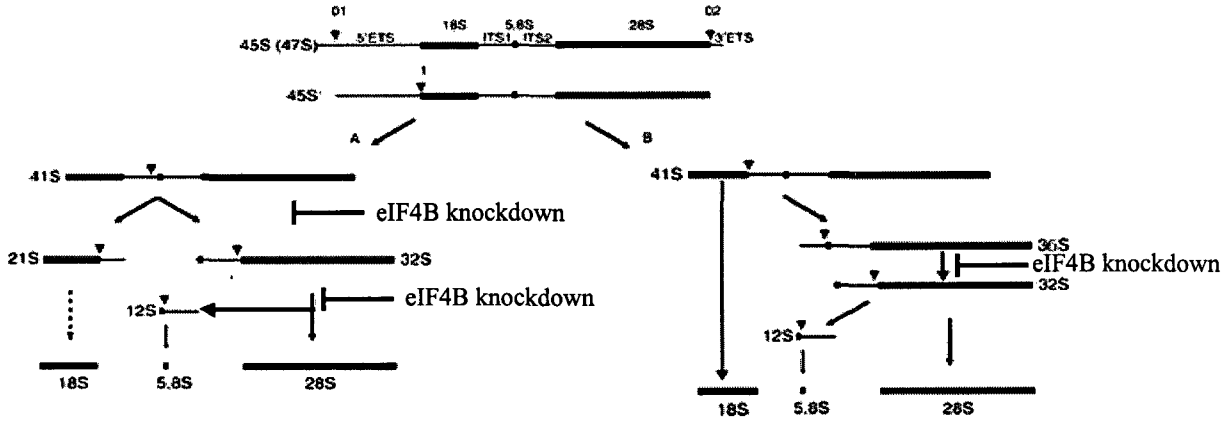

Diagram3.1 Alternative pathways in rRNA processing (adopted with alterations from (Rouquette et al., 2005)). Cleavage sites (▼) and steps presumably inhibited by eIF4B knockdown are indicated.

The levels of 18S, 12S (Fig.7B) and 5.8S (data not shown) corroborate the notion that the production of 28S and 5.8S but not of 18 S rRNA is affected by eIF4B down-regulation. To show total loading of RNA, the membrane was stained with Blot See Blue reagent. Only the final products, 28 and 18 S rRNA, which were present in greater proportion, were stained strongly (Fig.7B, right panel). The observed inhibition of rRNA production mediated by eIF4B silencing is different from that exerted by DDX21 depletion. The differential effect on 28S and 18S suggests an attenuated processing of 32S and 36S rRNAs downstream from initial cleavage of the 47/45S and 41S rRNA precursors. Accumulation of unprocessed 32S rRNA may be causative to accumulation of 41S and 45/47S precursors after 2 hours of chase. An alternative pathway is then turned on leading to efficient excision of 18S from pre-accumulated 41S rRNA precursor. The disappearance of 32S rRNA from eIF4B-silenced cells after 4 hours of chase may be associated with a slow conversion of 32S into 28S and 12S rRNAs, or degradation of 32S. This phenomenon is similar to the effects caused by mutation of the DOB1(MTR4)

gene which encodes an essential RNA helicase in yeast and which was initially identified in a screen for dependence on overexpression of eIF4B (de la Cruz et al., 1998). These data suggest that eIF4B silencing inhibits rRNA processing and causes accumulation of precursors of 28S and 5.8S rRNAs. Due to multiplicity of pathways that may co-exist in the same cell, more experiments should be conducted to pinpoint rRNA processing step(s) affected in eIF4B-silenced cells.

3.2 Discussion

The established eIF4B silencing system has uncovered an intriguing case of translation initiation factor-mediated regulation within the process of ribosomal biosynthesis.

The data presented in Chapter 3 demonstrate the involvement of eIF4B in an *in vivo* translation initiation and ribosomal biogenesis in mammalian cells. We base these conclusions on several observations. 5'TOP luciferase reporter expression is selectively suppressed in eIF4B-silenced cells and this effect can be reverted by exogenous non-phosphorylatable and phosphomimetic eIF4B Ser422 mutants (Fig.3.3 D and E). Under serum starved condition, knock down of eIF4B causes accelerated decay of ribosomal proteins and attenuates their *de novo* synthesis upon re-feeding (Fig.3.4). Polysomal profiles from mock and eIF4B-silenced cells point at the involvement of eIF4B in an *in vivo* translation initiation and might be also suggestive of a role in ribosomal biosynthesis (Fig.3.5). In addition, nucleolar helicase DDX21 was identified in complexes containing eIF4B and partial co-localization of these two proteins in nucleolar periphery could be shown by confocal microscopy (Fig.3.6). Finally, 28S rRNA production was inhibited in eIF4B-silenced cells (Fig.3.7). We propose that reduced proliferation and anchorage independent growth of eIF4B-silenced HeLa cells (Fig,3.2) can be explained by the combined effect of eIF4B on translation initiation and ribosomal biosynthesis.

The role of eIF4B in the initiation of translation is not well understood. The better characterized activity of the protein is the contribution to eIF4A helicase activity. eIF4B has been shown to be essential for eIF4A-mediated RNA unwinding *in vitro* (Lawson et al., 1989). However, later studies demonstrated that eIF4A can function independently of co-factors, but its processivity is significantly improved in the presence of eIF4B or a functionally related protein eIF4H (Rogers et al., 2001; Rogers et al., 1999). *In vitro*

comparative studies with human and yeast homologues of eIF4B demonstrated a dual action of the protein (Altmann et al., 1995). Surprisingly, both homologues have shown complementary RNA annealing activity, but only human eIF4B stimulated helicase activity of eIF4A *in vitro.*

There is little sequence similarity between eIF4B homologues from different species. For instance, due to the lack of sequence and size similarity the plant homologue of eIF4B was at first identified as "eIF4G" (Browning et al., 1989). The eIF4B deficiency in yeast could be complemented with human, but not with *Drosophila* homologue of the protein (Altmann et al., 1993; Hernandez et al., 2004). Still, the activities of mammalian and yeast eIF4B proteins *in vitro* do not entirely overlap (Altmann et al., 1995). The reported differences in biochemical properties observed in an *in vitro* studies are also reflected in works describing the effects of eIF4B in living cells. Several *in vivo* studies about the physiological role of eIF4B in yeast, fly and mammalian cells have been published. In yeast, eIF4B is not essential, since disruption of Tif3/Stm1 gene results in viable, however, slow growing cold- and temperature-sensitive strains (Altmann et al., 1993; Coppolecchia et al., 1993). Despite the fact that Drosophila homolog of eIF4B has been shown to stimulate cap-dependent translation *in vitro*, the silencing of eIF4B in *Drosophila* cells caused almost no inhibition in general protein synthesis (Hernandez et al., 2004). In the same study, the overexpression of eIF4B in cultured cells and eye imaginal disk caused accelerated proliferation and appearance of abnormal extramitotic cells in the differentiated tissue, correspondingly. The studies of eIF4B overexpression effects on translation in mammalian cells generated controversial results (Holz et al., 2005; Milburn et al., 1990; Naranda et al., 1994).

In spite of low sequence similarity, the eIF4B homologues from different species share overall comparable domain structure (Diagram.3.2). They contain N-terminal RRM (RNA Recognition Motif) domain, middle DRYG region and C-terminal ARM (Arginine Rich Motif) domain. The RRM and ARM domains contribute to RNA binding, whereas DRYG motif has been shown to be important for interaction with eIF3 and self-association of eIF4B (Methot et al., 1994; Methot et al., 1996b).

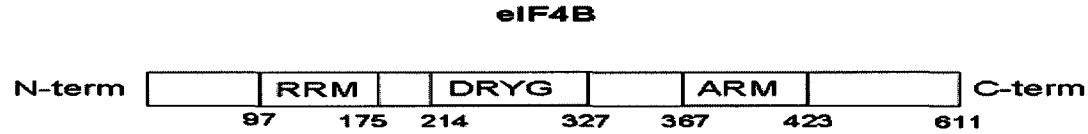

Diagram 3.2. eIF4B domain structure. Amino acids positions flanking the RRM, DRYG and ARM domains are indicated

The region important for eIF4A activity resides within C-terminal region of eIF4B adjacent to the ARM domain, however deletion of DRYG or point mutations in N-terminal RRM domains have also drastically reduced the ability of eIF4B to stimulate eIF4A helicase activity *in vitro*. Overexpression of eIF4B in COS1 cells has been shown to inhibit the translation of a DHFR reporter gene. However, the truncation mutant containing only N-terminal region with the RRM domain was sufficient for inhibition of DHFR translation (Naranda et al., 1994). The latter observation suggests that the effect on translation was independent of eIF4A co-factor activity, since the region responsible for eIF4A helicase stimulation resides in the C-terminal portion of eIF4B (Methot et al., 1994).

The RRM domain found in eIF4B is a highly conserved motif present in many other proteins. This domain consists of two ribonucleoprotein consensus sequences (RNP-CS) and is shared by RNA-binding proteins of splicosome (snRNPs), translational factors (eIF4B and PABP), components of rRNA transcription and processing complexes (e.g. nucleolin), and RNA chaperone-like factors (e.g. La) (Methot et al., 1994; Milburn et al., 1990).

eIF4B overexpression in yeast has been reported to suppress a temperature-sensitive mutation of DOB1 gene coding for an essential RNA helicase involved in the late stage of 60S ribosomal subunit biosynthesis (de la Cruz et al., 1998). Depletion of dob1p resulted in decreased levels of 60S subunit leaving 40S levels unchanged. The overexpression of other components of eIF4F (eIF4E, eIF4G or eIF4A) did not suppress the dob1p mutation, ruling out the possibility of eIF4B overexpression acting through

more efficient recruitment of 40S to mRNA and hence bypassing the 60S deficit.. The human homologue of dob1p has been also identified in association with pre-ribosomal particles (Nagahama et al., 2006). Some human factors involved in ribosome biogenesis, including fibrillarin and eIF6, can complement, at least partially, yeast strains with mutations in their orthologues (Jansen et al., 1991; Sanvito et al., 1999). Hence, an elaborate system for biogenesis is probably conserved in eukaryotes, including humans. Several lines of evidence indicate that eIF4B functions in ribosome biogenesis in human cells. First, eIF4B precipitated complex containing the nucleolar helicase DDX21 (Fig.3.6A) and pre-ribosomal particles were reported to contain eIF4B (Sekiguchi et al., 2006). Second, we were able to show that small fraction of eIF4B is present in nucleolar periphery (Fig.3.6C). In spite of predominantly cytoplasmic localization of eIF4B, the existence of nuclear pool of this protein has been previously documented (Beausoleil et al., 2004). Third, eIF4B down-regulation delays synthesis of ribosomal proteins (Fig.3.4B-D) and production of 28S and 5.8S rRNA from 32S and 36S precursors (Fig.3.B and C), most likely contributing to the attenuated proliferation and to the growth inhibition in soft agar assay of HeLa cells (Fig.3.2). When the structure of the nucleolus is viewed in the electron microscope, at least three morphological regions can be distinguished: the fibrillar centres (FCs), which are surrounded by the dense fibrillar component (DFC), and the granular component (GC), which constitutes the remainder of the nucleolus. Separate steps during the maturation of pre-rRNA into ribosomal subunits can be correlated with nucleolar structures. Pulse-labelling studies indicate that rRNA gene transcription occurs either in FCs, or at the FC/DFC boundary, although in some cases transcribed genes may extend into the DFC. Consistent with this model, more FCs are detected in nucleoli when the level of transcription by RNA polymerase I increases. As judged by the concentration of processing factors in this region, much of the cleavage and modification of rRNAs occurs in the DFC, whereas later steps during protein assembly on the ribosomal subunits occur in the GC (Lamond and Sleeman, 2003). The transport of pre-rRNAs within the nucleolus does not occur randomly, but appears as a radial flow starting from the fibrillar centers toward the nucleolar periphery (Thiry et al., 2000). The ordered processing steps are believed to progress concomitantly. Hence, the localization of nuclear eIF4B to the nucleolar periphery is in agreement with its role in a

late step(s) of rRNA processing. In addition, mTOR is the major regulator of ribosomal biogenesis (Mayer and Grummt, 2006) and eIF4B is phosphorylated on Ser422 by S6K in rapamycin-dependent manner(Chapter 2 and (Raught et al., 2004)). It was long believed that mTOR regulates 5'TOP mRNA translation through S6K activity-mediated ribosomal protein S6 phosphorylation (Jefferies et al., 1994). However, recent studies have demonstrated that 5'TOP mRNA translation retains rapamycin-sensitivity in S6K1/2 knockout cells, or in non-phosphorylatable S6 mutant knock-in cells (Pende et al., 2004; Ruvinsky et al., 2005). Nonetheless, rapamycin treatment and serum starvation have been demonstrated to inhibit eIF4B synthesis in murine cells (Bilanges et al., 2007). Thus, the possibility that mTOR inhibition by rapamycin or serum starvation causes translational inactivation of 5'TOP mRNAs in part through the suppression of eIF4B expression cannot be ruled out. The restoration of 5'TOP-luciferase reporter expression by transfections of eIF4B Ser422Ala or Ser422Glu mutants in eIF4B-silenced cells is in line with this assumption (Fig.3.3 compare D and E). The phosphorylation state of eIF4B Ser422 did not affect the rescue results, however, it cannot be ruled out that another subset of mRNAs is regulated by this phosphorylation, since it was shown to enhance eIF4B interaction with eIF3. Furthermore, eIF4B contains multiple phosphorylation sites and their involvement in eIF4B activities remains to be determined. We did not compare the levels of ribosomal protein coding mRNAs in mock and eIF4B-silenced cells. However, previous studies conducted in vertebrate cells have demonstrated that the ribosomal protein-mRNAs are relatively stable and the overall regulation of ribosomal protein synthesis is primarily at the level of translation. The 5' terminal oligopyrimidine (TOP) sequence, which is a ubiquitous feature of all vertebrate ribosomal protein-mRNAs, is required for controlling their translational efficiency (Meyuhas, 2000). Pateamine A-induced RNA-mediated sequestration of eIF4A has been shown to inhibit translation initiation (Bordeleau et al., 2006). Interestingly, in this report the Pateamine A treatment was also hallmarked with eIF4A associated with heavier polysomal fractions in HeLa cells. Our results demonstrate that eIF4B silencing causes a similar effect on sucrose density gradient distribution of eIF4A (Appendix 2). The stoichiometry of ribosomal components can be achieved also by turnover of ribosomal proteins that are not assembled into ribosomes, but, of course, this represents a mechanism of last resort

because it is energetically wasteful for the cell. Evidence for efficient degradation of unassembled ribosomal proteins has been demonstrated in both yeast and mammalian cells when excess ribosomal proteins are produced or when ribosomal RNA synthesis is inhibited (Perry, 2007). However, given the well characterized stimulatory effects of eIF4B on translation *in vitro,* and the data from the 5'TOP-luciferase reporters suggest that the differences in ribosomal protein synthesis observed in our study are unlikely to be consequential to the inhibition of rRNA processing.

Given the dependence of cancer cell growth on ribosomal biogenesis, high eIF4B expression in cancer cells may contribute to proliferation, a hypothesis supported by the fact that the eIF4B is overexpressed in lung cancers (Comtesse et al., 2007). Moreover, our preliminary data suggest that moderate level of eIF4B overexpression in 3T3 cells potentiates Ras-mediated transformation as these cells gain the capacity to grow in soft agar (see Appendix 3).

The outcome of our studies discovering a novel role for a translation initiation factor 4B in ribosomal biogenesis, unexpected at first sight, has at least one well documented paradigm. In fact, the eukaryotic translation initiation factor 6 (eIF6) was first purified and identified as ribosome dissociating activity stimulating translation in a partially fractionated wheat germ cell-free system (Russell and Spremulli, 1979). Due to significant sequence identity, molecular cloning of human eIF6 also identified putative orthologues of the gene in *Saccharomyces cerevisiae*, *Drosophila*, and the nematode *Caenorhabditis elegans* (Si et al., 1997). Later *in vitro* studies in reticulocyte lysates suggested that eIF2:GTP:Met-tRNA, ternary complex binding to 40S ribosomal subunit is highly dependent on eIF6 which facilitates this process (Raychaudhuri et al., 1984). However, later reports suggested that eIF6 is involved in biogenesis of 60S ribosomal subunit in yeast and mammalian cells (Basu et al., 2001; Sanvito et al., 1999). The disruption of yeast eIF6 gene (also called IIH or TIF6) was lethal and could be complemented by expression of human orthologue, suggesting an evolutionarily conserved function of eIF6 in ribosome biogenesis. The ability of at least two eIFs to regulate both translation initiation and ribosomal biogenesis suggests an interesting control mechanism allowing cells to adjust translation initiation levels to the ribosomal biosynthesis and vice versa.

In eukaryotes, ribosomal biogenesis is a very complex process. The major site of ribosomal biogenesis is the nucleolus, where ribosomal particles are assembled, modified and processed. However, ribosomal protein synthesis and some steps in ribosome maturation take place in the cytoplasm (Rouquette et al., 2005). In yeast, the maturation of rRNA and its incorporation into ribosomal subunits involves at least 170 accessory proteins comprising endo- and exoribonucleases, putative ATP-dependent RNA helicases, 'chaperones' or 'assembly factors'; and about as many small nucleolar ribonucleoprotein particles (snoRNPs) (Fromont-Racine et al., 2003). These non-ribosomal factors have homologues in other species, and some share properties with components of mammalian pre-ribosomal complexes (Takahashi et al., 2003). The incomplete human nucleolar proteome has been recently shown to contain more than 700 proteins (Leung et al., 2006). The various rRNAs and ribosomal proteins are produced in equimolar amounts and their synthesis is tightly regulated by a variety of growth conditions (Fromont-Racine et al., 2003). Depletion of factors essential for ribosome biogenesis in eukaryotic cells results in lethal phenotypes marked with disbalanced 40S/60S ratio or simultaneous suppression of both subunits' production. Alternatively, defective subunits (due to incomplete assembly, processing or modification) can be produced along with active ones and then undergo either slow maturation or degradation. It is tempting to postulate that yeast homologue of eIF4B also plays a role in ribosome synthesis. The fact that eIF4B gene disruption in yeast does not lead to a lethal phenotype suggests an auxiliary rather than an essential role of the factor. It might be virtually dispensable under normal growth conditions but its importance might increase during some types of stress (e.g. suboptimal temperature, starvation etc.). The slow growth phenotype observed in eIF4B knockout yeast (2 fold in generation time under permissive conditions) was not recapitulated in eIF4B-silenced HeLa cells used in our study. The effect in our cells was much more modest (the doubling time was increased in eIF4B-silenced cells by ~15%). The polysomal profiles from eIF4B knockout yeast grown under restrictive temperatures (18^0C) were interpreted as a defect in translation initiation (Coppolecchia et al., 1993). However, these data could be also interpreted as a defect in the processing of pre-ribosomal particles resulting in depletion of ribosomal subunits, since 40S and 60S subunits were not well resolved in this report and 80S monosomes are

indiscernible by this technique from 90S premature ribosomal particles. Our data suggest that both translation initiation and ribosomal biosynthesis are inhibited in eIF4B-silenced cells and these effects could be better shown in starved cells or in cells recovering from prolonged starvation.

Despite the fact that eIF4B could be cross-linked to the mRNA close to 5' cap structure, it is unclear how eIF4B assists to the helicase activity of eIF4F and eIF4A since no direct interaction could be shown between eIF4B and eIF4A in mammalian cells. A recent report has shown that vhs (virion host shutoff) protein of Herpes Simplex Virus binds to eIF4B, and that this interaction strongly stimulates the RNase activity of vhs (Doepker et al., 2004). Stimulation of two different RNA-directed catalytic activities (helicase and nuclease) by eIF4B may be puzzling at first sight, however could be explained if eIF4B played a role of an RNA-chaperone-like factor in the cells. Indeed, in a recent review, eIF4B has been categorized as an "orphan RNA-chaperone" and is enlisted in the RNA-chaperone database created by the authors (Rajkowitsch et al., 2007). Given the fact that eIF4B lacks RNA-binding sequence specificity, the factor might need tethering to the site of action through an interacting partner and/or RNA-directed enzyme (e.g. helicase, ribosome, nuclease etc.). Once recruited to the RNA, eIF4B may perform its known activities as an ATP-independent single stranded RNA binding protein, a strand exchanger or an annealase (Altmann et al., 1995; Methot et al., 1994). Another RRM-containing RNA chaperone-like protein, the La autoantigen, is also thought to be involved in the translational regulation of 5'TOP mRNAs. La protein has been shown to bind 5'TOP sequences in both human and *Xenopus* cells. Overexpression of La in *Xenopus* growth-arrested cells shifts the ribosomal protein L4 mRNA from subpolysomes to polysomes, whereas in proliferating cells, expression of truncated form of La caused translational repression of the L4 transcript. However, conflicting data were obtained in human cells. Phosphorylation of La Ser633 is important for nucleoplasmic localization of the protein. When non-phosphorylatable Ser633Ala mutant of La is expressed in cells it translocates to nucleoli and cytoplasm. Moreover, it is this variant of La that TOP mRNAs preferentially bind. However, increased binding of La Ser633Ala to L37 mRNA caused sub-polysomal accumulation of the transcript. Hence, La is apparently playing a role in TOP mRNA translation, but its precise function in this

process is yet to be discovered (Hamilton et al., 2006). In our study, the phosphorylation state of eIF4B Ser422 did not affect the rescue of 5'TOP-luciferase reporter expression, however, it cannot be ruled out that another subset of mRNAs is regulated by this phosphorylation, since it was shown to enhance eIF4B interaction with eIF3 (Holz et al., 2005; Shahbazian et al., 2006). Furthermore, eIF4B contains multiple phosphorylation sites and their effect on the function of eIF4B remains to be determined. Interestingly, RNA-chaperones were identified as proteins induced as a part of cold- and heat-shock responses in bacteria (Nonaka et al., 2006; Rajkowitsch et al., 2007). At low temperature, RNA folding problem becomes more dominant, because non-native RNA structures, representing kinetic traps, are more stable under these conditions. Hence cold and temperature sensitive phenotype of eIF4B knockout yeast may be attributed to the RNA-chaperone activity of eIF4B.

The novel role of eIF4B in ribosomal biogenesis by regulating ribosomal protein and rRNA processing underscores the importance of eIF4B in mammalian celsl. However, more experiments should be done in order to elucidate the molecular mechanisms underlying the effects of eIF4B silencing on ribosomal biosynthesis. Recent studies show that cancer progression depends on ribosome biogenesis (Holland et al., 2004). Compounds such as rapamycin, which are known to inhibit ribosome biogenesis and translation initiation, are showing promise as an effective anticancer drugs in clinical trials (Petroulakis et al., 2006). In conjunction with its function as a translation initiation factor, eIF4B is likely to be an important modulator of cell growth and proliferation by regulating ribosome biogenesis. The viability of eIF4B-silenced mammalian cancer cells indicates, however, that interference with eIF4B RNA-chaperone activity impedes cell proliferation but is not toxic to the cells. Hence, reduction of RNA-chaperone activity of eIF4B is likely to be better tolerated by normal slowly dividing or differentiated cells, and interference with this activity might be potentially promising in therapy of the over-proliferative diseases dependent on high rates of translation, such as cancers.

3.4 Experimental procedures

3.4.1 Plasmids

The pTER-4B plasmid was designed as follows. The oligos (Invitrogen) containing eIF4B targeting sequence 5'GGACAGGAAGTGAGTCATC3' (encompassing Ser422) were ligated into pTER-zeo plasmid (a kind gift of Dr. Reuven Agami) as described elsewhere (van de Wetering et al., 2003). Expression vectors coding for Flag-eIF4B (wt, Ser422Ala, and Ser422Glu) were previously described (Raught et al., 2004). Luciferase bicistronic plasmids Renilla-HCV IRES- Firefly and Renilla-Polio IRES-Firefly were described elsewhere (Kruger et al., 2001; Park et al., 2005). Luciferase and Stem-Loop-Luciferase plasmids were previously published (Yang et al., 2004). Plasmid containing EF2 5'UTR TOP sequence was published elsewhere (Schwab et al., 1999). pRL-TK (Renilla luciferase reporter) for transfection efficiency was from Promega.

3.4.2 Generation of stable pTER cell lines.

HeLa cells were cultured in DMEM with 10% FCS. pcCDNA6TR/Bla (Invitrogen) was used in accordance with the manufacturer's instructions to generate blasticidin-resistant cells expressing the Tet repressor. Blasticidin-resisant cells were then transfected with pTER and pTER–4B. Blasticidin/Zeocin doubly resistant clones were tested for their ability to downreguate eIF4B in response to tetracycline by immunoblotting. No tetracycline-inducible clones could be isolated and the pTER-4B clone 8 was used as constitutively silenced cells. The pcCDNA6TR/pTER transfected cell line, termed pTER or mock, was used as a negative control in all experiments.

3.4.3 Cell proliferation and Soft agar assay

Mock and eIF4B-silenced cells were harvested by trypsinization and seeded in 6-well plates (5×10^4 cells/well). Cells were microscopically counted in triplicates for five consecutive days in hemocytometer. Doubling time was calculated according to the following formula:

$$\text{Doubling time} = \frac{5\text{x}24 \text{ hours}}{\sqrt{(N_5 / 50000)}}$$

Where 5x24 is total time of proliferation during 5 days in hours, N_5 is number of cells after 5 days of growth and 50000 is the initial number of cells plated.

For soft agar assay, 1.5 ml of 0.5% Agar Nobel in DMEM medium supplemented with 10% FBS was placed in six-well plates and allowed to gel at room temperature. Subconfluent mock and eIF4B-silenced cultures were harvested by trypsinization, resuspended in DMEM containing 20% FBS, and mixed in 1:1 proportion with 0.7% agar solution in DMEM (kept heated at 42^0C) to a final concentration of 3000 cells/well in DMEM containing 10% FBS and 0.35% agar. The cells in suspension containing agar were promptly overlaid on the first layer and allowed to gel in incubator for 16 hours and then were covered with regular serum rich media and grown for 14 days. Media were replaced every 3 days. Triplicate wells were prepared for each group of mock and eIF4B-silenced cells. Colonies were visualized with 0.5% crystal violet solution in 50% methanol and photographed.

3.4.4 Western blot analysis

Proteins were denatured by addition of 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 5% SDS, 10 mM EDTA, 0.5 M DTT, 0.25 % bromophenol blue, 50% glycerol) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (BioRad). The membrane was blocked in 5% BSA in (TBS containing 0.1% Tween-20) for 1 hr and probed overnight at 4°C with the appropriate antibody. The signal was detected with secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:10,000 and developed with chemiluminescence substrate (Amersham). Antibodies were purchased or were from the following sources: α-eIF4B (Methot et al., 1996a); α-actin and α-Flag(M2) (Sigma); α-eIF4E (BD Biosciences); α-L11, -L18, -L32 (described previously in (Nadano et al., 2000); α-L7a (S. Fumagalli, University of Cincinnati, OH, USA); α-EF1α (Upstate Biotechnologies); α-eIF4AI (Ferraiuolo et al., 2004); α-S6 (Cell Signaling Technology); α-GFP (Roche Molecular Biochemicals).

3.4.5 Luciferase assays

Cells were seeded in 6-well plates (5×10^5 cells/well), transfected with 2-2.5 μg of total reporter DNA. Amount of pRL-TK (Renilla luciferase reporter) for transfection efficiency (Promega) co-transfected with monocistronic firefly luciferase reporters was 250 ng per sample. Cells were transfected and cultured for 48 hours in serum rich media or starved for last 24 hours (as indicated in Fig.3.3 legend). Transient transfections with Lipofectamine2000 reagent (Invitrogen) were performed according to the manufacturer's protocol. Cells were lysed, and luciferase assays were carried out according to a standard protocol for the firefly or dual luciferase assay system (Promega).

3.4.6 Sucrose gradient fractionation and polysome isolation

Mock and eIF4B-silenced cells were grown in 150-mm dishes to 80% confluency. Cells were washed three times in cold PBS containing 100 μg/ml cycloheximide and were scraped off the plate using a rubber policeman and 1 ml of the same solution. Cells were centrifuged for 10 min at 1,200 rpm and resuspended in 425 μl hypotonic lysis buffer (5 mM Tris-HCl, pH7.5; 2.5 mM $MgCl_2$; 1.5 mM KCl). Cells were transferred to a pre-chilled tube and incubated with 100 μg/ml cycloheximide, 2 mM DTT and 2 μl RNAsin Inhibitor (40 U/μl; Stratagene). Cells were incubated on ice for 5 min and vortexed. To each 425 μl of cells, 25 μl of 10% Triton X-100 and 25 μl of 10% sodium deoxycholate were added; cells were then vortexed and incubated on ice for 5 min. Cell extracts were centrifuged for 5 min at 14,000 rpm; the supernatants were collected and loaded onto a pre-chilled 10–50% sucrose gradient. Each gradient was formed by mixing 5.5 ml of 10% and 50% sucrose in a Beckman Centrifuge tube (14×89 mm; Beckman Instruments #3311372, CA, USA) using a Labconco pump (Kansas City, MO, USA). Gradients were placed in a Beckman SW40Ti rotor and centrifuged at 35,000 rpm for 2 hr at 4°C. Fractions were collected (24 fractions of 12 drops or 12 fractions of 24 drops each, depending on experiment) using a Foxy JR ISCO collector and UV optical unit type 11 (St-Lincoln, NE, USA).

3.4.7 Confocal microscopy

HeLa cells were co-transfected with DDX21-GFP (described in (Holmstrom et al., 2008) and Flag-eIF4B expression vectors. Cells were fixed, blocked, immunostained and washed as described elsewhere. Anti-Flag(M2) primary antibody was used to detect

eIF4B and was visualized with AlexaFluor 594 goat anti-mouse IgG secondary antibody (Molecular Probes, Invitrogen) using confocal laser scanning microscope. Cell images were analyzed for Flag-eIF4B and DDX21-GFP localization using Zeiss LSM data acquisition software.

3.4.8 Recombinant protein, affinity precipitation and MS

Escherichia coli BL21 (DE3) transformed with the pET-6His-eIF4B-Flag wt and pET-6His-eIF4B-Flag Ser422Asp point mutant constructs (cloned by Lisa Lindqvist from Pelletier lab, at McGill University) were induced with IPTG to produce recombinant eIF4B protein variants. Ni Sepharose™ 6 Fast Flow resin (GE Healthcare) was used to purify recombinant proteins from bacterial lysates according to manufacturer's instructions. The beads with recombinant protein attached were kept at -20^0 C in PBS with 50% glycerol. The amount of bound recombinant protein per µl of resin suspension was estimated by resolving on SDS-PAGE and comparison to BSA bands of known content (0.1-10 µgr/lane) run on same gel and visualized by Coomassie staining. HeLa extracts were prepared by lysing in CHAPS buffer (40 mM HEPES, pH 7.5, 120 mM NaCl, 10 mM pyrophosphate, 10 mM β-glycerolphosphate, 1 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 1 mM PMSF, 10 µgr/ml each of aprotinin, leupeptin, pepstatin) and peleting the cell debris by centrifugation. Equivalent of 5 µgr of column-bound eIF4B baits (wt and mutant) or same bed volume of empty beads (with no bait attached) were co-incubated with 2 mg of HeLa cell lysate overnight. The resultant complexes were washed and then eluted with 300 mM imidazole. Anti-Flag(M2) antibody covalently crosslinked to protein G sepharose beads with the imidoester crosslinker DMP (dimethyl pimelimidate, Pierce) was then used in the second purification step. The samples were boiled and affinity precipitated complex components were resolved by SDS PAGE and Coomassie stained. Visualized bands and corresponding areas from the control lane were excised for identification by MS. Excised gel fragments were washed, reduced, alkylated, trypsinized and peptides were applied to LC/MS for protein identification by product ion mass spectrometry.

3.4.9 Metabolic labeling

Mock and eIF4B-silenced cells were grown in 6-well plates in DMEM containing 10% FBS. Cells were then incubated in phosphate-free medium (Gibco) for 3.5 h. It was

replaced with fresh phosphate-free medium containing 40 μCi/ml [^{32}P] orthophosphate (PerkinElmer). Incubation at 37 °C with 5% CO_2 was continued for 1.5 h. Cells were then washed twice with a growth medium. The chase experiment was done by incubating the labeled cells in a regular growth medium at 0, 2, and 4 h. Total RNA was isolated using TRIzol Reagent (Invitrogen), and the RNA concentration was determined at 260 nm. Equal weight of RNA was resolved on a formaldehyde-containing 1% agarose gel and blotted onto a Hybond-N nylon membrane (Amersham Biosciences). The membrane was dried and placed in a PhosphoImager cassette for ~2 hours or exposed to an x-ray film overnight. Intensity of the radioactive bands was analyzed with Typhoon 8600 Phosphoimager (Amersham Biosciences). After analysis, the RNA blot was stained with Blot Stain Blue reagent (Sigma) to visually check loading.

3.5 Acknowledgements

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Chapter 4 -- Conclusion

4.1 General discussion

The experiments described in this thesis characterize the regulation of eIF4B by signaling pathways and reveal a novel role for the factor in the process of ribosomal biosynthesis. The serum-stimulated phosphorylation of eIF4B on Ser422 has been previously shown to be rapamycin sensitive and the kinase directly phosphorylating Ser422 was identified as S6K (Raught et al., 2004). In chapter 2, we demonstrated that a rapamycin-resistant component of eIF4B Ser422 phosphorylation exists. The phosphorylation is biphasic and persisted in S6K1/2 double knockout cells. The two phases have distinct pharmacological sensitivities and are dependent on temporally separable activation of MAPK and PI3K/mTOR signaling cascades. Catalytically active variants of RSK protein activated downstream of MAPK cascade are phosphorylating eIF4B on Ser422 *in vitro* and *in vivo*. The S6K and RSK proteins are members of the AGC protein kinase family, and require PDK1 phosphorylation for activation. Consistent with this requirement, phosphorylation of eIF4B Ser422 is abrogated in PDK1 null embryonic stem cells. RSK inhibitor (fmk) treatment of cells and siRNA targeting of RSK1/2 isoforms inhibits the early phase of serum-induced eIF4B Ser422 phosphorylation. In addition, RSK1/2 siRNA decreases cap-dependent translation of a Renilla-HCV IRES-Firefly bicistronic reporter. Furthermore, Ser422 phosphorylation causes increased interaction with eIF3. Finally, a non-phosphorylatable form of eIF4B Ser422, Ser422Ala, is constitutively uncoupled from eIF3, whereas a phosphomimetic mutant, Ser422Glu, is constitutively associated with eIF3. The latter result indicates that Ser422 is sufficient and necessary for interaction with eIF3. Given the fact that eIF3 is a 40S subunit-bound factor, we interpreted this interaction as potentially stimulatory for ribosomal recruitment. Furthermore, a recent study underscored the importance of Ser422 phosphorylation by demonstrating that expression of a phosphomimetic mutant stimulates cap-dependent translation of a bicistronic luciferase reporter (Holz et al., 2005).

In chapter 3, we have shown that cap-dependent translation of luciferase reporters containing synthetic structured and unstructured 5'UTRs is not affected in eIF4B-silenced cells. The only subclass of mRNAs suppressed by eIF4B silencing was identified as 5'TOP containing messages. However, we cannot rule out that the level of eIF4B silencing achieved in our study (~85-90%) was insufficient to cause translational

repression of other reporters. For instance, an earlier *in vitro* study has shown that eIF4B is necessary for 48S assembly on transcripts possessing even a low level of 5'UTR secondary structure but is dispensable for unstructured mRNAs (Dmitriev et al., 2003). The bacterially expressed recombinant protein poorly substituted for the native factor, suggesting that post-translational modifications absent in bacteria (e.g. phosphorylation) are crucial for the full activation of eIF4B. Hence, the effective levels of eIF4B necessary for the translation of individual transcripts might vary greatly, as a function of 5'UTR complexity and post-translational modifications of eIF4B. In our study, both non-phosphorylatable and phosphomimetic eIF4B Ser422 mutants were able to revert the 5'TOP reporter repression in eIF4B-silenced cells to a similar extent, suggesting that the repression was Ser422 phosphorylation-independent. The study of translational defects observed in PDK1 null embryonic stem cells has shown that polysomal distribution of mRNA has a similar sensitivity to rapamycin in PDK null and wild type cells, and the list of aberrantly regulated transcripts did not include those coding for ribosomal proteins (Tominaga et al., 2005). Taking into account that Ser422 phosphorylation is undetectable in PDK1 null cells, these data support the notion that translational regulation of rapamycin sensitive transcripts (many of which are 5'TOPs) is maintained in the absence of eIF4B Ser422 phosphorylation. A recent study by Pisarev et al. demonstrated that eIF3 and eIF4B along with other translation initiation factors promote recycling of post-termination complexes (PTCs), leading to subsequent assembly of 48S complexes on recycled transcripts (Pisarev et al., 2007). Thus, we cannot rule out the possibility that eIF4B phosphorylation on Ser422 is important for the recycling of PTCs. Nevertheless, the physiological significance of eIF4B Ser422 phosphorylation remains unresolved. The data previously obtained from the *in vitro* studies identified eIF4B as having *bone fide* translation stimulatory activity. Hence, our data suggest that unlike other translational activators such as eIF4E, which act to stimulate overall translation, eIF4B is likely to stimulate the cap-dependent translation of specific transcripts or mRNA subfamilies. This model is also supported by studies conducted in yeast (Altmann et al., 1993) and flies (Hernandez et al., 2004), since eIF4B gene disruption or silencing in these organisms did not significantly affect general translation rates (under optimal physiological conditions), but repression of specific transcripts could be shown.

As judged by immunoblotting results, the apparent steady-state levels of ribosomal proteins observed in our study were not significantly different in mock and eIF4B-silenced cells. However, using a serum starvation-refeeding regime, we were able to show the difference in the synthesis rate of proteins encoded by endogenous 5'TOP mRNAs. Upon starvation they disappeared faster and upon re-feeding they accumulated slower in eIF4B-silenced cells. An early study exploring the relative stabilities of individual HeLa ribosomal proteins concluded that most ribosomal proteins on cytoplasmic ribosomes have uniform, high stability (Lastick and McConkey, 1976). It is widely believed that mammalian cells initiate DNA replication at some relatively constant cell size. The time for S and G2 phases are relatively constant as the interdivision time varies (Cooper, 2004). It is possible that under normal growth conditions, ribosomal proteins and ribosomes are produced slower in eIF4B-silenced cells but eventually reach the same or similar threshold levels necessary for S-phase entry. This would also suggest that mock and eIF4B-silenced cells have slightly different rates of mass increase resulting in longer generation times in eIF4B-silenced cells. This is precisely what we have observed in our study: eIF4B-silenced cells proliferated slower on plasticware and in soft agar. Polysomal analysis suggested that translation initiation is affected in eIF4B-silenced cells, however, part of the effects could be also attributed to the difference in the overall number of ribosomes per cell. In normally growing cells, the amount of material in peaks corresponding to the free subunits and monosomes as well as heavy polysomes was similar in mock and eIF4B-silenced cells. However, significantly higher light polysomal peaks were detected in mock cells. Our search for novel eIF4B-interacting partners led to the identification of nucleolar helicase DDX21. As visualized by confocal microscopy, the tagged versions of eIF4B and DDX21 proteins partially co-localized in the nucleolar periphery. These results and previously reported co-purification of eIF4B with pre-ribosomal particles (Sekiguchi et al., 2006), stirred our attention to the possible involvement of eIF4B in the late steps of ribosomal biosynthesis. Pulse and chase experiments have confirmed this hypothesis, underscoring the importance of eIF4B in the process of rRNA processing. More specifically, the production of mature 18S rRNA was not significantly affected during the chase period, whereas label accumulation in the 28S rRNA was decreased by 45% within 4 hours of chase. The inhibition of 28S

rRNA production was concomitant with the appearance of a 36S rRNA species, which has an otherwise extremely low abundance in HeLa cells (Weinberg and Penman, 1970). The reports describing accumulation of 36S rRNA in mammalian cells are rare in the literature. One such paradigm describes the involvement of the Bop1 protein in rRNA processing in mouse cells (Strezoska et al., 2000). Interestingly, bop1 dominant negative mutant expression results in a specific inhibition of the synthesis of the 28S and 5.8S rRNAs without affecting 18S rRNA formation. In addition, general translation in cells expressing this form of bop1 was not significantly affected. A later publication by same group demonstrated that bop1 siRNA results in similar effects and is marked with accumulation of 36S rRNA (Strezoska et al., 2002). Due to the overall similarity of the effects, it is likely that eIF4B and Bop1 regulate the same steps in the process of ribosomal biosynthesis.

As discussed in chapter 3, another translation initiation factor, eIF6, has been shown to play a major role in ribosomal biosynthesis. Ribosome biosynthesis may consume up to 80% of the energy of rapidly proliferating eukaryotic cells (Warner, 1999). Hence, concomitant involvement of eIF4B and eIF6 in translation initiation and ribosomal biogenesis may represent an important regulatory mechanism allowing cells to co-ordinate these two processes and to avoid energetically wasteful expenditure.

4.2 eIF4B in cellular transformation

Overexpression of many translation initiation factors has been demonstrated in different types of cancer (reviewed in (Mamane et al., 2006)). In addition, experimental models in which overexpression of certain eukaryotic translation initiation factors results in cellular transformation (e.g. eIF4E, eIF4G, eIF2α Ser51Ala non-phosphorylatable mutant, several eIF3 subunits etc) provide evidence that elevated levels of translation factors associated with cancers are causative and not consequential. For instance, eIF4E overexpression is observed in a large number of malignansies: e.g. colon, breast, bladder, lung, prostate, cervix, gastrointestinal tract, head and neck, Hodgkin's lymphoma and neuroblastoma (reviewed in (Mamane et al., 2006). eIF4G is most frequently overexpressed in squamous cell carcinomas of the lung. The eukaryotic translation initiation factors 4A1, 2B and 4B as well as the poly(A)-binding protein PABPC1 were also found to be overexpressed in

lung cancers (Comtesse et al., 2007). Upregulation in the level of eIF4A mRNA has been reported in human melanoma and hepatocellular carcinoma cells (Eberle et al., 1997; Shuda et al., 2000). Overexpression of eIF4 family members in human malignancies is bound to lead to increased eIF4F formation and consequently to enhanced translation initiation and cell growth. Our preliminary data suggest that 3T3 mouse fibroblasts expressing elevated levels of exogenous eIF4B are more prone to Ras-induced transformation, as they gain the ability to grow in soft agar (Appendix 3). The ability of Ser422 non-phosphorylatable (S422A) and phosphomimetic (S422E) point mutants to promote cellular transformation is under current investigation in our lab. The results of this study may shed light on the biological significance of this phosphorylation.

4.3 Conclusion

Although we have unraveled a novel regulatory link between the MAPK and PI3K/mTOR signaling pathways and the translational machinery and also discovered a new functional facet of eIF4B in the process of ribosomal biogenesis, much work remains to be done to fully understand the role of eIF4B in mammalian cells. The eIF4B-stimulated promotion of unrelated RNA-directed enzymatic activities (such as ATP-dependent RNA unwinding, as well as endo- and exonuclease activities) offers an interesting possibility that its RNA-chaperone activity might be involved in many other cellular processes.

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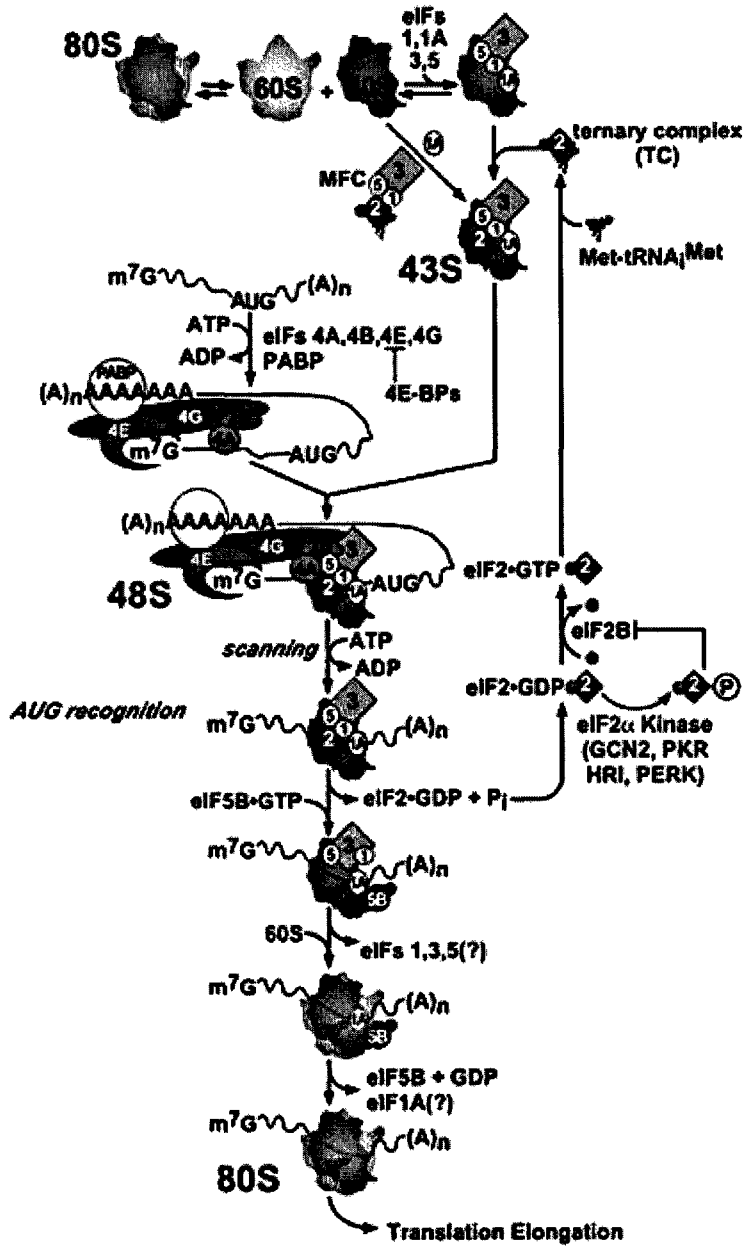
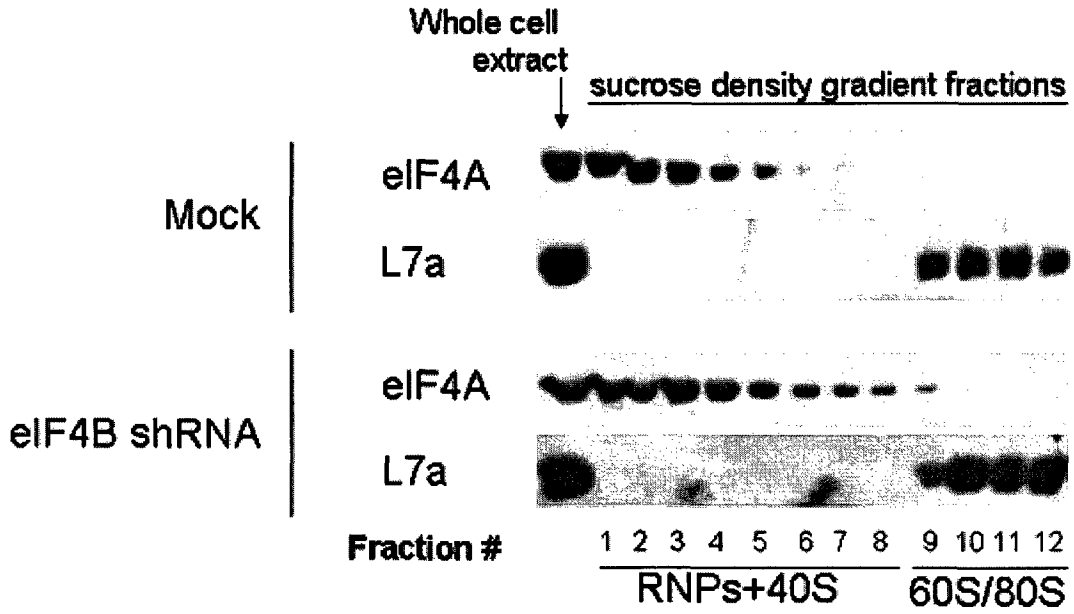


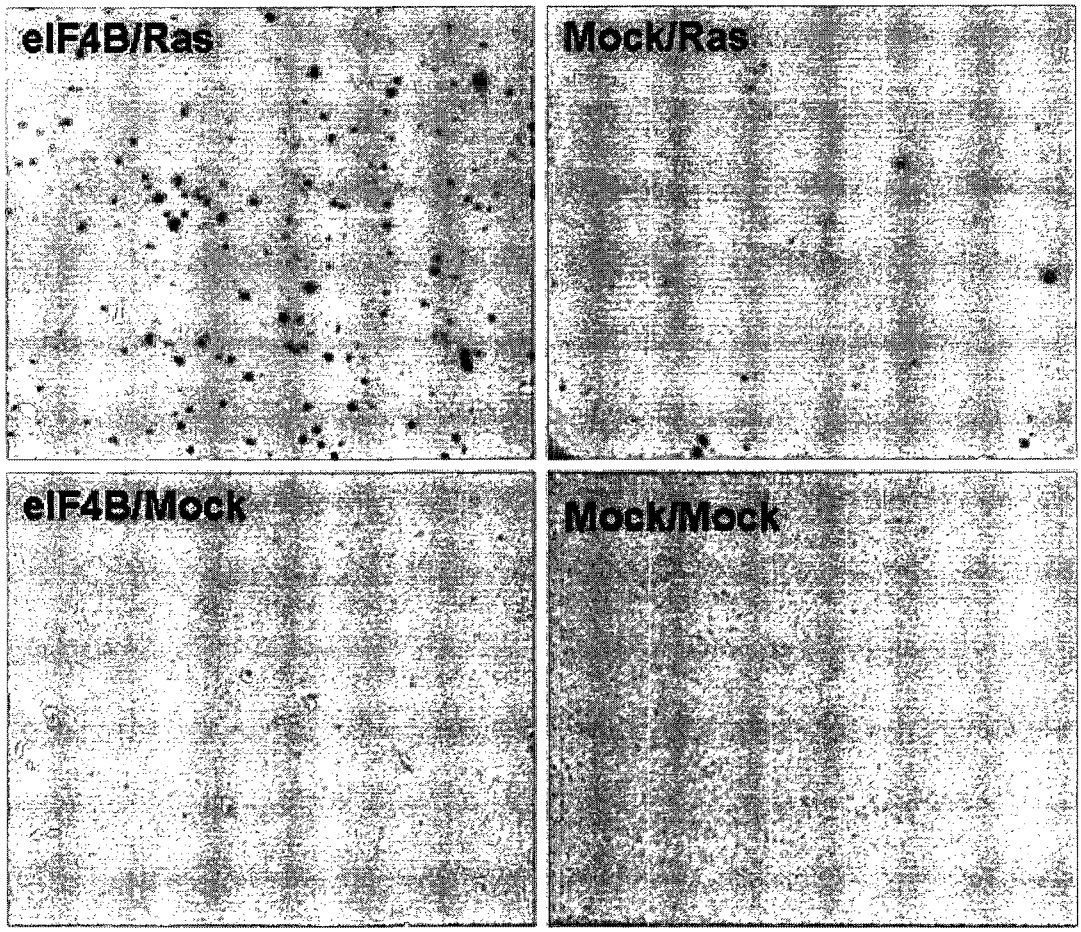
Appendix 1.

Eukaryotic translation initiation pathway (reproduced from Mathews et al. 2007)



Appendix 2.

Western blots demonstrating the position of migration of eIF4A and L7a in sucrose density gradients fractions collected from mock and eIF4B-silenced cells.



Appendix 3.

Effect of eIF4B overexpression on Ras-dependent transformation. 3T3 cells were trasfected with pcDNA3-Flag-eIF4B construct or empty vector (mock) and selected with G418. Single colonies were picked up and propagated. A clone expressing Flag-eIF4B or empty vector expressing cells were infected with pBabe-Ras or empty virus (mock) construct and a soft agar assay was performed. 14 day later colonies were stained with crystal violet and photographed.

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